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# EFFECTS OF GRAZING ON MICROBIAL FUNCTIONAL GROUPS INVOLVED IN SOIL N DYNAMICS

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*Abstract.* Enhancement of soil nitrogen (N) cycling by grazing has been observed in many grassland ecosystems. However, whether grazing affects the activity only of the key microbial functional groups driving soil N dynamics or also affects the size (cell number) and/or composition of these groups remains largely unknown. We studied the enzyme activity, size, and composition of five soil microbial communities (total microbial and total bacterial communities, and three functional groups driving N dynamics: nitrifiers, denitrifiers, and free $N_2$ fixers) in grassland sites experiencing contrasting sheep grazing regimes (one light grazing [LG] site and one intensive grazing [IG] site) at two topographical locations. Enzyme activity was determined by potential carbon mineralization, nitrification, denitrification, and $N_2$ fixation assays. The size of each community (except $N_2$ fixers) was measured by the most-probable-number technique. The composition of the total soil microbial community was characterized by phospholipid fatty acid analysis (PLFA), and the genetic structure of the total bacterial community was assessed by ribosomal intergenic spacer analysis. The genetic structures of the ammonia-oxidizing, nitrate-reducing, and $N_2$-fixing communities were characterized by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) or by polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) targeting group-specific genes. Greater enzyme activities, particularly for nitrification, were observed in IG than in LG sites at both topographical locations. The numbers of heterotrophs, nitrifiers, and denitrifiers were higher in IG than in LG sites at both topographical locations. The amplitude of changes in community size was higher than that of community enzyme activity. Phospholipid and nucleic acid analyses showed that the composition/structure of all the communities, except nitrate reducers, differed between IG and LG sites at both locations. For each community, changes in activity were correlated with changes in the occurrence of a few individual PLFAs or DNA fragments. Our results thus indicate that grazing enhances the activity of soil microbial communities but also concurrently induces changes in the size and composition/structure of these communities on the sites studied. Although the generality of our conclusions should be tested in other systems, these results are of major importance for predicting the effects of future disturbances or changed grazing regimes on the functioning of grazed ecosystems.

*Key words: carbon mineralization; denitrification; free $N_2$ fixation; grasslands; grazing; microbial functional communities; microbial diversity; nitrification; nitrogen cycle.*

## Introduction

Soil microorganisms are of paramount importance for the functioning and stability of ecosystems. In particular, a number of microbial functional groups, in addition to plants, are responsible for the cycling of nitrogen (N). Nitrifiers, denitrifiers, and $N_2$ fixers largely determine N availability in soils (in terms of both inorganic N quantity and $NO_3^-/NH_4^+$ balance), and N losses by nitrate leaching or gaseous N emissions.

In grasslands, herbivores can modify soil N cycling (Ruess 1986, McNaughton et al. 1988; for a review, see Bardgett and Wardle [2003]), which strongly influences plant growth (e.g., Leriche et al. 2001, 2003). Several studies report enhanced soil N transformation rates in response to long-term (i.e., years to decades)

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PLATE 1. View of the study sites at the downslope location. Photo credit: F. Louault.

grazing, but negative effects have also been reported (Bardgett and Wardle 2003). Denitrification has been found to increase in response to grazing in grasslands of Yellowstone National Park (Frank and Groffman 1998*a*, Frank et al. 2000) and in a seminatural grassland (Le Roux et al. 2003), but no effect was observed in other grasslands (Parsons et al. 1991, Groffman et al. 1993). Enhanced nitrification in response to grazing has been reported in many grassland ecosystems (Holland et al. 1992, Groffman et al. 1993, Seagle and McNaughton 1993, Frank et al. 2000, Le Roux et al. 2003). To our knowledge, the impact of grazing on free $N_2$ fixation has not been directly quantified.

Furthermore, grazer-induced changes in community activities could result from (1) changes in the physiological activity of individual cells (i.e., mean activity per cell), (2) changes in the community size (cell number), and/or (3) changes in the community composition. Indeed, the synthesis of key enzymes of the microbial functional groups involved in N cycling, particularly nitrifiers and denitrifiers, are inducible (e.g., Sundemeyer and Bock 1981, Zumft 1997), so that the size and activity of each functional group are not necessarily tightly coupled. In addition, within a given functional group, different populations can exhibit different physiological characteristics (e.g., Prosser 1989, Degrange et al. 1997) so that community composition can influence community activity. However, information on how grazers affect the size and composition of key microbial functional groups driving N dynamics is very scarce (Clegg et al. 1998, Webster et al. 2002). In particular, the influence of grazing per se (i.e., without the confounding effect of inorganic N fertilization) on the size and composition of the different microbial functional groups involved in soil N dynamics has not been studied to date. This restricts our understanding of the actual effects of grazers on grassland functioning and our ability to predict grassland response to changes in grazing intensity or management practices, because the composition of microbial communities can determine their resistance and resilience to disturbances (Griffiths et al. 2000).

The objectives of this study were to (1) compare the enzyme activities of the total soil microbial community and three functional groups involved in N cycling (nitrifiers, denitrifiers, and free $N_2$ fixers) between intensive grazing (IG) and light grazing (LG) sites in a seminatural grassland ecosystem, (2) compare the size and composition of these microbial communities between IG and LG sites, and (3) test if changes in community activity were independent of, or correlated with, changes in community size and composition. The implications of our findings for better understanding the effect of grazing on soil N cycling processes at the study site are discussed.

## Materials and Methods

### *Study site*

The study was carried out in a seminatural grassland (i.e., permanent pasture [see Appendix A]) at Theix, France (45°43′ N, 3°1′ E, at 870 m above sea level) consisting of a brown and slightly acidic sandy soil developed on a granite bedrock (see Plate 1). Soil characteristics differed slightly between topographical locations, but were not significantly affected by the grazing regime at each location (for soil characteristics, see Le Roux et al. 2003). Mean annual rainfall and temperature are 770 mm and 8°C, respectively. Before establishment of the study site in 1989, the grassland had experienced a moderate grazing pressure for more than 35 years. In 1989, two sites (500 $m^2$ each) were fenced at an upslope location and two sites (500 $m^2$) at a downslope location along a topographical transect. Different grazing regimes have been prescribed for 13 years on the sites: ewes were allowed to graze once (light grazing [LG]) or four times (intensive grazing [IG]) per year. The common grazing event occurred in April. For each grazing event, eight ewes per plot were allowed to graze until the mean height of the sward reached 6 cm in the IG plot (typically a few days). Ewes were kept permanently in the enclosures, allowing redistribution of N to the soil as labile forms in urine and dung. In addition, the IG plots experienced one mowing event each June, which mimicked some export by herbivores without redistribution to the soil. The mean amount of herbage used (grazed or cut) over the 1989–2001 period was 10.4 and 1.2 Mg dry mass·$ha^{-1}$·$yr^{-1}$ in IG and LG sites, respectively (F. Louault, *unpublished data*). No fertilizer was used in any treatment.

### *Soil sampling*

Soil samples (0–8 cm layer) were collected using corers (8.6 cm diameter) on 22 October 2001. Nine soil samples were taken randomly on IG and LG sites at the two topographical locations. The 36 samples (two treatments × two locations × nine replicates) were placed in plastic bags and transported to the laboratory, where soil was immediately sieved (2 mm mesh size), homogenized, and subdivided into two subsamples stored at either +4°C or −20°C. The moisture content and water holding capacity of the sieved soils were determined by gravimetric and pressure plate techniques, respectively.

### *Enzyme activities*

*C mineralization.*—The substrate-induced respiration rate, i.e., C mineralization potential, 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. Glucose (1 mL) was added to give a final concentration of 3 mg glucose/g 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 analyzed at 1, 3, 5, and 7 h for $CO_2$ concentration using a gas chromatograph (P200 Micro; Agilent Technology, Massy, France). C mineralization rate was expressed as μg C·$h^{-1}$·$g^{-1}$ dry soil.

*Denitrification.*—Denitrification enzyme activity (DEA), i.e., denitrification potential, was measured on fresh soils over a short period according to Smith and Tiedje (1979) and Tiedje et al. (1989). Soil (10 g equivalent oven-dried) was placed into 150 mL plasma flasks, and 6 mL distilled water containing $KNO_3$ (200 μg $NO_3^-$-N/g dry soil), glucose (0.5 mg C/g dry soil), and glutamic acid (0.5 mg C/g dry soil) was added. Additional water was provided to achieve 100% water holding capacity and flasks were sealed with rubber stoppers. The atmosphere of each flask was evacuated and 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 (200 μL) were taken at 4 h and 6 h and immediately analyzed for $N_2O$ using a gas chromatograph (Varian STAR 3400 CX; Varian, Walnut Creek, California, USA) equipped with a $^{63}Ni$ electron capture detector. Denitrification enzyme activity was expressed as μg N·$h^{-1}$·$g^{-1}$ dry soil.

*Nitrification.*—Nitrification enzyme activity (NEA), i.e., nitrification potential, was measured according to Lensi et al. (1986). 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 $NO_3^-$ content. This subsample was supplied with 6 mL of a suspension of a denitrifying organism (*Pseudomonas fluorescens*, optical density at 580 nm = 2) in a solution containing glucose and glutamic acid (final soil C concentration for each was 0.5 mg C/g 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 (final concentration 200 μg N/g dry soil). Water was added to achieve 70% water-holding capacity. After aerobic incubation (7 h at 26°C), which allows nitrate to accumulate, the soil samples were enriched with *Pseudomonas fluorescens* and incubated as described above. $N_2O$ was analyzed on a Varian STAR 3400 gas chromatograph. Nitrification rate (μg N·$h^{-1}$·$g^{-1}$ dry soil) was computed by subtracting the nitrate initially present in the soil from that present after aerobic incubation.

*$N_2$ fixation.*—Nitrogenase activity, i.e., $N_2$ fixation potential, was determined using the acetylene ($C_2H_2$) reduction technique (Hardy et al. 1968, Turner and Gibson 1980). The usefulness and limitations of this technique are discussed by Vessey (1994) and Minchin et al. (1994). Fresh soil (equivalent to 10 g oven dried) was placed in a sterile 150 mL flask with a rubber stopper. A solution containing glucose (4 mL, to make

1 mg C/g dry soil) and disodium malate (1 mg C/g dry soil) was added. The gaseous atmosphere was replaced with a 90:10 mixture of air:acetylene and the flasks were incubated for 5 d at 26°C. Gas (400 μL) was sampled daily and $C_2H_4$ concentration determined using gas chromatography with a flame ionization detector (Girdel 3000 C1, Suresnes, France). The $N_2$ fixation rate was determined from the kinetics observed between 24 and 72 h of incubation, during which the rate of $N_2$ fixation was maximal. $N_2$ fixation rate (μg $N \cdot h^{-1} \cdot g^{-1}$ dry soil) was calculated using a conversion factor of 1/3 $N_2$ reduced per $C_2H_2$ reduced (Burris 1974).

*Enumeration of bacteria*.—Numbers of heterotrophic bacteria, ammonia and nitrite oxidizers, and denitrifiers were determined using the most probable number (MPN) technique (Alexander 1982). Enumeration of heterotrophic and denitrifying bacteria was made on fresh soil (equivalent to 10 g oven dried) according to Cannavo et al. (2002). For ammonia oxidizers and nitrite oxidizers, soil samples (equivalent to 10 g oven dried) were homogenized in 50 mL of NaCl (8 mg/L) for 1.5 min with a Waring blender (Eberbach Corporation, Ann Arbor, Michigan, USA). Soil suspensions were serially diluted 10-fold and 750 μL of serial dilutions was placed in 4 × 6 wells of microtitre plates containing 750 μL of the double strength mineral salts medium (Schmidt and Belser 1982) containing either 0.5 mg/mL $(NH_4)_2SO_4$ or 0.34 mg/mL $NaNO_2$ for enumeration of ammonia and nitrite oxidizers, respectively. For each dilution, eight wells were inoculated and plates were incubated at 28°C in the dark for 10 wk. Growth of ammonia oxidizers was revealed by the presence of $NO_2^-$ and/or $NO_3^-$ using Morgan's reagent. Growth of nitrite oxidizers was revealed by the absence of nitrite using Griess-Ilosvay's reagent. The most probable numbers of heterotrophic, denitrifying, and nitrifying microorganisms were estimated by Cochran's method (1950).

### *Assessment of soil microbial composition by PLFA analysis*

The biomass and composition of the soil microbial community were assessed by analyzing the ester-linked phospholipid fatty acids (PLFA) composition of the soil (Grayston et al. 2001). PLFAs were extracted from 1.5 g sieved and frozen soil samples as described by Frostegård et al. (1991). The separated fatty acid methyl-esters were identified and quantified by chromatographic retention time and mass spectral comparison on a Hewlett Packard 5890 II gas chromatograph (Hewlett Packard, Albertville, Minnesota, USA) equipped with a 5972A mass selective detector (MSD II), using standard qualitative bacterial acid methyl ester mix (Supelco UK, Poole, Dorset, UK) ranging from C11 to C20. For each sample, the abundance of individual fatty acid methyl-esters was expressed as μg PLFA/g dry soil. All the individual PLFA provide information on the composition of the microbial community. More particularly, the fatty acids i15:0, ai15:0, 15:0, i16:0, 16:1ω7t, i17:0, ai17:0, 17:0, cy17:0, 18:1ω7, and cy19:0 were chosen to represent bacterial PLFA, and the polyenoic, unsaturated PLFA 18:2ω6 to represent fungal biomass (Federle 1986, Frostegård et al. 1993). The monoenoic and cyclopropane unsaturated PLFAs 16:1ω7t, 16:1ω7c, cy17:0, 18:1ω7, and cy19:0 were chosen to represent Gram-negative bacteria (Zogg et al. 1997). The branched, saturated PLFAs 10Me16:0, i15:0, ai15:0, i16:0, i16:1, i17:0, ai17:0, br17:0, and 10Me17:0 were chosen to represent Gram-positive bacteria, and the methylated, branched, saturated PLFA 10Me18:0 to represent actinomycetes (Zogg et al. 1997).

### *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, California, USA).

*Total bacterial community*.—The genetic structure of the total bacterial community was characterized by automated rRNA intergenic spacer analysis (A-RISA) according to Ranjard et al. (2001). All soil DNA samples were amplified in duplicate using the primers S-D-Bact-1522-b-S-20 and L-D-Bact-132-a-A-18 (Ranjard et al. 2001). Reaction mixtures (50 μL) for PCR (polymerase chain reaction) contained 5 μL of 10× dilution buffer (20 mmol/L Tris-HCl, pH 7.5, 100 mmol/L KCl, 15 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol [DTT], 0.1 mmol/L EDTA, 0.5% Tween 20 [vol/vol], 0.5% Nonidet P40 [vol/vol], 50% glycerol [vol/vol]), 1 μg T4 gene 32 protein (Boehringer, Mannheim, Germany), 0.5 μmol/L of primers, 200 μmol/L of each dNTP, 2.0 U of Expand High Fidelity DNA polymerase (Boehringer, Mannheim, Germany), and 25 ng of purified soil DNA. Amplification was performed in a Perkin Elmer Cetus 2400 (Perkin Elmer Applied Microsystems, Foster City, California, USA) after a hot start at 94°C for 3 min, followed by 25 cycles consisting of 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min; and extension of incomplete products for 5 min at 72°C. The duplicate PCR products were pooled, concentrated (freeze dried) to a volume of ~30 μL, and then examined by A-RISA using a capillary sequencer (MegeBACE 1000; Amersham Pharmacia Biotech, Piscataway, New Jersey, USA).

*Nitrate reducers*.—The nitrate-reducing community was characterized by PCR-RFLP (restriction fragment length polymorphism) analysis targeting the *narG* gene, encoding the catalytic subunit of nitrate reductase. Briefly, amplification of soil DNA was carried out using the primers *narG*1960f and *narG*2650r (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 previously by Philippot et al. (2002). The presence and size of the amplification products were determined by agarose (2%) gel electrophoresis of the reaction product. For RFLP fingerprinting analysis, gel slices containing the *narG* PCR products were excised and DNA purified using the Qiaex II kit (Qiagen, Courtaboeuf, France). Excised and purified PCR products were digested with *Alu*I restriction enzyme at 37°C for 12 h and separated by electrophoresis on a native 6% acrylamide gel. Although only one restriction enzyme was used, the choice of *Alu*I has been determined after *in silico* restriction analysis of the *narG* genes from more than 15 taxonomically diverse bacteria (Archaea, Firmicutes, Actinomycetes, and Gram-negative bacteria). Among all the enzymes tested, *Alu*I was capable of discriminating most of the tested *narG* genes. Combination of *Alu*I RFLP analysis and sequencing of cloned *narG* PCR (Philippot et al. 2002, Cheneby et al. 2003) confirmed the validity of the use of *Alu*I for RFLP analysis. GelCompar software (Applied Maths, Kortrijk, Belgium) was used to analyze restriction profiles.

*Ammonia oxidizers*.—The structure of the ammonia oxidizer community was analyzed by PCR-DGGE (denaturing gradient gel electrophoresis). The 16S rRNA gene amplifications of extracted soil DNA were achieved by a primary amplification with CTO189f and CTO654r primers (Kowalchuk et al. 1997) that are specific for betaproteobacterial ammonia oxidizers, and with a secondary nested amplification using eubacterial 357f-GC and 518r primers (Muyzer et al. 1993). PCR was carried out in 50-μL reaction volumes with a PCRExpress thermal cycler (Hybaid, Middlesex, UK). The reaction mixture contained 20 ng of template DNA, 0.2 μmol/L of each primer, 1× PCR buffer (Bioline, London, UK), 250 μmol/L each deoxynucleoside triphosphate, 1.5 mmol/L $MgCl_2$, (20 ng bovine serum albumin was added to the primary PCR only) and 1 U of Biotaq DNA polymerase (Bioline). The thermocycling conditions were as described previously by Freitag and Prosser (2003). CTO and eubacterial primers amplified 465-bp and 161-bp fragments, respectively. The PCR products were examined by electrophoresis on agarose gels stained with ethidium bromide.

DGGE analyses of PCR products were carried out using the D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, California, USA). Briefly, 8% polyacrylamide gels containing a gradient of 30–60% denaturant were prepared using a gradient maker (Fisher Scientific, Loughborough, UK), 100% denaturing solution being defined as 7 mol/L urea and 40% formamide. Gels were run for 16 h at 75 V in 1× TAE buffer at a constant temperature of 60°C. Following electrophoresis, gels were silver stained and scanned. Complex banding patterns of ammonia oxidizing communities were digitally analyzed using Phoretix 1D gel analysis software (version 4.0; Phoretix International, Newcastle/Tyne, UK) as described previously (McCaig et al. 2001).

*$N_2$ fixers*.—The genetic structure of the free $N_2$ fixing community was characterized by PCR-RFLP targeting the *nifH* gene according to Poly et al. (2001). We used 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 *nifH* gene. The final reagent concentrations for PCR were 0.5 μmol/L primers, 200 μmol/L each dNTP, 2.0 U Taq DNA polymerase (Invitrogen, Cergy Pointoise, France), manufacturer's buffer 1× containing 1 μg T4 gene 32 protein (Qbiogen, Carlsbad, California, USA). PCR conditions consisted of 30 cycles at 94°C (1 min), 1 min for the annealing step at 55°C and 72°C (2 min), with a 5-min extension at 72°C for the last cycle. Amplicon (20 ng) was directly used for restriction enzyme cleavage (*Nde*II, *Mnl*I). Digestion was performed for 12 h at 37°C according to manufacturer's instructions (Qbiogene, Carlsbad, California, USA). Digested DNA samples were analyzed by electrophoresis in 5% polyacrylamide gels (19:1) (Bio-Rad Laboratories, Hercules, California, USA). Runs were performed in 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 1× SYBRGreenI (FMC BioProducts, Rockland, Maine, USA). GelCompar software (Applied Maths, Kortrijk, Belgium) was used to analyze the restriction patterns.

### *Statistical analyses*

In the present study, a pseudoreplicated experimental design was used. This design thus limits the applicability of our results, i.e., we do not test here general patterns about grazing effects sensu stricto, but rather test patterns observed at the particular sites studied (i.e., two locations only). Two-factor analysis of variance was performed to determine the effects of grazing regime, topographical location, and grazing × location interaction on the activity and cell number of each microbial community. For each variable, Duncan's and Fisher's tests were used to determine if means differed significantly.

To analyze PLFA data and genetic fingerprints, the intensity and relative position of each PLFA/DNA band in all lanes (i.e., soil samples) were determined according to a reference lane. The total band intensity for each lane was normalized among lanes and data were standardized by log transformation when necessary. Principal-component analysis (PCA) on covariance matrix was first performed on all (36) soil samples. Two-factor analysis of variance was performed on the coordinates of samples on the first axis generated by PCA to test the effects of grazing regime, topographical location, and grazing × location interaction. In addition, PCA restricted to soil sampled at either upslope or downslope location was performed to focus on the effect of grazing. In this case, the Monte Carlo

TABLE 1. Effects of grazing regime, topographical location, and grazing × location interaction on enzyme activities and cell numbers of soil microbial communities.

| Source of variation | Carbon mineralization | Nitrification | Denitrification | Free $N_2$ fixation | No. heterotrophs | No. ammonia oxidizers | No. nitrite oxidizers | No. denitrifiers |
|---|---|---|---|---|---|---|---|---|
| Grazing regime | 0.016 | 0.0007 | 0.0007 | NS | 0.0003 | 0.0003 | <0.0001 | 0.001 |
| Topographical location | 0.0016 | 0.033 | 0.008 | NS | 0.0024 | 0.022 | 0.0012 | 0.0003 |
| Grazing × location | NS | NS | NS | NS | 0.041 | NS | 0.011 | NS |

*Notes:* Data are *P* values from the ANOVA. NS = not significantly different ($P > 0.05$).

test was carried out with 10 000 random permutations to test the significance of grazing effect on community composition. For each community, a stepwise, multiple-regression analysis was used to test the correlation between changes in activity and changes in key variables (i.e., individual PLFAs, A-RISA fragments, DGGE bands, or RFLP fragments) within the community composition matrix.

## RESULTS

### *Enzyme activities*

The effects of topographical location and grazing regime on enzyme activity were significant for C mineralization, nitrification, and denitrification, whereas the effect of grazing × location interaction was not significant (Table 1). For a given grazing regime, enzyme activities were generally higher at the downslope than upslope elevation locations. Soil C mineralization, nitrification, and denitrification were significantly higher at IG than LG sites, excepted at the downslope location for C mineralization (Fig. 1).

Nitrogenase activity ranged from 0.04 to 0.12 μg $N \cdot h^{-1} \cdot g^{-1}$ dry soil between sites and had a high spatial variance within a given site (Fig. 1). The effects of grazing regime, topographical location, and grazing × location interaction on free $N_2$ fixation were not significant (Table 1; grazing effect close to significant at $P = 0.065$).

### *Cell numbers*

The effects of topographical location and grazing regime on the numbers of heterotrophs, ammonia oxidizers, nitrite oxidizers, and denitrifiers were significant (Table 1). The effects of grazing × location interaction were not significant for the number of ammonia oxidizers and denitrifiers, but were significant for the number of heterotrophs and nitrite oxidizers (Table 1). Cell numbers of the four communities were significantly greater in IG than in LG sites, except for heterotrophs and ammonia oxidizers at the downslope location (Fig. 2).

Denitrification and nitrification enzyme activities were significantly and nonlinearly correlated ($P < 0.01$ and 0.001, respectively) to the number of denitrifiers and ammonia oxidizers, respectively (Fig. 3). Ammonia oxidizers were used here rather than nitrite oxidizers because ammonia oxidation is often reported to

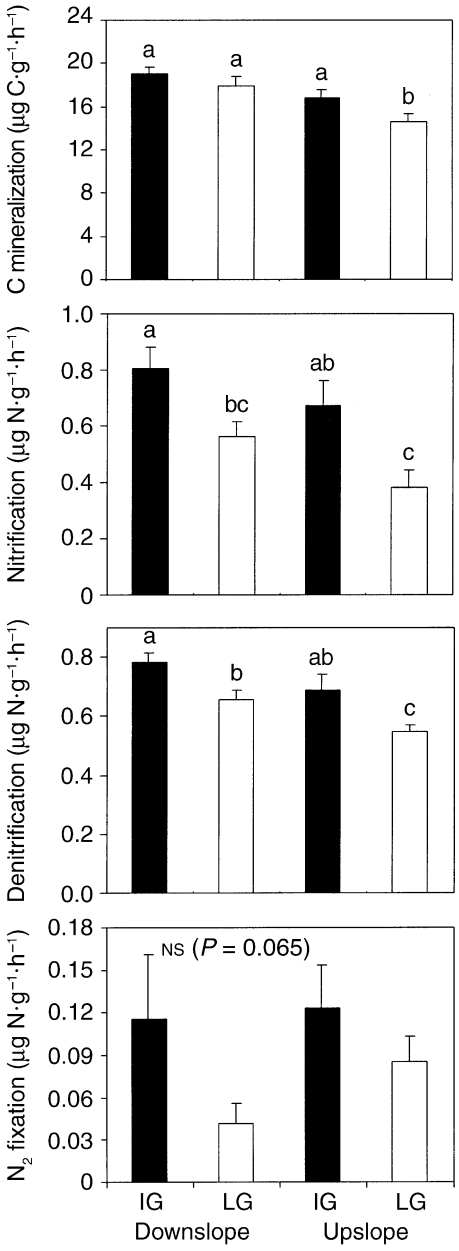


FIG. 1. C mineralization, nitrification, denitrification, and free $N_2$ fixation enzyme activities in soils under intensive grazing (IG) and light grazing (LG) at two topographical locations (upslope and downslope). Error bars represent 1 SE; $n = 9$. Values with different letters differ significantly at level $P \leq 0.05$. NS = not significantly different.

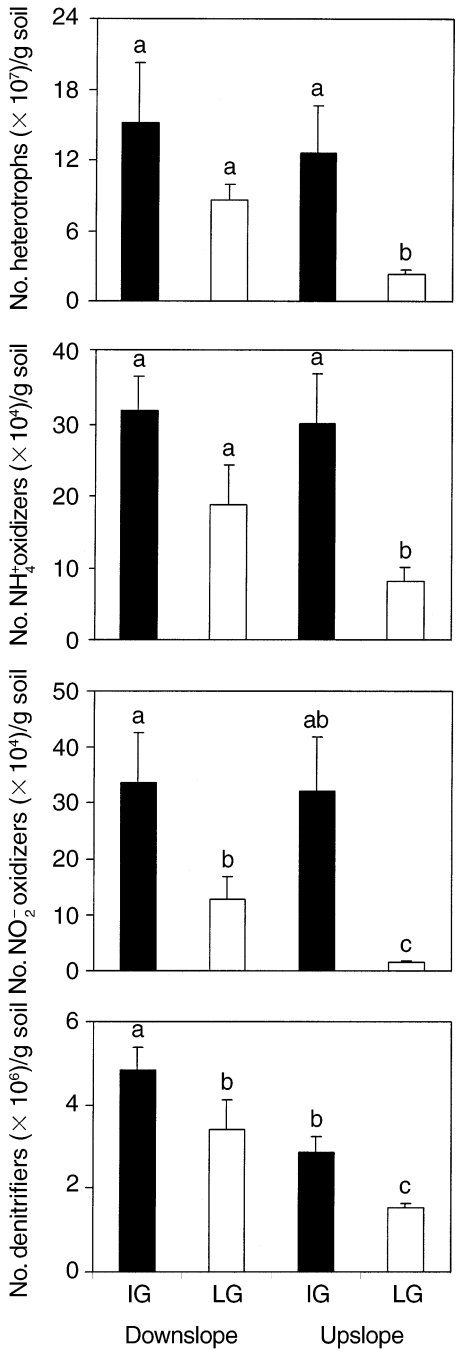


FIG. 2. Most-probable numbers of heterotrophs, ammonia oxidizers, nitrite oxidizers, and denitrifiers in soils under intensive grazing (IG) and light grazing (LG) at two topographical locations (upslope and downslope). Error bars represent 1 SE; $n = 9$. Values with different letters differ significantly at level $P \leq 0.05$.

be the limiting step of nitrification. The amplitude of changes in cell number between IG and LG sites was higher than that in enzyme activity. No significant correlation was found between C mineralization activity and number of heterotrophs (not shown).

*PLFA analysis of microbial community composition*

Concentrations of fungal PLFAs did not differ significantly with grazing intensity or topographical location (Table 2 and Fig. 4). Total bacterial PLFA was not significantly affected by grazing regime ($P = 0.064$), despite a tendency for the intensively grazed sites to contain greater concentrations of bacterial PLFA than the LG sites at both topographical locations (Fig. 4). Concentrations of PLFAs indicative of Gram-negative and Gram-positive bacteria were not significantly affected by grazing intensity (Table 2, data not shown). However, IG sites contained significantly greater actinomycete biomass than LG sites on both the upslope and downslope locations (Table 2 and Fig. 4). Concentrations of PLFAs indicative of total bacteria, Gram-negative bacteria, Gram-positive bacteria, and actinomycetes were all significantly greater in the downslope location (Table 2 and Fig. 4).

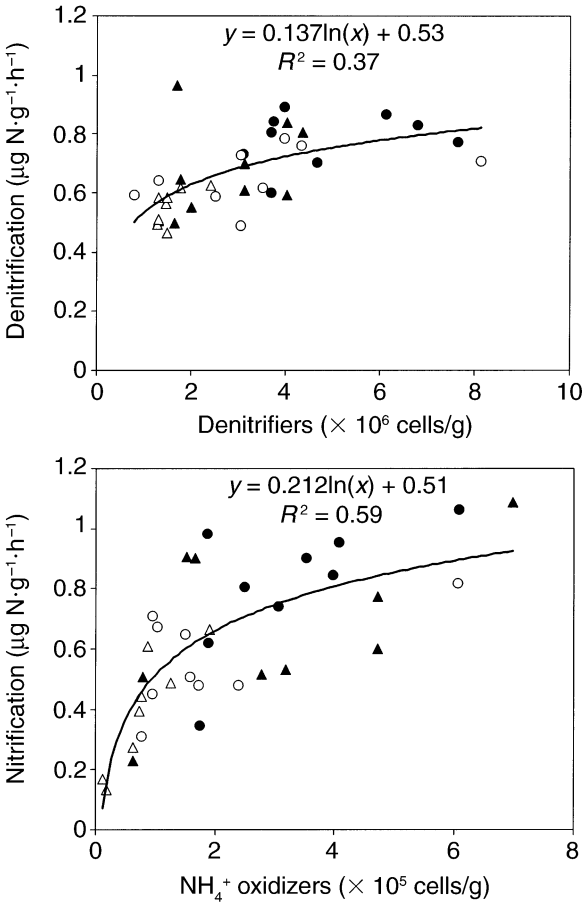


FIG. 3. Relationships between (top) denitrification enzyme activity and the number of denitrifiers and (bottom) nitrification enzyme activity and the number of ammonia-oxidizing bacteria in soils of the four sites studied (solid circles, intensive grazing downslope; open circles, light grazing downslope; solid triangles, intensive grazing upslope; open triangles, light grazing upslope).

TABLE 2. Effects of grazing regime, topographical location, and grazing × location interaction on the amounts of phospholipid fatty acids (PLFAs) of microbial taxonomic groups.

| Source of variation | Fungi | Bacteria | Gram negative bacteria | Gram positive bacteria | Actinomycetes |
|---|---|---|---|---|---|
| Grazing regime | NS | NS | NS | NS | <0.001 |
| Topographical location | NS | 0.003 | 0.008 | 0.005 | 0.017 |
| Grazing × location | NS | NS | NS | NS | NS |

*Notes:* Data are *P* values from the ANOVA. NS = not significantly different ($P > 0.05$).

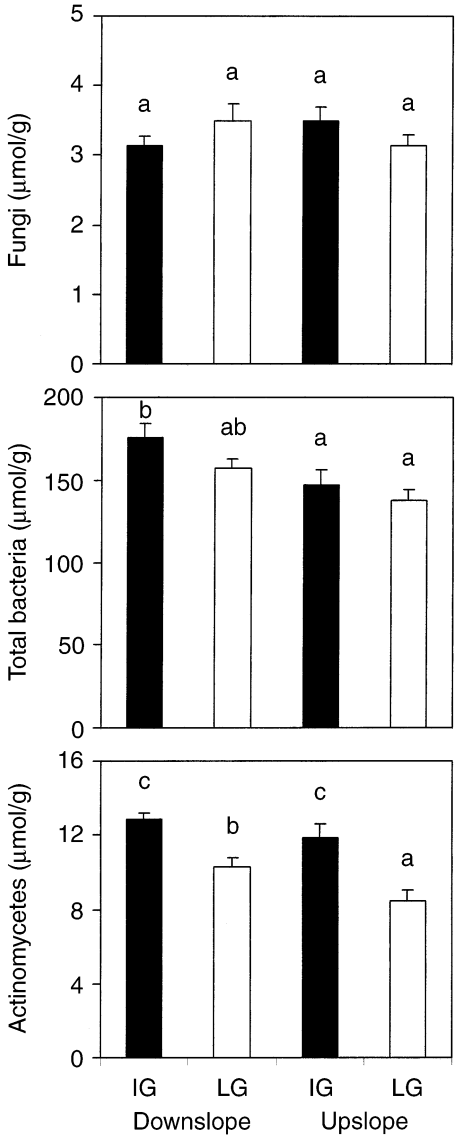


FIG. 4. PLFA (phospholipid fatty acid) amounts of (top) fungi, (middle) total bacteria, and (bottom) actinomycetes in soils under intensive grazing (IG) and light grazing (LG) at two topographical locations (upslope and downslope). Error bars represent 1 SE; $n = 9$. Values with different letters differ significantly at level $P \leq 0.05$.

Principal-component analysis of the PLFA data revealed that grazing intensity was the major factor influencing the composition of the total microbial community (discrimination by grazing mainly on the first axis; Table 3, figure not shown). Only results of principal-component analysis restricted to either upslope or downslope location are presented in Fig. 5 to focus on the effect of grazing regime, i.e., the main factor studied. At both upslope and downslope locations, the composition of the microbial community differed significantly between the intensive and light grazing sites (Fig. 5; $P = 0.0002$ and 0.0001 at downslope and upslope locations, respectively). Higher concentrations of seven PLFAs (in particular the Gram-negative fatty acids 17:0cy and cy 19:0, and the actinomycete phospholipid 10Me18:0) were observed in IG as compared to LG sites at both upslope and downslope locations, whereas higher concentrations of four PLFAs (in particular the Gram-negative fatty acids 16:1ω7c and 18:1ω7) were observed at LG than at IG sites at both locations.

### *Genetic structures of the total and functional bacterial communities*

Grazing intensity, and, to a lesser extent, topographical location, significantly influenced the composition of the total bacterial, ammonia oxidizing, and free $N_2$ fixing communities (Table 3, figures not shown). In contrast, principal-component analysis of genetic fingerprints revealed that the composition of the nitrate reducing community (for images of genetic fingerprints, see Appendix B) was mainly influenced by topographical location (Table 3, figure not shown, but see Appendix E). Only results of principal-component analysis restricted to either upslope or downslope location and for total bacterial, ammonia oxidizing and free $N_2$ fixing communities are presented to focus on the effect of grazing.

The total bacterial communities significantly differed between the intensive and light grazing sites at both locations (Fig. 6; $P = 0.0002$ and $<0.0001$ at downslope and upslope locations, respectively). Thirteen and 15 bands mainly discriminated IG vs. LG bacterial communities at the downslope and upslope locations, respectively. Among these bands, five were typical of IG communities and five were typical of LG

TABLE 3. Effects of grazing regime, topographical location, and grazing × location interaction on the structure of total microbial (PLFA data), total bacterial (RISA data), and functional bacterial (RFLP and DGGE data) communities.

| Source of variation | Total microbial | Total bacterial | Ammonia oxidizers | Nitrate reducers | Free $N_2$ fixers |
|---|---|---|---|---|---|
| Grazing regime | <0.0001 | <0.0001 | 0.0008 | NS | 0.0001 |
| Topographical location | NS | 0.0001 | NS | <0.0001 | NS |
| Grazing × location | NS | NS | NS | NS | NS |

*Notes:* Data are *P* values from the ANOVA. NS = not significantly different ($P > 0.05$). Abbreviations are: PLFA, phospholipid fatty acid; RISA, rRNA intergenic spacer analysis; RFLP, restriction fragment length polymorphism; DGGE, denaturing gradient gel electrophoresis.

communities (i.e., discriminated IG vs. LG sites at both downslope and upslope locations).

The composition of the ammonia oxidizing community (for images of genetic fingerprints see Appendix C) significantly differed between the intensive and light grazing sites (Fig. 7; $P < 0.0001$ and 0.018 at downslope and upslope locations, respectively). Four DNA bands were characteristic of the LG treatment at both downslope and upslope locations, whereas two other bands were characteristic of the IG treatment at both downslope and upslope locations.

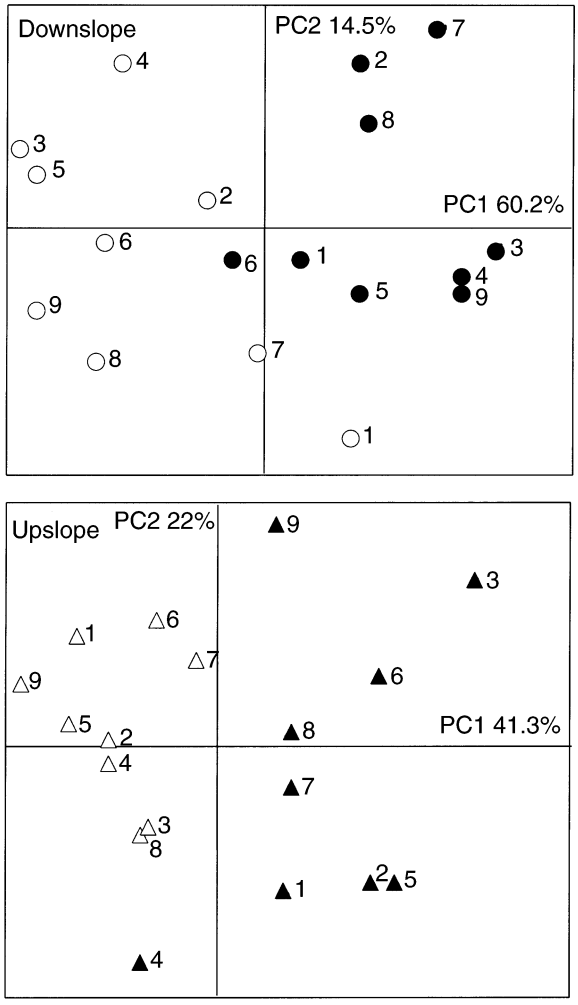


FIG. 5. Principal-components plots generated from the phospholipid fatty acid (PLFA) profiles of the microbial communities in soils under intensive grazing (IG) and light grazing (LG) at two topographical locations (upslope and downslope). Symbols are as in Fig. 3. Permutation tests indicate that the grazing effect is significant both at downslope ($P = 0.0002$) and upslope ($P = 0.0001$) locations.

Genetic fingerprints of the $N_2$ fixing communities are presented in Appendix D. Principal-component analysis discriminated the communities between the intensive and light grazing sites (Fig. 8; $P = 0.0006$ and 0.0015 at downslope and upslope locations, respectively). The same six restriction fragments mainly discriminated IG vs. LG sites at both downslope and upslope locations (four fragments and two fragments characterizing IG and LG sites, respectively [Appendix E]).

The activity of each microbial community was correlated to its composition (Fig. 9), and 80% of the variance observed in activity of the total heterotrophic community was related to changes in the levels of five individual PLFAs or five RISA fragments. Similarly, 80% of the variance observed in enzyme activity of the nitrifying, denitrifying, and $N_2$ fixing communities was related to changes in the levels of seven AOB (ammonia oxidizing bacteria) DGGE bands, eight *narG* RFLP fragments, and nine *nifH* RFLP fragments, respectively (Fig. 9). These correlations show that grazing had concurrent, consistent effects on community activity and structure. However, it is impossible to separate changes in community composition that actually contribute to changes in activity from those that only reflect response to environmental change paralleling changes in activity. In addition, genetic fingerprinting revealed no clear changes in community complexity (i.e., number of bands/fragments detected) with grazing intensity, except for ammonia oxidizers. In this case, a negative correlation was observed between nitrification rate and the number of bands identified from DGGE profiles (Fig. 10). This indicates that samples exhibiting the highest activity rates also exhibited the lowest community complexity.

## DISCUSSION

High spatial variations in enzyme activity (Parkin et al. 1987), cell number, and community structure (Ca-

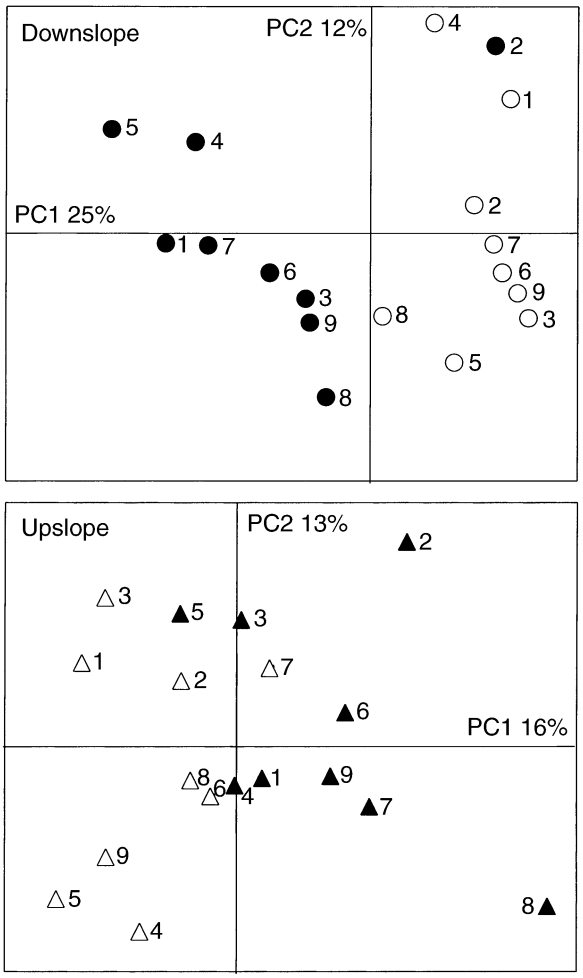


FIG. 6. Principal-components plots generated from ARISA (automated rRNA intergenic spacer analysis) profiles of the total bacterial communities of soils under intensive grazing (IG) and light grazing (LG) at two topographical locations (upslope and downslope). Symbols are as in Fig. 3. Permutation tests indicate that the grazing effect is significant both at downslope ($P = 0.0002$) and upslope ($P < 0.0001$) locations.

vigelli et al. 1995) of microbial communities have often been reported in field studies (but see Felske and Akkermans 1998). Clegg et al. (2000) and McCaig et al. (2001) reported that the variation in the total microbial community structure between replicate soil samples could be as great as the variation between treatments in upland grasslands, which could obscure any treatment effects. However, the variation in enzyme activity, cell number, and community structure between replicates within a given plot was generally much less than the variation between plots in our study. This allowed us to (1) identify highly significant differences in the enzyme activity, size, and composition of total soil microbial community and three functional groups involved in N cycling between IG and LG sites; and (2) test if activity changes were independent of, or correlated with changes in community size and/or composition.

### *Grazing induces changes in activities of soil microbial communities*

Potential C mineralization was slightly higher on IG than LG plots, particularly in the downslope location. Although grazing effects on C mineralization and microbial biomass are idiosyncratic (Stark and Grellmann 2002, Bardgett and Wardle 2003), Frank and Groffman (1998*b*) showed that herbivores slightly enhanced po-

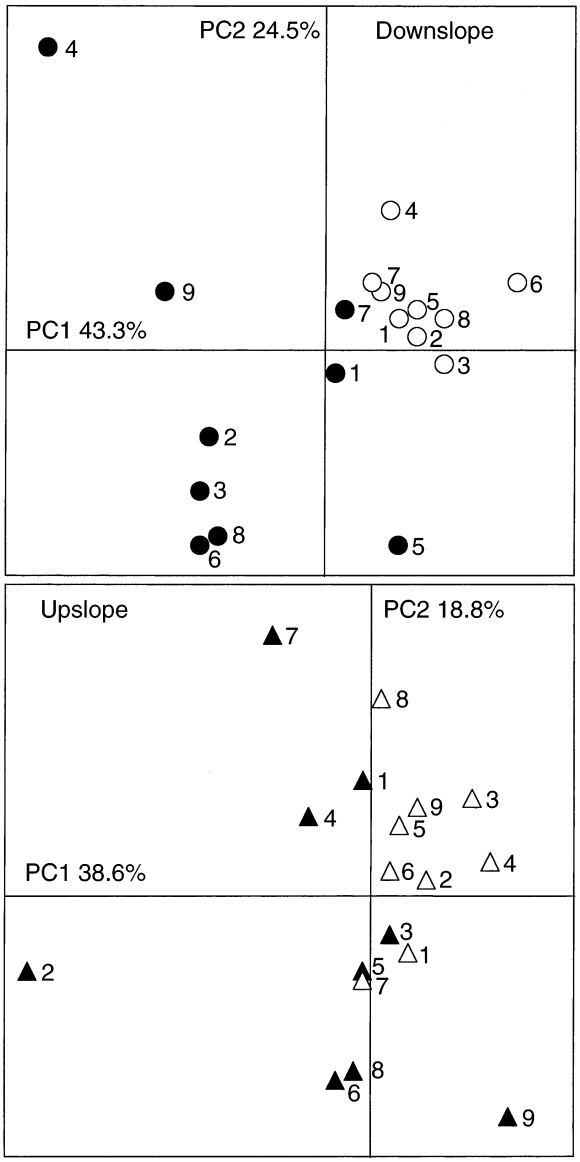


FIG. 7. Principal-components plots for the ammonia oxidizer communities of soils under intensive grazing (IG) and light grazing (LG) at two topographical locations (upslope and downslope). Symbols are as in Fig. 3. Permutation tests indicate that the grazing effect is significant both at downslope ($P < 0.0001$) and upslope ($P = 0.018$) locations.

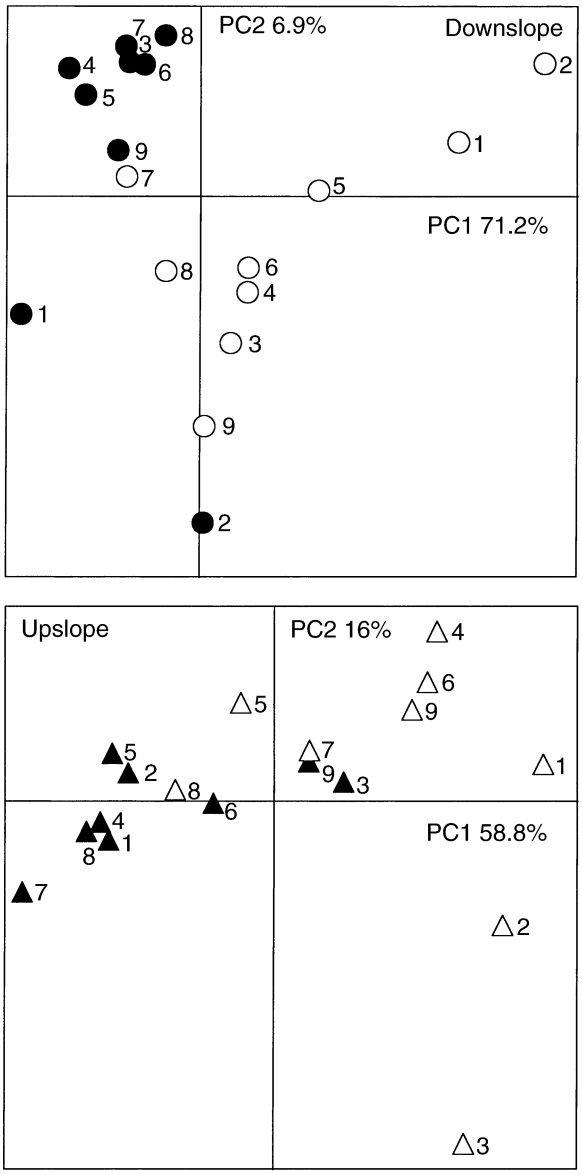


FIG. 8. Principal-components plots for the free $N_2$ fixer communities of soils under intensive grazing (IG) and light grazing (LG) at two topographical locations (upslope and downslope). Symbols are as in Fig. 3. Permutation tests indicate that the grazing effect is significant both at downslope ($P = 0.0006$) and upslope ($P = 0.0015$) locations.

tential C mineralization in Yellowstone grasslands, and Bardgett et al. (1997) found that C mineralization was positively related to sheep grazing intensity in a temperate grassland ecosystem. Altered quantity and quality of the belowground organic matter by grazers may explain the higher potential C mineralization rate in intensively grazed systems (Frank and Groffman 1998*b*). Indeed, grazing could increase root/shoot allocation, root exudation, possibly primary production, and thus the availability of labile organic substrates

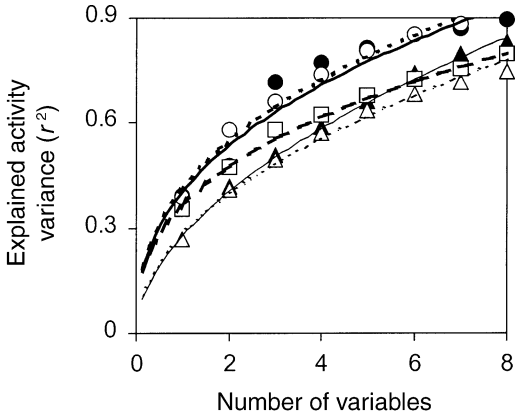


FIG. 9. Correlation between the activity levels and structures of microbial communities estimated from stepwise multiple regression analyses (model form: activity = $a_0 + a_1X_1 + a_2X_2 + \ldots + a_nX_n$). For each community, the explained variance in activity ($r^2$ of the model) is plotted against the number, $n$, of variables used (solid circles and bold line, C mineralization correlated to the occurrence of individual PLFAs; open circles and dashed line, C mineralization correlated to the occurrence of individual RISA fragments; solid triangles and thin line, nitrification correlated to the occurrence of individual AOB DGGE [ammonia-oxidizing bacteria; denaturing gradient gel electrophoresis] bands; open triangles and thin dashed line, $N_2$ fixation correlated to the occurrence of individual *nifH* RFLP [restriction fragment length polymorphism] fragments; open squares and long-dash line, denitrification correlated to the occurrence of individual *narG* RFLP bands). Lines correspond to fitted power functions.

belowground (Bardgett et al. 1998, Frank and Groffman 1998*b*, Bardgett and Wardle 2003).

Denitrification enzyme activity was significantly greater in IG than in LG plots. This is consistent with previous results showing that grazing increases denitrification in Yellowstone grasslands (Frank and Groffman 1998*a*, Frank et al. 2000), grazed ecosystems of China (Du and Wang 2001), and humid African sa-

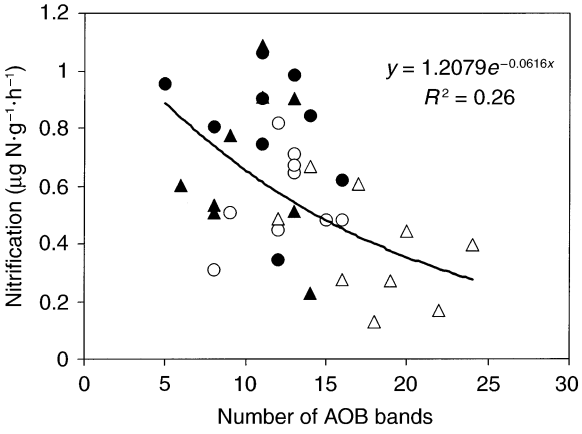


FIG. 10. Relationship between nitrification enzyme activity and the number of bands detected from genetic fingerprinting of the ammonia-oxidizing community (symbols are as in Fig. 3).

vannas (Le Roux et al., *in press*). However, no effect was observed in grass–clover swards (Parsons et al. 1991) or a tallgrass prairie (Groffman et al. 1993). In particular, measurements made during another field campaign in spring (May 2001) also showed higher denitrification and nitrification enzyme activities in IG than in LG sites (Le Roux et al. 2003). Thus, although DEA and NEA can vary substantially with time (Groffman and Tiedje 1989) and grazing-induced changes in DEA and NEA are not necessarily observed in all seasons (Luo et al. 1999), the results obtained in May indicate that the stimulation reported here remains during most of the annual cycle. Because denitrifiers are heterotrophs, stimulation of denitrification may be due, as for C mineralization, to increased labile C availability. Other factors, such as altered soil structure (Du and Wang 2001), altered soil moisture regime (Naeth and Chanasyk 1995), or input of easily available nitrogen forms in urine and dung could also stimulate denitrification. In the present study, denitrification activity was proportional to C mineralization ($C_{min}$; for all sites: denitrification = 0.021 $C_{min}$, $P = 0.0001$, $r^2 = 0.67$, $n = 36$), suggesting that the same factor, i.e., labile C availability, could regulate those activities. This could also explain the trend toward higher fixation rates in IG than in LG sites, because C availability, along with mineral N availability, is a major factor controlling free $N_2$ fixation.

Nitrification enzyme activity was much higher on IG than LG plots. Enhanced nitrification in response to grazing has been reported in tall grass prairies (Groffman et al. 1993), Yellowstone grasslands (Frank et al. 2000), seminatural grasslands (Le Roux et al. 2003), shortgrass and tallgrass prairies of the Serengeti (Seagle and McNaughton 1993), and grasslands of South Dakota (Holland et al. 1992). A linear relationship between potential denitrification and potential nitrification with a high intercept was observed in our study (for all sites: denitrification = 0.46 nitrification + 0.40, $P = 0.0001$, $r^2 = 0.43$, $n = 36$). The nitrification-to-denitrification ratio was thus affected by grazing (from 0.69 ± 0.10 and 0.87 ± 0.09 in LG upslope and LG downslope, to 0.99 ± 0.12 and 1.02 ± 0.07 in IG upslope and IG downslope [mean ± 1 SE], respectively), as already observed by Le Roux et al. (2003). This suggests that environmental factors explaining the increase in nitrification differed from those explaining the increase in denitrification (e.g., effect of grazer wastes that are rich in labile forms of N).

### *Grazing induces changes in numbers of soil microorganisms*

The numbers of heterotrophs, denitrifiers, ammonia oxidizers, and nitrite oxidizers were higher in IG than in LG plots. This is consistent with previous studies showing that microbial cell number and/or biomass are higher in frequently disturbed than in weakly disturbed plots. For instance, Bardgett et al. (1997) showed that cessation of grazing resulted in a significant drop in soil microbial biomass in a temperate grassland ecosystem. Similarly, MPN estimates of ammonia oxidizers are generally lower in undisturbed than frequently disturbed, e.g., tilled or N-fertilized, soils (Bruns et al. 1999). Grazer-induced changes in availability of nutrients and labile organic substrates can explain such changes in number/biomass of microorganisms (Bardgett and Wardle 2003). To our knowledge, information on the effect of grazing and/or N fertilization on the size of the $N_2$ fixing community is very scarce.

In our study, denitrification and nitrification enzyme activities were strongly correlated with the numbers of denitrifiers and ammonia oxidizers, respectively. In contrast, Mendum et al. (1999) and Phillips et al. (2000) found no correlation between nitrification rates and numbers of ammonia oxidizers in several agricultural soils. The amplitude of changes in cell numbers between IG and LG plots was higher than changes in activities. Although MPN values only refer to cultivated microorganisms and underestimate the actual bacterial numbers when compared to molecular methods (Degrange and Bardin 1995, Phillips et al. 2000), they still provide useful information on the effects of disturbances on numbers of microorganisms belonging to a functional community. Our data suggest that changes in microbial cell numbers can play a major role in explaining the effect of grazing on N-related soil processes.

### *Grazing induces changes in microbial community composition and genetic structure*

*Total microbial and total bacterial communities*.— The effect of grazing on the total soil microbial (bacteria plus fungi) or total bacterial communities has been quantified in some studies, but in situations where confounding factors such as inorganic N fertilization or soil type obscured the grazing effect. The removal of grazing while also withholding fertilizer and lime additions affected the soil fungal:bacterial biomass ratio assessed by PLFA analysis in an upland grassland (Bardgett et al. 1996). Clegg et al. (1998, 2000) showed that management (mixed effect of grazing and fertilizer input) significantly modified the total soil microbial community at an upland grassland site in the United Kingdom. Similarly, McCaig et al. (1999) and Grayston et al. (2001) found significant changes in the structure of the total microbial and total bacterial communities between unimproved (lightly grazed, not fertilized) and improved (intensively grazed, fertilized) upland grasslands. However, inorganic N fertilization per se affects the community structure for total soil microorganisms, eubacteria, and actinomycetes in grasslands (Clegg et al. 2003). To our knowledge, this is the first comparison of the composition of soil microbial communities between sites differing by grazing regime per se (i.e., without concurrent changes in N fertilization other than

urine and feces inputs by grazers, and for the same background soil characteristics).

The abundance of large taxonomic microbial groups such as fungi, Gram-positive bacteria and Gram-negative bacteria did not differ between IG and LG sites. In contrast, clear differences were detected when focusing on actinomycetes or when using PLFA or RISA fingerprints as indices of community structure. The changes in the composition of soil microbial/bacterial community observed by PLFAs or genetic fingerprints show a profound effect on the composition of the soil microbiota. Furthermore, differences between IG and LG sites were qualitatively very similar at the upslope and downslope locations (same PLFAs or A-RISA bands discriminating IG vs. LG sites). Our results thus show that grazing has major, consistent effects on soil microbial communities in the grasslands studied, and that the observed changes in the bacterial functional groups involved in soil N dynamics occurred in a background of change in the dominant soil bacterial populations.

*Bacterial functional groups involved in soil N dynamics*.—The composition of the nitrate-reducing community was only marginally different between IG and LG sites, whereas clearer differences were observed between the two topographical locations. In contrast, the composition of the two other functional groups studied, i.e., nitrifiers and $N_2$ fixers, was mainly determined by the grazing regime. To our knowledge, the effect of grazing on the composition of the $N_2$ fixing community has not been previously tested and the main factors influencing *nif*H gene diversity still remain to be identified (Zehr et al. 2003). Only a few studies have analyzed the effect of management on ammonia-oxidizer communities in grazed grasslands. For instance, Webster et al. (2002) showed that the composition of the ammonia-oxidizing community differed between improved (intensively grazed and fertilized) and unimproved (lightly grazed and not fertilized) grassland pastures but grazing effects were obscured by confounding factors such as N fertilization and soil characteristics. Inorganic N fertilization strongly affects the ammonia oxidizer community structure in grasslands (Bruns et al. 1999, Phillips et al. 2000, Clegg et al. 2003). We show here that grazing per se has a profound effect on the genetic structure of the ammonia oxidizing community at the sites studied. In particular, dominant sequences differed between IG and LG plots. Furthermore, IG soils exhibited a reduced complexity (assessed from DGGE bands) as compared to LG soils. This is consistent with the findings of Webster et al. (2002) who showed that the complexity of ammonia oxidizer populations was greater in unimproved than managed soils. This could be explained by selection, under intensive grazing, of only a few strains that may be favored by the high, grazer-induced N recycling and labile N availability. For example, ammonia oxidizers are either sensitive to or tolerant to high concentrations of ammonia, potentially leading to selection of the latter group in soils with persistent high ammonia supply, increasing their relative abundance and decreasing the community complexity. In addition, changes in nitrification enzyme activity were correlated with changes in the occurrence of a few DGGE bands, although it is impossible to separate changes in community composition that contribute to changes in activity from those that only reflect response to environmental change paralleling changes in activity. However, our results suggest that changes in nitrification enzyme activity could be partly due to changes in the composition of the ammonia-oxidizing community. Such concurrent changes in the activity and genetic structure of the ammonia-oxidizing community have been reported in response to application of swine manure to soils (Ceccherini et al. 1998). In contrast, Avrahami et al. (2002) observed increased nitrification activity after ammonium addition to soil without any changes in the community structure of ammonium oxidizers. Identification and characterization of the physiological traits of dominant nitrifying populations under intensive versus light grazing is thus needed for better understanding of the effect of grazers on soil N dynamics.

## *Conclusion*

Our results show that rough indicators, such as potential carbon mineralization rate or total amount of fungal and bacterial PLFAs, sometimes used as surrogates for microbial biomass, can be insensitive or weakly sensitive to disturbance regimes and environmental conditions, whereas other characteristics of microbial communities are more sensitive. The use of a combination of traditional and molecular methods investigating changes in the activity, size, and composition/genetic structure of key microbial functional groups is a powerful approach to understanding the effect of disturbances on soil microbiota in natural and managed ecosystems, although few studies have achieved such a combination so far (Phillips et al. 2000, Torsvik and Övreas 2003). In this study, grazing was found to (1) promote N-related soil microbial enzyme activities, as already observed for other N-rich grassland ecosystems, but also (2) increase strongly the size of the corresponding microbial functional groups, and (3) modify significantly the composition of these functional groups. Our results thus show for the first time that enhancement of microbially driven N processes by grazing per se can partly be explained by increased cell numbers, and that changes in community composition can also have a major role. In particular, a reduction of the complexity of the ammonia oxidizing community was observed in IG sites as compared to LG sites. The observed changes in the size and composition of microbial functional groups demonstrate that grazing deeply affects these groups far beyond purely physiological aspects at the grassland sites studied. Although

the generality of our conclusions should be tested in other systems, they are important for predicting the effects of changed grazing regimes on grassland ecosystem functioning and grassland response to future disturbances. Indeed, purely physiological effects are likely to be more reversible than changes in community composition in the context of intensification/deintensification of grassland management, and changes in the composition of the soil microbiota can in turn modify the response of the soil system to further disturbances (Griffiths et al. 2000).

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## APPENDIX A

A photograph showing a view of the grassland sites upslope is provided in ESA's Electronic Data Archive: *Ecological Archives* M075-002-A1.

## APPENDIX B

A photograph of PCR-RFLP analysis of *narG* sequences from the intensive grazing (IG) and light grazing (LG) grassland sites at two topographical locations (upslope and downslope) with restriction enzyme *Alu*I is presented in ESA's Electronic Data Archive: *Ecological Archives* M075-002-A2.

## APPENDIX C

A photograph of DGGE analysis of 16S rRNA gene sequences of ammonia oxidizers from the intensive grazing (IG) and light grazing (LG) grassland sites at two topographical locations (upslope and downslope) is presented in ESA's Electronic Data Archive: *Ecological Archives* M075-002-A3.

## APPENDIX D

A photograph of PCR-RFLP analysis of *nifH* sequences from the intensive grazing (IG) and light grazing (LG) grassland sites at two topographical locations (upslope and downslope) with restriction enzymes *Nde*II and *Mnl*I is presented in ESA's Electronic Data Archive: *Ecological Archives* M075-002-A4.

## APPENDIX E

A table of size (in bp) of bands specific of either IG or LG treatments (i.e., bands discriminating IG vs. LG samples in PCA) for the nitrate-reducing and free $N_2$ fixing communities is presented in ESA's Electronic Data Archive: *Ecological Archives* M075-002-A5.