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Brief report

Differential response of archaeal and bacterial communities to nitrogen inputs and pH changes in upland pasture rhizosphere soil

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Summary

Grassland management regimens influence the structure of archaeal communities in upland pasture soils, which appear to be dominated by as yet uncultivated non-thermophilic *Crenarchaeota*. In an attempt to determine which grassland management factors select for particular crenarchaeal community structures, soil microcosm experiments were performed examining the effect of increased pH, application of inorganic fertilizer (ammonium nitrate) and sheep urine deposition on both archaeal and bacterial communities in unmanaged grassland soil. As grassland management typically increases pH, a further experiment examined the effect of a reduction in pH, to that typical of unimproved grassland soils, on archaeal and bacterial communities. The RT-PCR amplification of 16S rRNA followed by denaturing gradient gel electrophoresis analysis demonstrated a distinct and reproducible effect on bacterial communities after incubation for 28 or 30 days. In contrast, none of the treatments had a significant effect on the structure of the crenarchaeal community, indicating that these factors are not major drivers of crenarchaeal community structures in grassland soils.

Introduction

Archaea are prokaryotic organisms that are evolutionarily distinct from *Bacteria* and represent one of three primary domains of life (Woese *et al*., 1990). Cultivated representatives are typically isolated from environments extreme in temperature, salinity or anoxia. However, over the last decade, culture-independent molecular analyses have revealed uncultivated lineages of *Archaea* to be distributed ubiquitously in 'non-extreme' terrestrial and aquatic environments (DeLong and Pace, 2001). The largely non-thermophilic 'Group 1' lineage, which is associated with cultivated hyperthermophilic *Crenarchaeota*, appears to be the most ecologically diverse (DeLong, 1998). In upland pasture rhizosphere soil, archaeal communities are dominated by two distinct lineages of Group 1 *Crenarchaeota (*1.1b and 1.1c) (Nicol *et al*., 2003a). The substantial evolutionary distance between these two groups may be reflected in associated differences in physiology and ecosystem function. Nicol *et al*. (2003a) demonstrated that unmanaged ('unimproved') upland pastures, which receive nitrogen inputs largely from grazing sheep, have a different 1.1b crenarchaeal community structure to that of managed ('improved') pastures, which have additional inputs of inorganic nitrogen fertilizer, a different plant community composition, increased sheep grazing (and associated nitrogen deposition) and higher soil pH. However, lack of cultured representatives has prevented physiological characterisation of these organisms. In the soil environment, pH changes and nitrogen amendments with synthetic sheep urine or NH_4NO_3 have been shown to influence bacterial community structure (Williams *et al*., 2000; Peacock *et al*., 2001; Bååth and Anderson, 2003). However, the effect of these factors, the major factors imposed by grassland management, on soil archaeal communities dominated by crenarchaea has not been assessed.

The aim of this study was to examine whether differences in nitrogen inputs and pH were responsible for producing non-thermophilic *Crenarchaeota* community structures typical of improved and unimproved rhizosphere soil by amending unimproved soil with synthetic sheep urine (associated with increased levels of grazing), NH_4NO_3 and $Ca(OH)_2$ to increase soil pH, and amending improved soil with $Al_2(SO_4)_3$ to decrease soil pH to that typical of unimproved soil. Changes in archaeal and bac-

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terial community structures were characterized by RT-PCR amplification of 16S rRNA, using *Archaea*- and *Bacteria*-specific primers, respectively, from extracted 16S rRNA, which were then analysed by denaturing gradient gel electrophoresis (DGGE).

Results and discussion

Influence of synthetic sheep urine amendment of unimproved soil

Soil was sampled from unimproved grassland plots [National Vegetation Classification U4a (Rodwell, 1992)] at Fasset Hill, Sourhope Research Station, Borders region, Scotland (map reference NT 850 205) as previously described (Nicol *et al*., 2003a). Microcosms consisted of sterile glass Universal bottles (with loose-fitting caps to allow air exchange) containing 10 g of homogenized soil and were incubated for 28 days at 14°C in the dark. Microcosms were amended with 0.5 ml of synthetic sheep urine (2 × concentration) (Williams *et al*., 1999) to give a final nitrogen content of 500 kg ha^{-1}, equivalent to that following one average sheep urination (Haynes and Williams, 1993). Rapid hydrolysis of urea to ammonia in soil by urease activity (Sherlock and Goh, 1984) increases soil pH and conversion was complete within 10 h of incubation. Therefore, to determine whether any changes resulted from an increase in soil pH alone, a set of microcosms were amended with 0.05 g $Ca(OH)_2$ which increased soil pH to approximately 7.2, the value observed 24 h after addition of synthetic urine addition (and subsequent urea hydrolysis). All control microcosms were amended with 0.5 ml sterile dH_2O. Microcosms were sampled immediately (0 days) and at 28 days. All microcosm treatments were prepared in triplicate and destructively sampled with 5 g of soil stored at −20°C for subsequent molecular analysis, and 5 g was used immediately for soil characterization, including pH as described by Webster *et al*. (2002). Moisture content was maintained during incubation by weighing microcosms weekly and adding sterile dH_2O onto the soil surface to replace water lost through evaporation. Nucleic acid extraction, RT-PCR amplification and DGGE analysis were performed as described previously (Nicol *et al*., 2003b), except that the bacterial 16S rRNA was targeted using primer P2 to select for bacterial 16S rRNA, and primers P3/P2 (Muyzer *et al*., 1993) were used to amplify cDNA.

Denaturing gradient gel electrophoresis profiles of archaeal and bacterial communities in triplicate microcosms after incubation for 0 and 28 days are shown in Fig. 1. In the DGGE profiles of the archaeal communities (Fig. 1A), a marker consisting of DGGE fragments of selected clones was run alongside the environmental samples to indicate the lineage to which a particular band belonged and whether bands, previously identified as characteristic of improved and unimproved archaeal communities, were present (Nicol *et al*., 2003a). Primers SAf/PARCH519r used to generate DNA fragments contained degeneracies necessary to provide broad coverage of archaeal sequences and sometimes resulted in multiple bands (e.g. SUPA7) in DGGE analyses. The most intense band co-migrated with the marker SUPA5, which represents a crenarchaeal group widely distributed throughout UK upland pasture (Nicol *et al*., 2003a). Generally, in all three sets of microcosms incubated for 28 days, the relative intensity of bands decreased at positions at the lower half of the gel, positions occupied by 1.1c marker bands. Minor bands (in terms of percentage of total band volume in each lane) decreased or disappeared in all three sets of microcosm incubated for 28 days compared with day 0 control microcosms. However, no difference could be detected in these minor bands between control (water only), pH-control or urine-amended microcosms at 28 days. There was therefore no detectable effect of pH or urine amendment on archaeal communities.

In contrast, DGGE profiles of amplified bacterial 16S rRNA (Fig. 1B) showed reproducible differences following urine amendment and pH adjustment. DGGE profiles of control (water only) microcosms did not change during incubation but the relative intensity of some bands differed significantly in the synthetic urine profiles only (labelled U), in pH-adjusted microcosms only (examples labelled P), or in both pH-adjusted and urine-amended profiles (examples labelled B). Although this indicates that urine deposition itself influenced the bacterial community, it should be noted that there were small differences in the mean pH between both pH and urine microcosms (7.4 and 6.9 respectively). The possibility that this small difference in pH alone resulted in differences between these two sets of microcosms cannot be ruled out. Three DGGE bands representing bacterial strains which exhibit a potentially positive response to urine and/or an increase in pH were excised, reamplified and sequenced, and the closest match in the GenBank database determined by BLASTN searches (Table 1). Interestingly, band bU-IP1 which appeared relatively more intense in those microcosms amended with synthetic urine exhibited 100% identity over 117 nucleotides to sequences placed within the *Rhizobiales*, including those of known nitrite oxidizers.

Addition of ammonium nitrate in unimproved soil

Microcosms containing 10 g of homogenized soil were set up to examine community structure in unimproved soil after NH_4NO_3 additions at a soil pH typical of both unimproved (~ 4.5) and improved grassland soils (~ 6.5). The addition of 0.5 ml of 71 mM NH_4NO_3 solution to a 10 g soil

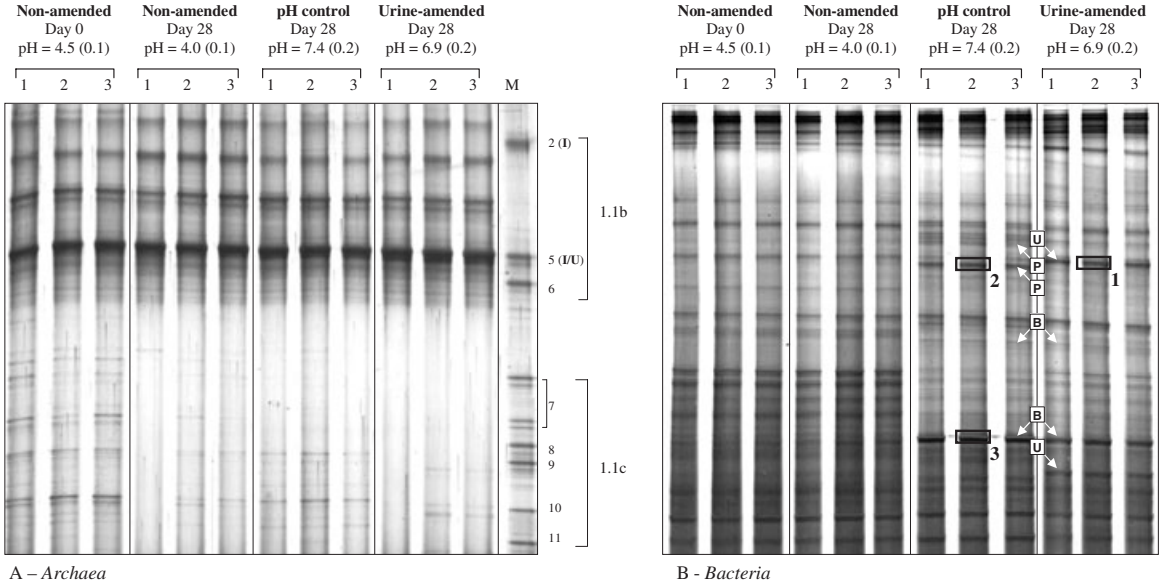


Fig. 1. DGGE profiles of archaeal and bacterial 16S rRNA RT-PCR products amplified from unimproved soil amended with synthetic urine and pH-adjusted after incubation for 28 days. Each lane represents an individual microcosm with archaeal and bacterial profiles derived from the same 16S rRNA extracts. pH values are the mean (and standard error) of the triplicate microcosms profiled.
A. *Archaea*. PCR products were obtained from archaeal cDNA using a nested PCR approach using primers Ar3F/Ar9R (Giovannoni *et al*., 1988; Jurgens *et al*., 1997) followed by SAf/PARCH519r (Øvreås *et al*., 1997; Nicol *et al*., 2003a) using conditions previously described (Nicol *et al*., 2003a). A marker lane composed of SAf/PARCH519r PCR products from cloned grassland archaeal 16S rRNA gene sequences SUPA2, 5, 6, 7, 8, 9, 10 and 11 (accession numbers AF512958, and AF512961 to AF512967) was run alongside the archaeal DGGE gel. The short 117 bp DGGE fragment (excluding primers) of SUPA2 represents 1.1b crenarchaeal strains which appear to be selected for in improved soil, and that of SUPA5 represents those 1.1b strains which have been shown to be present in both improved and unimproved grassland soil.
B. *Bacteria*. PCR products were obtained from bacterial cDNA using primers P3/P2 (Muyzer *et al*., 1993) using the same cycling conditions as for Ar3F/Ar9R. Bands highlighted are those that reproducibly appear more intense than in control profiles in microcosms that have been pH-adjusted only (P), synthetic urine-amended only (U) or both pH-adjusted and urine-amended (B). Bands of interest that were excised, reamplified and sequenced are highlighted with numbered boxes and prefixed with bU-IP (urine or increased pH) in the text for further discussion.

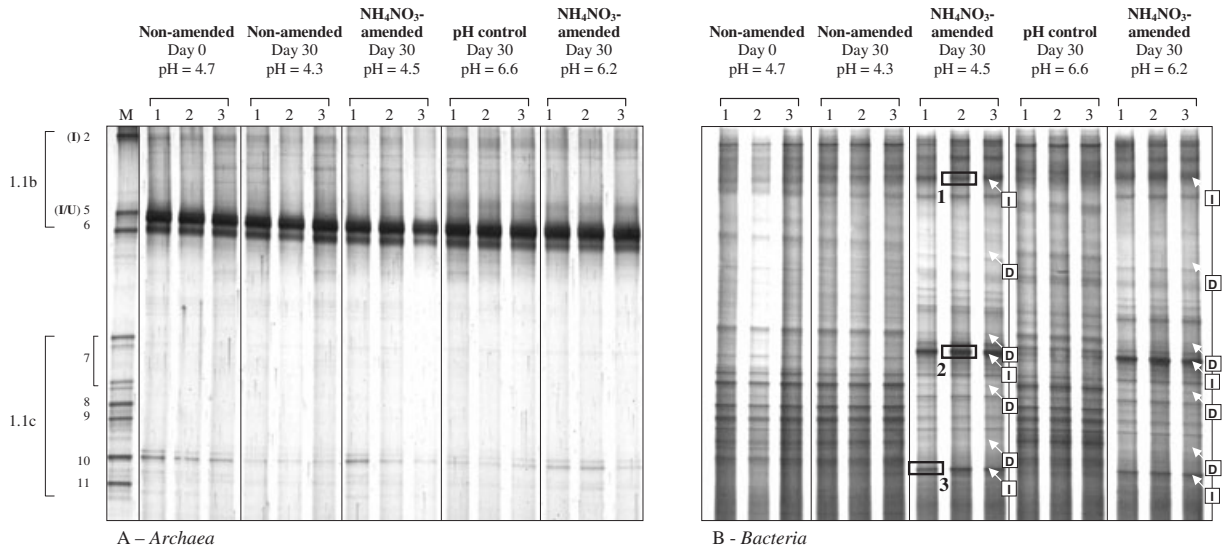
microcosm was equivalent to one application of 50 kg N ha^{-1} NH_4NO_3 fertilizer, assuming addition was to a depth of 5 cm and a soil density of 1 g cm^{-3}. To negate a decrease in soil pH due to the addition of NH_4NO_3, 0.01 g and 0.04 g $Ca(OH)_2$ were added to maintain pH values typical of unimproved and improved soils respectively. In addition, a set of microcosms, pH-amended by addition of 0.04 g $Ca(OH)_2$ was also established to examine the influence of pH on improved soil pH alone. All microcosms were incubated in the dark at 14°C for 30 days.

Archaeal DGGE profiles from triplicate microcosms were again dominated by a band co-migrating with SUPA5 and no obvious differences in profiles were seen in samples from microcosms incubated for 0 or 30 days (Fig. 2A). A different batch of unimproved soil was used in this experiment to that used in the synthetic sheep urine experiment and DGGE profiles showed some differences. This reflected recognized spatial heterogeneity of specific microbial communities within grassland rhizosphere soil (Webster *et al*., 2002; Nicol *et al*., 2003c), but profiles were characteristic of unimproved rhizosphere soil with a band co-migrating with the SUPA5 marker but lacking a SUPA2-type band.

Bacterial DGGE profiles from replicate microcosms were identical and unamended microcosms incubated for 0 and 30 days showed no differences (Fig. 2B). However, distinct shifts in the bacterial communities were observed following addition of NH_4NO_3. Denaturing gradient gel electrophoresis profiles from both sets of NH_4NO_3-amended microcosms were very similar, despite a difference in soil pH of approximately 1.7, and increases or decreases in relative intensity of bands, in comparison to control microcosms (both unamended and pH-adjusted), were the same for both 'unimproved' and 'improved' pH values (examples labelled I or D in Fig. 2B). Three bands which appeared to increase in relative (within-lane) intensity in response to NH_4NO_3-amendment were excised and sequenced (Table 1). Two exhibited closest similarity to gamma-*Proteobacteria* with one displaying 100% identity to mainly *Pseudomonas* spp., and one sequence possessed 100% similarity to a number of *Dactylosporangium* spp.

Table 1. Closest matches [name (accession number)] to excised, reamplified and sequenced 16S rRNA-derived DGGE bands (refer to Figs 1,2 and 3). Sequences were compared to those held in the GenBank database using the BLASTN search tool. Sequences were obtained from DGGE bands using a previously described method (Nicol *et al*., 2003b) with primer p2 used in all sequencing reactions. All sequences have been deposited in GenBank with the ascribed accession number.

Band [name (accession number)]	Associated amendment	Closest match in GenBank database [name (accession number)]	% Similarity	Division
bU-IP 1 (AY533526)	synthetic urine addition	>100 *Rhizobiales* sequences including: *Nitrobacter winogradskyi* (AY055796) *Bradyrhizobium japonicum* (AY169423) *Oligotropha carboxidovorans* (AB099660)	100% 117/117	*Proteobacteria*; alpha-subdivision
bU-IP 2 (AY533527)	pH increase	9 *Acidobacteria* sequences including: UA3 (AF200699) clone SMS9.16WL (AY043842) clone C46.02PG (AF431479)	97% 114/117	*Acidobacteria*
bU-IP 3 (AY533528)	pH increase + synthetic urine addition	4 alpha-*Proteobacteria* sequences including: *Inquilinus limosus* (AY043374) sequence PX3.14 (AY337602) strain 15158 (AF085496)	99% 116/117	*Proteobacteria*; alpha-subdivision
bAN 1 (AY533529)	ammonium nitrate addition	>50 *Pseudomonas* sequences including: *Pseudomonas stutzeri* (AJ312157) *Pseudomonas fluorescens* (AJ278814) *Pseudomonas putida* (AF288729)	100% 152/152	*Proteobacteria*; gamma-subdivision
bAN 2 (AY533530)	ammonium nitrate addition	*Cimanggu media* isolate 88 (AF229452)	98% (149/152)	*Proteobacteria*; gamma-subdivision
bAN 3 (AY533531)	ammonium nitrate addition	21 *Dactylosporangium* sequences including: *Dactylosporangium vinaceum* (X93196) *Dactylosporangium aurantiacum* (X93191) *Dactylosporangium matsuzakiense* (D86940)	100% 129/129	*Actinobacteria*
bDP (AY533532)	pH decrease	67 *Actinomycetales* sequences including: *Streptomyces brasiliensis* (X53162) *Cellulomonas biazotea* (X83802) *Promicromonospora pachnodae* (AF105422)	100% 129/129	*Actinobacteria*



A – *Archaea* B - *Bacteria*

Fig. 2. DGGE profiles of archaeal and bacterial 16S rRNA RT-PCR products amplified from unimproved soil amended with ammonium nitrate and pH-adjusted after incubation for 30 days. Each lane represents an individual microcosm with archaeal and bacterial profiles derived from the same 16S rRNA extracts. pH values are the means of the triplicate microcosms profiled (the standard error for all mean values was <0.05).
A. *Archaea*. M denotes SUPA marker lane where SUPA2 and SUPA5 represent archaeal sequences generally present in improved grassland rhizosphere soil (I) and in both improved and unimproved grassland rhizosphere soil (I/U) respectively.
B. *Bacteria*. Examples of bands which either increased or decreased in relative intensity to the controls (both unamended and pH) in all NH_4NO_3-amended microcosms profiles (both sets) are labelled I and D respectively. Bands of interest that were excised, reamplified and sequenced are highlighted with numbered boxes and prefixed with bAN (ammonium nitrate) in the text for further discussion.

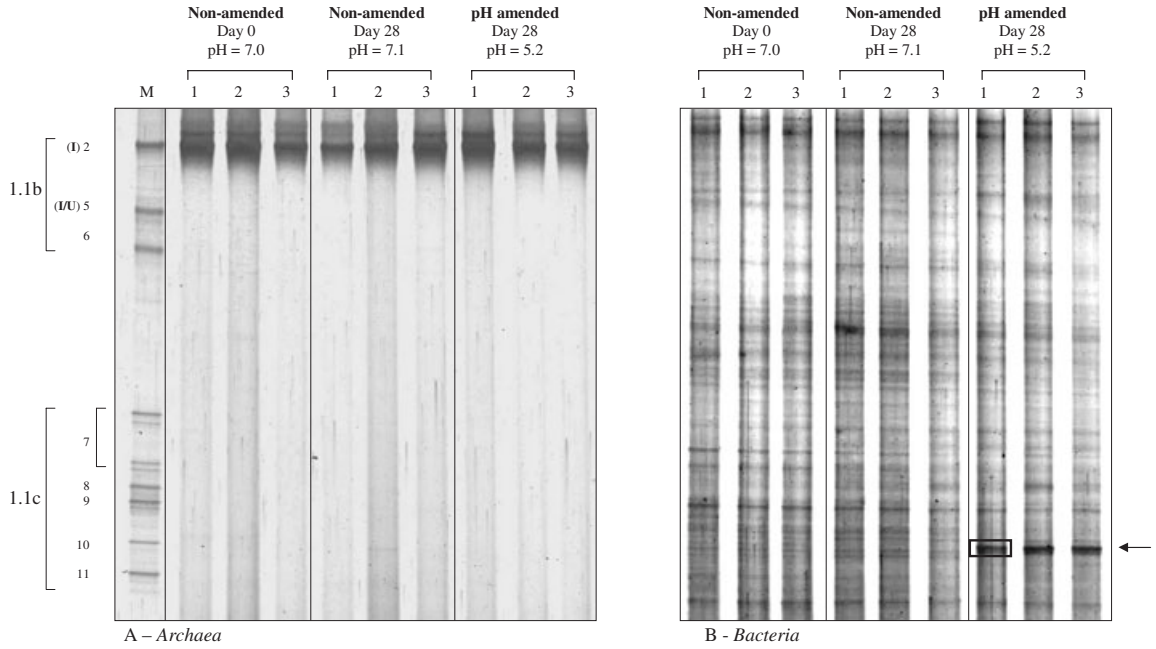


Fig. 3. DGGE profiles of archaeal and bacterial 16S rRNA RT-PCR products amplified from improved soil with a reduced pH after incubation for 28 days. Each lane represents an individual microcosm with archaeal and bacterial profiles derived from the same 16S rRNA extracts. pH values are the mean of the triplicate microcosms profiled (the standard error for all mean values was <0.05).
A. *Archaea*. M denotes SUPA marker lane where SUPA2 and SUPA5 represent archaeal sequences generally present in improved grassland rhizosphere soil (I) and in both improved and unimproved grassland rhizosphere soil (I/U) respectively.
B. *Bacteria*. The arrow highlights a band position of greater intensity in the pH-amended microcosms compared to control microcosms. The individual band highlighted with a box was excised, reamplified and sequenced and prefixed with bDP (decreased pH) in the text for further discussion.

Decrease in soil pH of improved soil

Improved grassland rhizosphere soil has a higher pH than soil from semi-improved and unimproved grassland (McCaig *et al*., 1999; Webster *et al*., 2002; Nicol *et al*., 2003a). Previous DGGE analyses have shown that archaeal communities in improved soil are characterized by a greater relative abundance of organisms possessing SUPA2-like 16S rRNA gene sequences compared with those in unimproved rhizosphere soil (Nicol *et al*., 2003a). In addition, archaeal profiles from soil obtained from one particular improved plot (plot 1) at Sourhope were consistently dominated by a SUPA2-type band to the exclusion of others (including SUPA5) (Nicol *et al*., 2003a,c). This plot had the highest soil pH (≥7.0) of all plots of improved grassland, suggesting that SUPA2 may be selected for by an increase in pH, compared to unimproved grassland soils. To determine whether a lower pH, associated with that of unimproved rhizosphere soil, decreased the contribution of bands co-migrating with SUPA2 and selected for increased activity of other *Archaea*, microcosms containing 10 g improved soil from plot 1 were amended with 0.08 g $Al_2(SO_4)_3$, reducing the pH from 7.0 to approximately 5.0 and incubated at 14°C for 28 days.

Archaeal DGGE profiles of control or pH amended microcosms did not change after incubation for 28 days (Fig. 3A) with profiles dominated by bands co-migrating with SUPA2. However, reproducible changes were again observed in bacterial profiles (Fig. 3B), with an increase in the relative intensity of one band in the pH-amended microcosms which exhibited 100% similarity to strains placed within the *Actinomycetales* (Table 1).

Differential response of archaeal and bacterial communities

Soil microcosm experiments were established to examine whether different nitrogen inputs and pH shifts were specific drivers of management-associated archaeal community structures. Both archaeal and bacterial community structures were profiled by RT-PCR of 16S rRNA (rather than DNA) to increase sensitivity and potentially target the predominantly active members of the microbial community (Felske and Akkermans, 1998). Greater sensitivity will arise through the higher copy number of 16S rRNA per cell compared to *rrn* operon copy number, and growth and activity may be detected earlier, through increases in ribosome content prior to cell division.

The amendment of natural, unimproved grassland soil with synthetic sheep urine, ammonium nitrate and an

increase in soil pH did not appear to influence archaeal communities in the soil microcosms. However, determining whether organisms have been negatively affected by particular amendments may be problematic when using 16S rRNA-based methodologies, as different organisms may have different rRNA turnover strategies. Large ribosome contents can be maintained during short periods of starvation (Wagner *et al*., 1995) and in excess of that required for specific protein synthesis rates (Flardh *et al*., 1992), whereas cells with low metabolic rates may maintain large ribosome contents to enable rapid response when nutrients become available (Alfreider *et al*., 1996). In contrast, DGGE profiles of the bacterial communities showed clear, reproducible differences in comparison to control microcosms in all experiments. The various amendments affected organisms belonging to a variety of major soil bacterial groups including alpha- and gamma-*Proteobacteria*, *Actinobacteria* and *Acidobacteria*. The relatively small amount of discriminatory sequence information obtained from short DGGE fragments spanning the v3 region limited taxonomic inference largely to a 'subdivision' level and highlighted the fact that individual bands or sequences may represent a large number of different strains in an environmental sample.

There are a number of possible reasons for the lack of change in predominantly active *Archaea*. Microcosms may not fully represent the field situation, and may have lacked an essential factor for archaeal growth or activity (e.g. root exudates). The crenarchaeal cells, which appear to dominate the archaeal community, may be inactive or dormant, despite high ribosomal copy number. The cell numbers of those organisms which respond to grassland management practices may have been too low for detection of changes after 28 days (e.g. SUPA2- and SUPA5-like organisms in unimproved and improved soils respectively). It is possible that seeding of microcosms with soil of a different type before amendment could provide a sufficient 'inoculum' to enable detection of changes in cell concentration. The soil bacterial community is much more diverse that the archaeal community and this is likely to reflect greater physiological diversity. Consequently, some members of the bacterial community are more likely to be affected by a particular amendment. To determine whether there had been an increase or decrease in overall archaeal numbers or activity, real-time PCR (e.g. Ochsenreiter *et al*., 2003) or probing of extracted 16S rRNA (e.g. Buckley *et al*., 1998) could be used to compare archaeal and bacterial contributions to prokaryotic activity. There is evidence that Group 1 crenarchaea are associated with plant roots (Simon *et al*., 2000; Chelius and Triplett, 2001), and future experiments will examine the potential role of the rhizosphere in influencing archaeal community structure in soil. Reverse transcriptase-PCR of 16S rRNA and DGGE analysis of the bacterial community was able to demonstrate clearly that the individual grassland management practices examined have a significant impact on microbial community structure in soil and provides tentative evidence that these factors are not major drivers of archaeal diversity in soil.

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