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Effects of management regime and plant species on the enzyme activity and genetic structure of N-fixing, denitrifying and nitrifying bacterial communities in grassland soils

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Summary

Management by combined grazing and mowing events is commonly used in grasslands, which influences the activity and composition of soil bacterial communities. Whether observed effects are mediated by management-induced disturbances, or indirectly by changes in the identity of major plant species, is still unknown. To address this issue, we quantified substrate-induced respiration (SIR), and the nitrification, denitrification and free-living N_2-fixation enzyme activities below grass tufts of three major plant species (*Holcus lanatus, Arrhenatherum elatius* and *Dactylis glomerata*) in extensively or intensively managed grasslands. The genetic structures of eubacterial, ammonia oxidizing, nitrate reducing, and free-living N_2-fixing communities were also characterized by ribosomal intergenic spacer analysis, and denaturing gradient gel electrophoresis (DGGE) or restriction fragment length polymorphism (RFLP) targeting group-specific genes. SIR was not influenced by management and plant species, whereas denitrification enzyme activity was influenced only by plant species, and management–plant species interactions were observed for fixation and nitrification enzyme activities. Changes in nitrification enzyme activity were likely largely explained by the observed changes in ammonium concentration, whereas N availability was not a major factor explaining changes in denitrification and fixation enzyme activities. The structures of eubacterial and free-living N_2-fixing communities were essentially controlled by management, whereas the diversity of nitrate reducers and ammonia oxidizers depended on both management and plant species. For each functional group, changes in enzyme activity were not correlated or were weakly correlated to overall changes in genetic structure, but around 60% of activity variance was correlated to changes in five RFLP or DGGE bands. Although our conclusions should be tested for other ecosystems and seasons, these results show that predicting microbial changes induced by management in grasslands requires consideration of management–plant species interactions.

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Introduction

Management by combined grazing and mowing events can deeply affect the biodiversity and functioning of grassland ecosystems (Collins *et al*., 1998). Herbivores, which have a more selective effect on vegetation than mowing, often enhance soil N cycling (McNaughton *et al*., 1997), which can result in improved N availability to plants (Risser and Parton, 1982; McNaughton *et al*., 1997; Hamilton and Frank, 2001). In particular, grazing can affect key microbially mediated processes involved in soil N cycling (i.e. nitrification, denitrification and N_2-fixation) that largely control the balance between the forms of soil mineral nitrogen (NO_3^- versus NH_4^+) available to plants and N conservation at the ecosystem level. Ecosystem-scale

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increases in denitrification and nitrification activities have been observed in grassland ecosystems in response to long-term grazing pressure (Parsons *et al.*, 1991; Groffman *et al.*, 1993; Frank and Groffman, 1998; Frank *et al.*, 2000; Le Roux *et al.*, 2003; Patra *et al.*, 2005), but studies on the effect of grazing on free-living N_2-fixation are limited (Patra *et al.*, 2005). Similarly, effects of mowing on N fluxes and N retention in grasslands have been reported (Maron and Jeffries, 2001). Grazing and mowing can also affect the size and composition of key microbial functional groups driving N dynamics, although most of published studies have not quantified the influence of grazing/mowing *per se* without the confounding effect of inorganic N fertilization (Clegg *et al.*, 1998; Webster *et al.*, 2002; but see Patra *et al.*, 2005).

Changes in the activity and genetic structure of these soil bacterial functional groups induced by management through grazing/mowing can be explained by different direct and indirect effects, including (i) input of urine and dung by herbivores (Ruess and McNaughton, 1987), (ii) changes in soil porosity through trampling of soil by animals (Abdelmagid *et al.*, 1987), (iii) changes in competition for N between microorganisms and the recently defoliated sward (Busso *et al.*, 2001) and (iv) changes in the input rates and quality of plant residues and root exudates due to defoliation (Paterson and Sim, 1999; Hamilton and Frank, 2001). However, grazing/mowing regime also strongly modifies the identity of major plant species (Collins *et al.*, 1998; Olff and Ritchie, 1998), and it has been shown that plant species can influence nitrification, denitrification and N_2-fixation enzyme activities (Wheatley *et al.*, 1990; Crush, 1998; Priha *et al.*, 1999; Van der Krift and Berendse, 2001; Briones *et al.*, 2002). Similarly, plant species can influence the structure of soil microbial communities (Ibekwe and Kennedy, 1998; Priha *et al.*, 1999; Briones *et al.*, 2002; Söderberg *et al.*, 2002). Thus, the response of nitrifying, denitrifying and N_2-fixing communities to long-term grazing/mowing pressure observed at the ecosystem level could be also indirectly driven or influenced by differences in the identity in major plant species. To our knowledge, this hypothesis has been tested once and only for nitrification and denitrification enzyme activities (Le Roux *et al.*, 2003).

The objectives of this study were (i) to unravel the direct effect of management regime and the effect of changes in the identity major plant species on the enzyme activity and genetic structure of bacterial functional groups involved in soil N dynamics (nitrifiers, denitrifiers and free-living N_2-fixers); those groups have different ecological requirements and it was expected that their response to management and plant species could differ, (ii) to test if changes in soil moisture and/or mineral N could explain the observed effects on community activity and structure and (iii) to test if changes in activity were correlated or not to changes in genetic structure. This was achieved by comparing enzyme activities and genetic structures of these functional groups below three dominant grass species, whose abundance was favoured, discouraged or unaffected by management through grazing/mowing, between intensively managed (I) or extensively managed (E) grassland sites. Soil moisture and nitrate and ammonium concentrations were measured on the same samples. The substrate-induced respiration (SIR) and the genetic structure of the eubacterial community were also measured to test whether the observed changes in the bacterial functional groups involved in soil N dynamics occurred in a background of changes in microbial biomass and in the dominant soil bacterial populations.

Results

Management and plant species effects on soil moisture and mineral N concentrations

The effect of plant species on soil nitrate concentration was significant, whereas the effects of management regime and management–plant interaction were not significant (Table 1, Fig. 1). Nitrate concentration values were always higher than 19 $\mu g\ N\ g^{-1}$ (Fig. 1). Nitrite concentration was always below detection limit. The effect of management on soil ammonium concentration was significant, whereas the effects of plant species and management–plant interaction were not significant (Table 1, Fig. 1). Ammonium values were low, particularly under plants on E plots ($< 0.12\ \mu g\ N\ g^{-1}$, as compared with values around $0.3\ \mu g\ N\ g^{-1}$ under plants on I plots). Soil moisture tended to be slightly higher under I than E treatments (Table 1, Fig. 1), but differences between I and E treatments were not significant for all species (Fig. 1). Soil moisture was higher under *Holcus lanatus* than other plant species, particularly for E conditions (Fig. 1).

Table 1. Results (*P*-values) of the two-way ANOVA for the effects of management regime, plant species, and management–species interaction on nitrate and ammonium concentrations, soil moisture, and enzyme activities of soil microbial communities.

	Nitrate	Ammonium	Moisture	SIR	Free-living N_2-fixation	Denitrification	Nitrification
Management regime	NS	0.0001	0.017	NS	0.0038	NS	0.0001
Plant species	0.028	NS	0.01	NS	0.029	0.037	NS
Management–species	NS	NS	NS	NS	0.0057	NS	0.024

NS, not significantly different at level $P = 0.05$.

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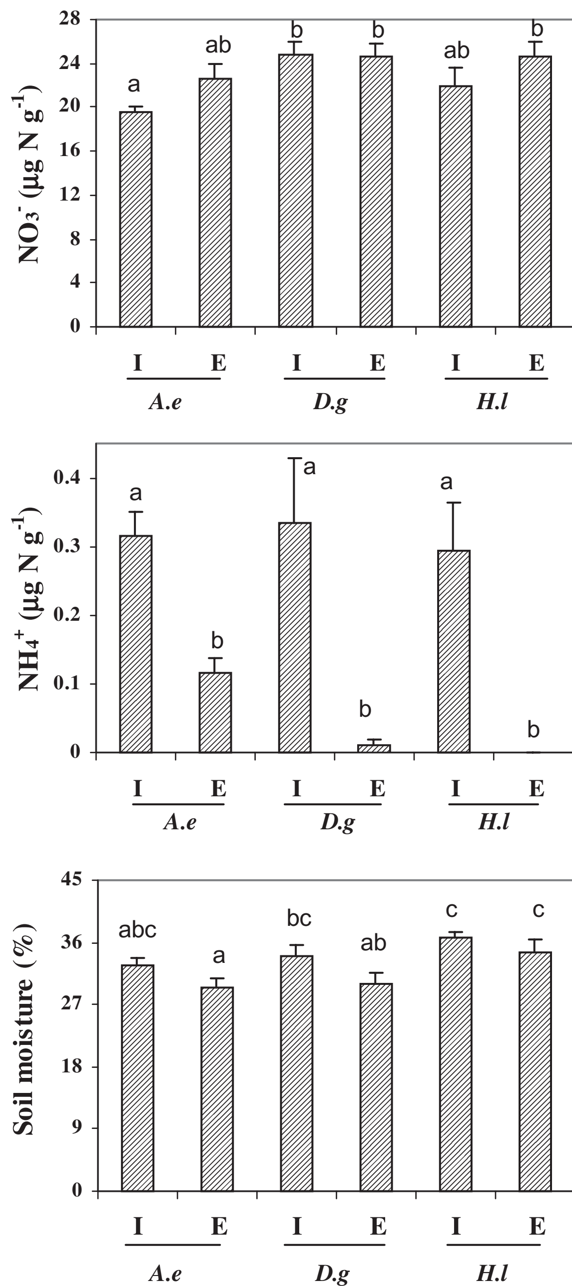

Fig. 1. (Top) Nitrate concentration, (Middle) ammonium concentration and (Bottom) moisture of soils sampled below tufts of three different grass species (*A.e., Arrhenatherum elatius; D.g., Dactylis glomerata; H.l., Holcus lanatus*) on intensively managed plots (I) and extensively managed plots (E). Bars are standard errors ($n = 6$). Values with different letters differ significantly at $P = 0.05$.

Management and plant species effects on enzyme activities

The effects of management regime, plant species and management–plant interactions on SIR were not significant (Table 1, Fig. 2). The effect of plant species on denitrification enzyme activity was significant, whereas the effects of management regime and management–plant interaction were not significant (Table 1, Fig. 2). In particular, denitrification enzyme activity was lower below *Arrhenatherum elatius* than the two other plant species (Fig. 2). Management regime had a strong effect on free-living N_2-fixation and nitrification enzyme activities and a significant management–plant interaction effect was also observed for each activity (Table 1, Fig. 2). Free-living N_2-fixation was higher in I than E sites for soils below *A. elatius*, whereas no significant effect of management was observed under *Dactylis glomerata* and *H. lanatus* (Fig. 2). Nitrification enzyme activity was higher in I than E sites for soils below *A. elatius* and *D. glomerata*, whereas no significant effect of management was observed under *H. lanatus* (Fig. 2).

Considering mean values of the six management–plant species treatments, a strong correlation was observed between nitrification enzyme activity and ammonium concentration (NEA = 0.870 [NH_4^+] + 0.085; $r^2 = 0.77$, $P = 0.02$). All the other correlations tested between activities and environmental variables were not significant.

Management and plant species effects on genetic structures of bacterial communities

Two-way analysis of similarities (ANOSIM) showed that management regime had a significant overall effect on the genetic structures of the four communities studied ($P < 0.001$ for each), with generally strong effects (R-values ranging from 0.37 to 0.88). Plant species also had a significant and strong overall effect on the genetic structure of nitrate reducers ($P < 0.001$; $R = 0.92$), but weak ($P < 0.001$; $R = 0.22$) to medium ($P < 0.001$; $R = 0.31$) overall effects on the genetic structures of the eubacterial and ammonia oxidizing communities, and no significant effect on the genetic structure of the free-living N_2-fixing community.

The genetic structures of the eubacterial community and free-living N_2-fixing community were mainly influenced by management regime (Fig. 3; Table 2) (R-values ranging from 0.54 to 0.87 for all the pairs of I versus E treatments). At the same time, the genetic structures of these two communities were either significantly, but weakly, or not significantly influenced by plant species (Table 2). The genetic structure of the nitrate reducing community was strongly influenced by both management regime and plant species (Fig. 3; Table 2) (R-values ranging from 0.83 to 1.0 for all the pairs of treatments). The genetic structure of the ammonia oxidizing community was influenced by both management regime and plant species (Fig. 3; Table 2). In this case, a management–plant interaction effect was observed: management regime significantly influenced the genetic structure of the community below *A. elatius* and *D. glomerata* ($R = 0.6$ in

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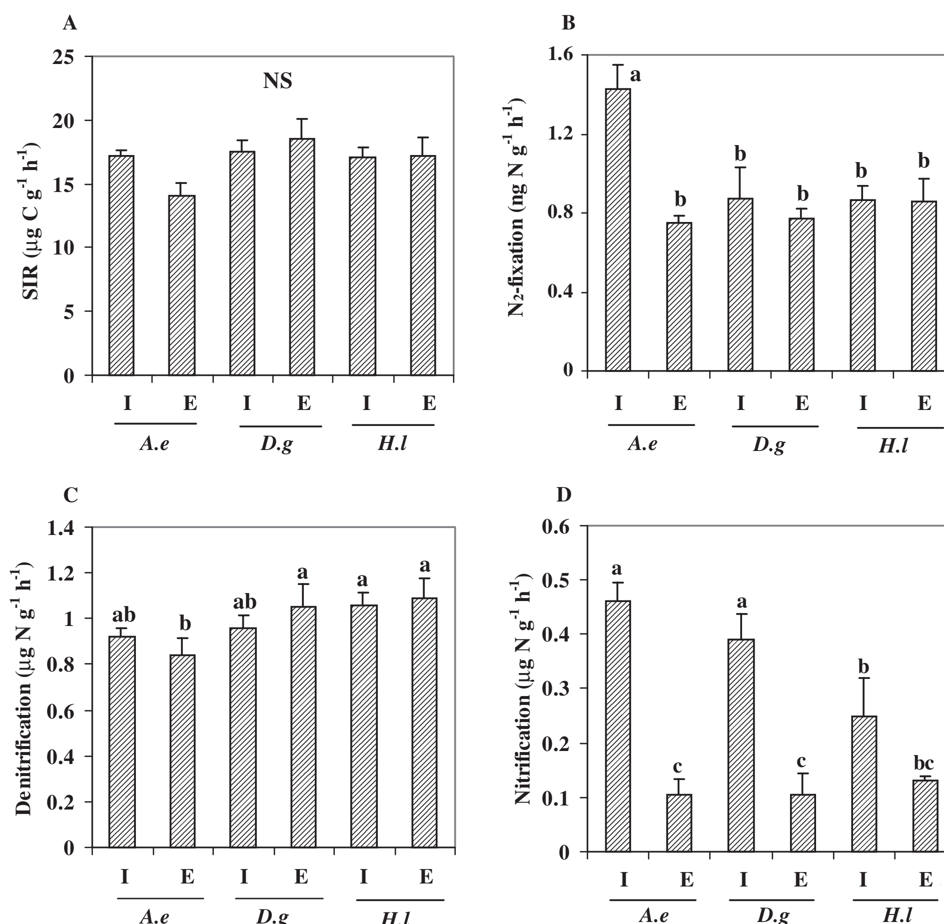

Fig. 2. (A) Substrate-induced respiration, SIR; (B) free-living N_2-fixation enzyme activity; (C) denitrification enzyme activity; and (D) nitrification enzyme activity in soils sampled below tufts of three different grass species on intensively and extensively managed plots (abbreviations as in Fig. 1). Bars are standard errors ($n = 6$). Values with different letters differ significantly at $P = 0.05$.

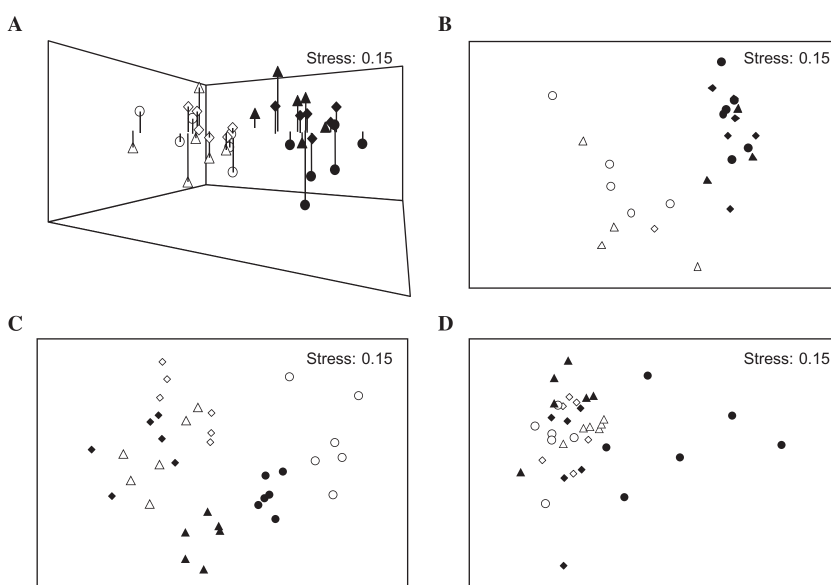

Fig. 3. Non-metric MDS ordinations of the genetic structures of (A) the eubacterial community, (B) the free-living N_2-fixing community, (C) the nitrate reducing community, and (D) the ammonia oxidizing community, for the soils sampled below tufts of three different grass species on intensively and extensively managed plots (●: intensive management – *A. elatius*; ○: extensive management – *A. elatius*; ▲: intensive management – *D. glomerata*; △: extensive management *D. glomerata*; ◆: intensive management – *H. lanatus*; ◇: extensive management – *H. lanatus*). The stress (distortion factor between actual similarity rankings and the corresponding distance rankings in the ordination plot) is indicated. Two-dimensional MDS for eubacterial community would lead to a too high distortion.

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Table 2. Results (*P*-values) of one-way ANOSIM for comparisons of the genetic structures of the eubacterial community or three functional communities between key pairs of (management regime–plant species) treatments.

	Eubacteria	Free-living N_2-fixers	Nitrate reducers	Ammonia oxidizers
A.e. I–*A.e.* E	$P = 0.002$	$P = 0.002$	$P = 0.002$	$P = 0.002$
D.g. I–*D.g.* E	$P = 0.002$	$P = 0.002$	$P = 0.002$	$P = 0.002$
H.l. I–*H.l.* E	$P = 0.002$	$P = 0.006$	$P = 0.002$	NS
A.e. I–*D.g.* I	$P = 0.019$	NS	$P = 0.002$	$P = 0.002$
A.e. I–*H.l.* I	$P = 0.002$	NS	$P = 0.002$	$P = 0.002$
D.g. I–*H.l.* I	$P = 0.013$	NS	$P = 0.002$	$P = 0.05$
A.e. E–*D.g.* E	NS	NS	$P = 0.002$	$P = 0.002$
A.e. E–*H.l.* E	$P = 0.017$	$P = 0.024$	$P = 0.002$	$P = 0.026$
D.g. E–*H.l.* E	$P = 0.035$	NS	$P = 0.002$	$P = 0.004$

A.e., *Arrhenatherum elatius*; *D.g.*, *Dactylis glomerata*; *H.l.*, *Holcus lanatus*; NS, not significantly different at level $P = 0.05$.

both cases), whereas no significant effect of management was observed under *H. lanatus*. The effect of plant species on the genetic structure of the ammonia oxidizing community was generally significant but weaker than the effect of management (*R*-values ranging from 0.19 to 0.53 for all the pairs of different plant species within a given management regime).

Correlations between changes in enzyme activity and changes in genetic structure for each bacterial functional group

The changes in N_2-fixation and denitrification enzyme activities were not correlated to changes in the bulk genetic structures of N_2-fixing and nitrate reducing communities ($P = 0.74$ and 0.095 respectively; Fig. 4). However, changes in nitrification enzyme activity were significantly ($P = 0.012$), although weakly ($\rho = 0.16$), correlated to changes in the bulk genetic structure of the ammonia oxidizing community (Fig. 4).

In contrast, for each functional group, a high proportion of variance in enzyme activity was explained by changes in the levels of a few individual DGGE (denaturing gradient gel electrophoresis) bands or RFLP (restriction fragment length polymorphism) fragments (Fig. 5). For example, 60%, 56% and 58% of the variance observed in enzyme activity of the nitrifying, denitrifying and N_2-fixing communities, respectively, was related to changes in the levels of five DGGE bands or RFLP fragments (Fig. 5). Given the amount of variance explained, these correlations were not due to a random sampling effect.

Discussion

Relative effect of management and plant species on enzyme activities

Management regime did not significantly influence SIR and denitrification enzyme activities. This is consistent with previous observations, on the same site, that management induced non-significant or weak changes in SIR and denitrification enzyme activities (Patra *et al.*, 2005), although the effect on denitrification was clearer than in the present study. Grazing effects on C mineralization and microbial biomass in grasslands are idiosyncratic (Stark and Grellmann, 2002; Bardgett and Wardle, 2003). Similarly, denitrification has been found to increase in response to grazing in different grassland ecosystems (Frank and Groffman, 1998; Frank *et al.*, 2000; Le Roux *et al.*, 2006), but no effect was observed in other systems (Parsons *et al.*, 1991; Groffman *et al.*, 1993). In contrast, nitrification enzyme activity was significantly influenced by management regime, consistent with previous observations on our site (Patra *et al.*, 2005) and in several other grassland ecosystems (Holland *et al.*, 1992; Groffman *et al.*, 1993; Frank *et al.*, 2000). At least, free-living N_2-fixation enzyme activity was influenced by management regime, although the interaction effect with plant species can explain the high spatial variance in fixation activity reported by Patra and colleagues (2005). The effects of management on soil moisture were weak (+2–3%) and not significant for all plant species, so that moisture was not a major factor driving the observed effects. Patra and colleagues (2005) suggested that enhanced nitrification on I plots could result from higher N availability. Our mineral N data support this view, because (i) ammonium levels were low and probably limiting for nitrifiers in those soils and (ii) enhanced nitrification enzyme activity was strongly related to the higher ammonium concentration levels observed in I treatment than in E treatment. Such higher ammonium concentrations in rhizospheric soil under clipped than unclipped plants were observed by Hamilton and Frank (2001), who discussed possible mechanisms. In contrast, nitrate concentrations were high and not affected or weakly affected by management in our study, suggesting that other factors such as C or oxygen availability could explain the higher fixation activity observed for I than E treatment under *A. elatius*.

Plant species did not significantly influence SIR, but significantly influenced denitrification enzyme activity as

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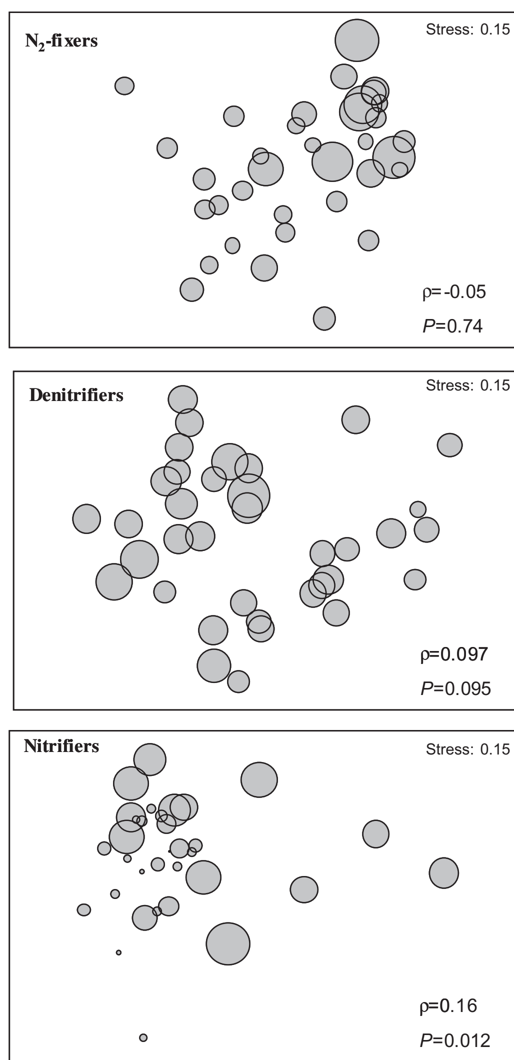

Fig. 4. Correlation between (Top) the enzyme activity and genetic structure for N_2-fixers, (Middle) the denitrification enzyme activity and genetic structure for nitrate reducers, and (Bottom) the nitrification enzyme activity and genetic structure for ammonia oxidizers. The correlation is visualized in bubble plots by representing activity values as the size of a symbol and superimposing these symbols on the community structure ordination of the same soil samples (i.e. Fig. 3B–D for N_2-fixers, denitrifiers and nitrifiers respectively). For each functional group, the significance level of the correlation (p) and the level of correlation between the rank similarity matrices obtained for activity and genetic structure (Spearman rank correlation coefficient, ρ) are given.

reported in previous studies (Wheatley *et al.*, 1990; Crush, 1998; Priha *et al.*, 1999; Hery *et al.*, 2005). Plant species also affected nitrification and free-living N_2-fixation enzyme activities on intensive management in this study, but not under extensive management. Several studies have already reported that plant species can influence nitrification (Wheatley *et al.*, 1990; Steltzer and Bowman, 1998; Priha *et al.*, 1999; Van der Krift and Berendse, 2001; Briones *et al.*, 2002; Barnard *et al.*, 2004). Other studies have found evidence that large N inputs are explained by free-living N_2-fixation in the rhizosphere of some plant species (Bredja *et al.*, 1994). The influence of plant species on nitrification, denitrification and free-living N_2-fixation can be explained by differences in plant traits influencing competition for mineral N (Roswall, 1982; Verhagen *et al.*, 1994), modifications of local water, pH and oxygen statuses (Klemedtsson *et al.*, 1987; Hojberg and Sorensen, 1993), and/or quantitative and qualitative differences in root exudates and plant litter inputs (Steltzer and Bowman, 1998; Bürgmann *et al.*, 2005). Our soil mineral N data show that the lower mean denitrification enzyme activity observed below *A. elatius* than below other plant species, and the higher fixation enzyme activity observed for *A. elatius* under intensive management than for other plant–management treatments were not due to changes in N levels, but rather probably to differences in C and/or oxygen availability. The higher moisture observed under *H. lanatus* than under other species could partly explain the enhanced denitrification enzyme activity observed below this species.

Relative effect of management and plant species on structures of bacterial communities

Management regime significantly influenced the genetic structure of eubacterial community, as reported for soil bacterial or microbial communities in fertilized grassland ecosystems (Bardgett *et al.*, 1997; Clegg *et al.*, 1998;

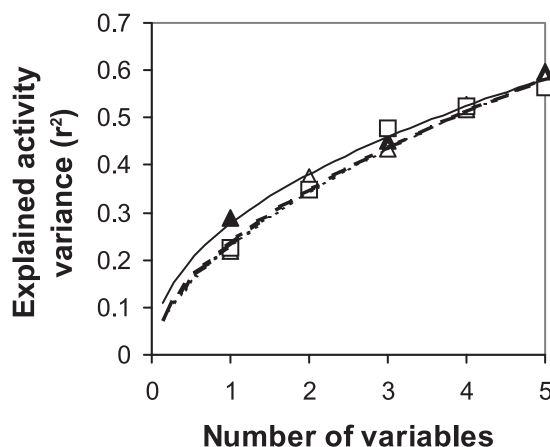

Fig. 5. Correlation between enzyme activity levels and parts of the structures of microbial functional groups estimated from stepwise multiple regression analyses (model form: Activity = $a_0 + a_1X_1 + \ldots + a_nX_n$). For each community, explained activity variance (r^2 of the model) is given in relation to the number, *n*, of variables used (▲, —: nitrification enzyme activity correlated to the occurrence of individual AOB DGGE bands; □, – –: denitrification correlated to the occurrence of individual *narG* RFLP bands; and △, - - -: N_2-fixation correlated to the occurrence of individual *nifH* RFLP fragments). Lines correspond to fitted power functions.

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McCaig *et al*., 1999; Grayston *et al*., 2001). In addition, management significantly influenced the genetic structure of free-living N_2-fixing, nitrate reducing and ammonia oxidizing communities, which is consistent with previous studies on fertilized grasslands (Clegg *et al*., 1998; Webster *et al*., 2002). Modifications of C and N availability in soil can explain the selection of specific bacterial populations by management regime (Williams *et al*., 2000). In our study, ammonium level was strongly affected by management and could be a major factor explaining changes in community diversity below a given plant species, particularly for nitrifiers that use ammonium as a key substrate. In contrast, soil moisture and nitrate levels were not significantly affected by management regime and did not explain management effect on community diversity.

Plant species significantly influenced the genetic structure of soil eubacterial, nitrate reducing, and free-living N_2-fixing communities, as reported in other studies for microbial or bacterial community (Ibekwe and Kennedy, 1998; Priha *et al*., 1999; Söderberg *et al*., 2002), nitrate dissimilating community (Clays-Josserand *et al*., 1999) and ammonia oxidizing community (Briones *et al*., 2002). In our study, mineral N levels alone could not explain most of the observed effects of plant species or management–plant interaction on community structure, and other factors probably largely explained these effects.

Correlations between changes in enzyme activity and changes in genetic structure

A key issue in microbial ecology is to test the intensity of the link between the diversity and functioning of microbial communities. For each functional group studied, changes in enzyme activity were not correlated (free-living N_2-fixers and denitrifiers) or marginally correlated (nitrifiers) to overall changes in genetic structure. Thus, most of changes in community diversity were not associated with concurrent changes in community activity. A major practical problem when linking diversity to activity of microbial communities is that we can hardly target all the organisms belonging to a given functional group due to the non-perfect specificity and exhaustivity of primers, so that a perfect detection of functional groups is not possible. In addition, only ammonia oxidizers were targeted here, whereas no information is available on changes in the diversity of nitrite oxidizers. For one functional group (denitrifiers), we targeted nitrate reducers that include denitrifying but also non-denitrifying organisms. At least, limitations exist when using the acetylene reduction assay to assess nitrogenase activity (e.g. Vessey, 1994). Beyond such possible methodological limitations, four functional hypotheses can be made to explain the lack of strong correlations between changes in enzyme activity and overall changes in genetic structure: (i) most of the populations detected were not active and did not have a key role in community functioning, which could be tested by characterizing changes in the diversity of active populations through RNA-based approaches (e.g. Freitag and Prosser, 2003); (ii) a high level of functional redundancy exists within the free-living N_2-fixing, denitrifying, and to a lesser extent nitrifying communities (for which a significant correlation was observed); this would mean that the less diverse community, i.e. nitrifiers, is characterized by a lower level of functional redundancy; (iii) the relationship between changes in overall community structure and functioning was obscured by concomitant changes in soil environmental variables (Rich *et al*., 2003); and (iv) changes in community size could partly explain changes in community activity (Patra *et al*., 2005).

In contrast, for each functional group studied, most variance in enzyme activity was explained, statistically speaking, by changes in the abundance of a few populations (actually RFLP or DGGE bands). This shows that management–plant species had concurrent, consistent effects on community activity and components of the community. However, it is very difficult to separate changes in community composition that contribute to changes in activity from those that only reflect response to environmental changes paralleling changes in activity in field studies.

Conclusion

Although the generality of our conclusions should be tested for other periods over the year and in other ecosystems, our results show that the relative effect of management regime and plant species differed between the four bacterial communities studied during the growing season at our sites: the diversity of eubacteria and free-living N_2-fixers was essentially controlled by management regime, whereas the diversity of nitrate reducers and ammonia oxidizers depended on both management regime and plant species. Our results thus show that understanding the changes in the diversity of the nitrate reducing and ammonia oxidizing communities in response to management regime cannot be determined from mean values of processes and diversity at ecosystem scale, but that the interaction between management regime and plant functioning must be considered. Furthermore, the relative importance of management and plant species can differ between different characteristics for a given community: the diversity of N_2-fixers was mainly controlled by management regime, whereas the activity of this community was controlled by both management and plant species. This led to a large disconnection between the observed changes in community enzyme activity and concurrent changes in community structure.

Because there is no necessary strong coupling between phylogenetic and functional relatedness, we did

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not affiliate the key sequences obtained for each functional group within a phylogenetic framework by cloning–sequencing of gene fragments. Rather, the next step of the study will be to characterize the functional diversity and traits of microorganisms that explain how changes in key soil variables induced by management and/or plant species affect the activity and genetic structure of soil microbial functional groups. In this context, analysing the role of the functional diversity of ammonia oxidizers with regards to ammonium levels will be of prime importance.

Experimental procedures

Study site

The study was carried out in a permanent pasture at Theix (45°43′ North, 3°1′ East, at 870 m a.s.l., France). A brown and slightly acidic sandy soil developed on a granite bedrock. Soil texture, pH, and total C and N concentrations were not significantly affected by the management regime (Le Roux *et al*., 2003). Mean annual rainfall and temperature are 770 mm and 8°C respectively. Before establishment of the study sites in 1989, the grassland had experienced a moderate grazing/mowing pressure for more than 35 years. In 1989, 500 m^2 sites were fenced, and two management regimes have been prescribed from 1989 to 2001 on the sites: ewes were allowed to graze once (extensively managed plots, E) or four (intensively managed plots, I) times per year. Ewes were kept permanently in the enclosures, allowing redistribution of N to the soil as labile forms in urine and dung. In addition, the I plots experienced one mowing event each June. The mean amount of herbage grazed or cut over the 1989–2001 period was 10.4 and 1.2 t DM ha^{-1} $year^{-1}$ in I and E sites respectively (F. Louault, unpublished). No fertilizer was used in any treatment.

Soil sampling

In October 2001, i.e. 3 weeks after a grazing event on I and E sites, the relative importance of the management regime *per se* versus plant species identity on microbial characteristics was tested by sampling soil beneath six grass tussock individuals of three dominant species on each of the two sites: *A. elatius, H. lanatus* and *D. glomerata*. Botanical surveys showed that the fractional cover of *A. elatius* and *H. lanatus*, respectively, increased from 2.3% to 15.0% and decreased from 21.9% to 11.2% from E to I treatment, whereas the fractional cover of *D. glomerata* was not influenced by the management regime. The total fractional cover for the three plant species was around 40% at each site. Soil samples (0–8 cm layer) were collected using a 8.6-cm-diameter corer. The six replicates were randomly taken per species leading to a total of 36 soil samples (two treatments × three plant species × six replicates). Target tussocks corresponded to large (at least 15 cm diameter for basal area) monospecific tufts at the same growing stage with neighbours of the same species. No legumes known to be specifically associated to symbiotic N_2-fixers (*Trifolium repens* at these sites) were present in a 50 cm area around the tufts sampled. Fresh soils were sieved using 2 mm mesh size, homogenized and subdivided into two subsamples stored at either +4°C or −20°C.

Soil moisture, and nitrate and ammonium concentrations

The moisture and water holding capacity of the sieved soils were determined by gravimetric and pressure plate techniques respectively. Fresh soil equivalent to 20 g oven-dried soil was shaken with 60 ml of 2 M KCl for 30 min. After filtration, ammonium, nitrate and nitrite concentrations were determined spectrophotometrically (San Plus System, Skalar) according to Nacro and colleagues (1996).

Enzyme activities

The SIR rate, a proxy for microbial biomass, was measured according to Anderson and Domsch (1978). Fresh soil equivalent to 10 g oven-dried soil was placed in a sterile 150 ml plasma flask with a rubber stopper. One millilitre of glucose was added to give a final concentration of 3 mg glucose g^{-1} dry soil. Additional water was added to achieve 70% of the water holding capacity. The plasma flasks were closed and incubated at 26°C for 7 h. Gas samples were analysed at 1, 3, 5 and 7 h for CO_2 concentration using a gas chromatograph (P200 Micro, Agilent Technology, Massy, France).

Nitrogenase activity was determined using the acetylene (C_2H_2) reduction technique (Hardy *et al*., 1968). Fresh soil equivalent to 10 g oven-dried was placed in a sterile 150 ml flask. Four millilitres of a solution containing glucose (1 mg C g^{-1} dry soil) and disodium malate (1 mg C g^{-1} dry soil) was added to insure non-limiting C availability. The flask atmosphere was replaced with a 90:10 mixture of air : acetylene and the flasks were incubated for 1 day at 26°C (time chosen according to the kinetics observed for some samples). Gas was sampled and C_2H_4 concentration determined using gas chromatography with a flame ionization detector (Girdel 3000 C1, Suresnes, France). N_2-fixation was calculated using a conversion factor of 1/3 N_2 reduced per C_2H_2 reduced (Burris, 1974).

Denitrification enzyme activity was measured in fresh soils over a short period according to Smith and Tiedje (1979). Ten grams of equivalent oven-dried soil was placed into 150 ml plasma flasks, and 6 ml of distilled water containing KNO_3 (200 μg NO_3^- N g^{-1} dry soil), glucose (0.5 mg C g^{-1} dry soil) and glutamic acid (0.5 mg C g^{-1} dry soil) was added. Additional water was provided to achieve 100% water holding capacity and flasks were sealed. The atmosphere of each flask was replaced by a 90:10 He–C_2H_2 mixture to provide anaerobic conditions and inhibit N_2O-reductase activity. During incubation at 26°C, gas samples were taken at 4 and 6 h (linearity during the first 6 h was checked for some samples) and immediately analysed for N_2O using a gas chromatograph (Varian STAR 3400 CX, walnut Creek, CA, USA).

Nitrification enzyme activity was measured according to the method proposed by Lensi and colleagues (1986) that is an alternative to classical methods implying extraction and filtration steps. For each fresh soil sample, two subsamples equivalent to 10 g oven-dried were placed in 150 ml plasma flasks. One subsample was used to estimate the initial soil

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NO_3^- content. This subsample was supplied with 6 ml of a suspension of a denitrifying *Pseudomonas fluorescens* ($OD_{580} = 2$) in a solution containing glucose and glutamic acid (for each: 0.5 mg C g^{-1} dry soil). The atmosphere of the flask was replaced by a He–C_2H_2 mixture (90:10) and N_2O accumulation was measured until soil NO_3^- was converted fully to N_2O. The other subsample was used to determine potential NO_3^- accumulation. In this case, 4 ml of a $(NH_4)_2SO_4$ solution was added (200 μg N g^{-1} dry soil). Water was added to achieve 70% water holding capacity. After aerobic incubation during 7 h at 26°C which allows nitrate to accumulate (linearity during the first 7 h was checked for some samples), the soil samples were enriched with *P. fluorescens* and incubated as described above. N_2O was analysed on a Varian STAR 3400 gas chromatograph. Nitrification enzyme activity was computed by subtracting the nitrate initially present in the soil from that present after aerobic incubation.

Genetic structures of bacterial communities

For each sample, DNA was extracted from 0.5 g sieved and frozen soil using the FastDNA SPIN Kit for Soil (BIO 101 Systems, Qbiogene, Carlsbad, CA, USA) yielding to around 4 μg DNA g^{-1} soil. Twenty nanogram DNA were used as templates.

The genetic structure of the eubacterial community was characterized by automated rRNA intergenic spacer analysis (A-RISA) according to Ranjard and colleagues (2001). All soil DNA samples were amplified by polymerase chain reaction (PCR) in duplicate using the primers S-D-Bact-1522-b-S-20 and L-D-Bact-132-a-A-18 (Ranjard *et al*., 2001). The composition of reaction mixtures and conditions for PCR amplification are given in Patra and colleagues (2005). The duplicate PCR products were pooled, concentrated (freeze dried) to a volume of approximately 30 μl and then examined by A-RISA using a capillary sequencer (MegaBACE 1000, Amersham Pharmacia Biotech, Piscataway, NJ, USA).

The genetic structure of the free-living N_2-fixing community was characterized by PCR-RFLP targeting the *nifH* gene according to Poly and colleagues (2001). The term 'free-living' is used here, but note that this approach takes into account free-living forms of potentially symbiotic bacteria. We use 20 ng soil genomic DNA as a template for PCR with primers PolF and PolR (Poly *et al*., 2001) to amplify a 360 bp region of the *nifH* gene. The final reagent concentrations and conditions for PCR amplification are detailed in Patra and colleagues (2005). Twenty nanograms of amplicon was directly used for restriction enzyme cleavage (NdeII, MnlI). Digestion was performed for 12 h at 37°C according to manufacturer's instructions (Qbiogene, Carlsbad, CA, USA). Digested DNA samples were analysed by electrophoresis in 5% polyacrylamide gels (19:1) (Bio-Rad Laboratories, Hercules, CA, USA) using a ProteanII XI electrophoresis system (Bio-Rad, Ivry sur Seine, France). The electrophoresis conditions were: 15 h at 35 V in 1× TBE buffer, followed by 30 min staining in 1xSYBRGreenI (FMC BioProducts, Rockland, ME, USA). Restriction profiles were analysed using GELCOMPAR software (Applied Maths, Kortrijk, Belgium).

The nitrate reducing community was characterized by PCR-RFLP analysis targeting the *narG* gene, encoding the catalytic subunit of nitrate reductase. Amplification of soil DNA was carried out using the primers narG1960f and narG2650r (Philippot *et al*., 2002) leading to PCR products of about 650 bp. Two PCR amplifications were performed for each sample in which 25 ng of template DNA was added in a 50 μl reaction mixture. The concentration of reagents in the PCR reaction mixture and thermocycling conditions were as described by Philippot and colleagues (2002). For RFLP fingerprinting analysis, gel slices containing the *narG* PCR products were excised and DNA purified using the Qiaex II kit (Qiagen, France). Excised and purified PCR products were digested with AluI restriction enzyme at 37°C for 12 h and separated by electrophoresis on a native 6% acrylamide gel. GELCOMPAR software was used to analyse the restriction profiles.

The structure of the ammonia oxidizing community was analysed by PCR-DGGE. Amplification of 16S rRNA gene fragments from extracted soil DNA was achieved by a primary amplification with CTO189f and CTO654r primers (Kowalchuk *et al*., 1997) that are specific for the majority of betaproteobacterial ammonia oxidizers, and with a secondary nested amplification using eubacterial 357f-GC and 518r primers (Muyzer *et al*., 1993). CTO and eubacterial primers amplified 465 bp and 161 bp fragments respectively. The composition of the reaction mixture is given in Patra and colleagues (2005) and the thermocycling conditions were as described previously by Freitag and Prosser (2003). DGGE analyses of PCR products were carried out using the D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) as detailed in Patra and colleagues (2005). Gels were silver stained and scanned following electrophoresis. Complex banding patterns of ammonia oxidizing communities were digitally analysed using Phoretix 1D gel analysis software (version 4.0, Phoretix International, Newcastle/Tyne, UK).

Statistical analyses

In the present study, a pseudo-replicated experimental design was used, i.e. true replicates were used to test the species effect but no replication of the management effect was included. However, no changes in soil texture, pH, total C and N, and topography exist between the sites studied here (Le Roux *et al*., 2003) and management effect at plot scale was tested for the same location during a previous study (Patra *et al*., 2005). Such a design is accepted in ecology (e.g. Oksanen, 2004) provided that the limits of the applicability of results are recognized, i.e. we do not test here general patterns about management–plant species effects *sensu stricto*, but rather test patterns observed at the particular sites studied. Two-factor analysis of variance (ANOVA) was performed to determine the effects of management regime, plant species and management regime–plant species interaction on the enzyme activity of each bacterial community and soil characteristics. For each variable, Duncan's and Fisher's tests were used to determine if means differed significantly.

To analyse genetic fingerprints, the intensity and relative position of each DNA band in all lanes were determined according to a reference lane. The total band intensity for each lane was normalized among lanes and data were square root-transformed. Bacterial community matrices were analysed using PRIMER software (versus 2, PRIMER-E Ltd,

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Plymouth, UK). Rank similarity matrices were computed for each bacterial community and used to construct 'maps' highlighting the similarity/dissimilarity of genetic structures among soil samples by non-metric multidimensional scaling (MDS) (Kruskal and Wish, 1978). Two- or three-dimensional maps were chosen so that the stress factor (i.e. distortion factor between actual similarity rankings and the corresponding distance rankings in the map) was sufficiently low. Two-way analysis of similarities (ANOSIM) was performed to test the overall effects of management regime and plant species on the genetic structure of each bacterial community. One-way ANOSIM was then performed to compare the genetic structures among each pair of management regime–plant species treatments. ANOSIM results in the computation of *P*-values (level of significance) and *R* statistic values (degree of discrimination between treatments: values around 0 and 1 for no discrimination and perfect discrimination respectively).

The correlation between changes in the enzyme activity of N_2-fixers, denitrifiers and nitrifiers and changes in the overall genetic structure of the corresponding functional group were tested by a two-step approach (graphically and then statistically). First, this correlation was visualized in bubble plots by representing activity values as a symbol of different sizes according to activity level and superimposing these symbols on the community structure ordination of the corresponding soil samples. Then, for each functional group, a rank correlation coefficient (here Spearman coefficient) and significance level (obtained by a permutation test using 5000 permutations) were computed to quantify the correlation between the rank similarity matrices obtained for activity and genetic structure (Clarke and Ainsworth, 1993). In addition, for each functional group, a stepwise, multiple regression analysis was used to test the correlation between changes in enzyme activity and changes in key variables (i.e. individual DGGE bands or RFLP fragments) within the community composition matrix.

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Supplementary material

The following supplementary material is available for this article online:

Appendix S1. PCR-RFLP analysis of *nifH* sequences for soils below tufts of three different grass species (*Arrhenatherum elatius*; *Holcus lanatus*; *Dactylis glomerata*) on intensively and extensively managed plots with restriction enzymes MnlI and NdeII.

Appendix S2. PCR-RFLP analysis of *narG* sequences for soils below tufts of three different grass species (*Arrhenatherum elatius*; *Holcus lanatus*; *Dactylis glomerata*) on intensively and extensively managed plots.

Appendix S3. DGGE analysis of 16S rRNA gene sequences of ammonia oxidizers for soils below tufts of three different grass species (*Arrhenatherum elatius*; *Holcus lanatus*; *Dactylis glomerata*) on intensively and extensively managed plots.

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