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Seasonal and management influences on bacterial community structure in an upland grassland soil

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Abstract

Floristically diverse *Nardo-Galion* upland grasslands are common in Ireland and the UK and are valuable in agricultural, environmental, and ecological terms. Under improvement (inputs of lime, fertiliser and re-seeding), they convert to mesotrophic grassland containing very few plant species. The effects of upland grassland improvement and seasonality on soil microbial communities were investigated at an upland site. Samples were taken at five times in one year in order to observe seasonal trends, and and bacterial community structure was monitored using automated ribosomal intergenic spacer analysis (ARISA), a DNA-fingerprinting approach. Differences in soil chemistry and bacterial community structure between unimproved and improved grassland soils were noted. Season was also found to cause mild fluctuations in bacterial community structure, with soil samples from colder months (October and December) more correlated with change in ribotype profiles than samples from warmer months. However, for the majority of seasons clear differences in bacterial community structures from unimproved and improved soils could be seen, indicating seasonal influences did not obscure improvement effects.

1. Introduction

Acidic grasslands are prevalent throughout upland areas in north-western Europe [1], and are of major agricultural importance [2]. *Nardo-Galion* grasslands are typical of many upland areas in Ireland and the UK, and are dominated by a diverse assemblage of pasture species, principally *Agrostis capillaris*, *Festuca ovina*, and *Galium saxatile*. In many of these areas there has been a drive to increase productivity through agricultural improvement, involving liming, fertilisation, re-seeding and increased grazing. This has resulted in broad-scale losses of plant diversity, specifically resulting in low diversity pastures typically dominated by just two species, *Lolium perenne* and *Trifolium repens*. Concern has been expressed over the environmental impacts of intensification upon these ecosystems, which can result in soil erosion, loss of organic matter, and contamination of soil, water and air with nutrients, pesticides and heavy metals [3]. Changes in plant community structure may also affect ecosystem functioning through alteration of biogeochemical cycling and decomposition processes [4-6].

In upland acidic grassland ecosystems, improvement results in significant changes to soil microbial community structures. A variety of approaches has been used to uncover differences both in broad-scale microbial parameters, such as microbial biomass and activity, and in molecular community structure of both bacterial and fungal communities [7-20]. These studies have indicated that improvement results in an increase in labile soil substrates and may result in bacterial domination of soil microbial biomass, while unimproved soils are rich in more resistant substrates and provide an environment more suited to fungi. Using a microcosm based approach, Kennedy et al. [21-22] found that bacterial communities

changed markedly in response to chemical improvement factors (nitrogen, lime), but were unaltered by plant rhizosphere effects. Fungal communities were found to be much more resilient to change than bacterial communities.

This study examines bacterial and fungal community structure in unimproved (U4a) and improved (MG7b) grasslands at an upland site in the Wicklow Mountains using automated ribosomal intergenic spacer analysis (ARISA), a powerful community fingerprinting approach. Although molecular studies of bacterial [19] and fungal [20] community structure have previously been carried out on this site, the use of ARISA in this study to target bacterial genes highly variable in sequence and size may result in better discrimination between community profiles [23]. This greater discrimination will provide more definitive information on the effects of improvement and seasonality on bacterial community structure in upland grassland soils.

Some studies have indicated seasonal changes impact upon soil microbial communities in agroecosystems [11,17,24-27]. However, most field-based studies of upland acidic grasslands have not incorporated the effects of seasonality on their findings, with most data collected resulting from single time point samplings. This is perhaps understandable as limited resources (of time, budget, and personnel) often prohibit extended sampling. However, a key question remains as to the extent of seasonal variation in soil microbial community structures in upland grasslands, and whether conclusions drawn from a single sampling can be extrapolated to reflect year-round patterns. In this study, soil samples were taken from each grassland type at five different dates over one year, to determine whether differences in microbial community structure between the two grassland types were evident, and if any such differences were affected by season, or if they remained consistent throughout the year.

2. Materials and methods

2.1. Soils and sampling

Soil was collected during 2001 from an area of unimproved *Nardo-Galion* grassland at Long Hill, Kilmacanogue, County Wicklow, Ireland (OS Coordinates O218 124). The site was 300 m above sea level and consisted of a peaty podzolic soil [28] formed over granite/quartzite bedrock [29], with an annual rainfall between 2000 and 2800 mm [30]. Enclosed in the south-east corner of the site was an improved area which was ploughed, rotavated, limed and re-seeded with *Lolium perenne* and *Trifolium repens* in 1993, and fertilised every April since then with nitrogen-phosphorus-potassium (NPK) fertiliser at a rate of approximately 150, 25 and 50 kg ha^{-1}, respectively. Approximately 5 tonnes ha^{-1} of lime have also been applied to the field each April. Floristic analysis was carried out according to the UK National Vegetational Classification (NVC) system [2], with the unimproved grassland classifying as a U4a grassland type, and the improved classifying as a MG7b grassland type.

Soil samples were taken using a soil corer to remove three replicate cores (4 cm diameter, 10 cm depth) at each sampling point. Five sampling points in each grassland type (U4a and MG7b) were selected. A reference sampling point in each grassland type was determined by measuring from a landmark to the centre of each grassland type, then measuring 2 m in each cardinal direction (north, south, east, west), in order to designate five sampling points. The same sampling points were used at each

season. Soil was sieved to <4 mm and stored at 4°C for less than 7 days for soil chemical analysis, and at -20°C for molecular analysis.

2.2. Soil physical and chemical analysis

Soil samples were analysed for pH_{water} by an electrometric method [31], using a single junction reference electrode (Orion Instruments, Boston, USA). Total nitrogen was determined by the Kjeldahl method [32], using a Kjeltec system 2000 Digestion apparatus and 2100 Distillation unit (Foss Tecator, Sweden). Phosphorus was extracted using the Morgan method [33], and was measured by the colorimetric ammonium molybdate-ascorbic acid method [34]. Soil potassium was extracted using the ammonium acetate centrifuge method [35], and analysed by atomic emission spectrometry on a Corning 410 flame photometer (Essex, UK). Soil temperature readings were obtained from Met Eireann [30] in 2001, the same year soil samples were collected. Soil temperature, air temperature, and rainfall were measured by Met Eireann at their Casement Aerodrome station in County Kildare (25 km northwest of our sampling site), and monthly averages were published on their website (www.meteireann.ie) in January 2002.

2.3. Total Soil DNA Extraction and Purification

Total soil DNA was extracted as described previously [19]. Briefly, soil (0.5 g) was added to tubes containing glass and zirconia beads, and extracted with CTAB (hexadecyltrimethylammonium bromide) buffer and phenol:chloroform:isoamylalcohol (25:24:1). Tubes were then shaken in a Hybaid Ribolyser at 5.5 m/s for 30 s before purification with chloroform:isoamylalcohol (24:1). DNA was further purified by incubation with lysozyme solution (100 mg/ml)

for 30 min at 37°C, followed by purification using a High Pure PCR Product Clean Up Kit (Roche Diagnostics GmbH, Penzberg, Germany). DNA was eluted in a final volume of 50 µl and was consistently suitable for PCR amplification without further treatment.

2.4. Bacterial community fingerprinting using ARISA analysis

The 16S-23S intergenic spacer region (IGS) from the bacterial rRNA operon was amplified using forward primer S-D-Bact-1522-b-S-20 (eubacterial rRNA small subunit, 5'-TGCGGCTGGATCCCCTCCTT-3') and reverse primer L-D-Bact-132-a-A-18 (eubacterial rRNA large subunit, 5'-CCGGGTTTCCCCATTCGG-3') [36]. Amplified sequences contained the IGS plus approximately 150 bp corresponding to the 20 nucleotides of the forward primer and about 130 bp of the 23S rRNA gene. The forward primer was labelled with Beckman Coulter fluorescent dye D4 (Research Genetics Inc., Alabama, USA). PCR reactions were performed in 50µl volumes containing 5 µl of 10× Mg-free PCR buffer, 1.25 mM MgCl2, 15 pmol of each primer, 200 µM of each dNTP, 1 µg BSA, ~10 ng extracted total soil DNA, and 2.5 U Taq DNA polymerase (Promega, WI, USA). Thermocycling conditions were as follows: a hot start at 94ºC for 2 min (1 cycle), after which 2.5 U Taq DNA polymerase were added; 94ºC for 3 min (1 cycle); 94ºC for 1 min, 55ºC for 30 s, 72ºC for 1 min (25 cycles); 72ºC for 5 min (1 cycle). PCR products were first visualised on a 1.2% agarose gel, then purified using a High Pure PCR product purification kit (Roche Diagnostics GmbH, Penzberg, Germany) and eluted in 50 µl of sterile water heated to 50ºC. The purified PCR products were quantified on a 1.2% agarose gel before mixing aliquots (0.5 –1 µl) with 38.4 µl of deionized formamide, 0.2 µl of Beckman Coulter size standard 600 (dye D1), and 0.4 µl of custom-made marker

(containing ribotypes ranging from 600-1000 bp in intervals of 20 bp, and 1000-1200 in intervals of 50 bp, all labeled with Beckman Coulter Dye D1) (BioVentures, Murfreesboro, TN, USA).

Intergenic spacer lengths were determined by electrophoresis using a Beckman Coulter (CEQ 8000) automated sequencer. Run conditions were 60ºC separation temperature, 4 kV voltage, and 120 min separation time to allow for separation of the larger ribotypes. Analysis of spacer profiles was performed using the Beckman Coulter CEQ 8000 fragment analysis software, algorithm v 2.1.3. A quartic polynomial model rather than the recommended cubic model was used for size standard calibration as this resulted in improved correlation between expected and actual size standard fragment sizes, particularly for ribotypes in the range 400–1200 bp. Ribotypes that differed by less than 0.5 bp in different profiles were considered identical.

2.5. Statistical Analysis

Data sets for pH, nitrogen, phosphorus, potassium, bacterial ribotype number, and relative abundances of individual bacterial ribotypes were analysed by one-way factorial analysis of variance (ANOVA) using Genstat v 6. The significance level was set at $p<0.05$.

A randomization test [21] was performed on bacterial ARISA profiles using Genstat v 6 to test the null hypothesis that there were no significant differences between samples from different grassland types or different seasons. Peak heights for each bacterial ARISA ribotype were first converted into proportions of the total for each replicate. Grassland type and seasons were then tested in pairwise comparisons

using a variation of the city-block (Manhattan) randomization test procedure, using a critical level of $p<0.05$.

Bacterial ARISA profiles were explored using canonical correspondence analysis (CCA) (Canoco for Windows, v 4.02) after initial analysis by detrended correspondence analysis (DCA) revealed that the data exhibited a unimodal, rather than linear, response to the environmental variables (grassland type and season). The resulting ordination biplot approximated the weighted average of each species (in this case, relative abundances of ribotypes) with respect to each of the environmental variables, which were represented as arrows. The length of these arrows indicated the relative importance of that environmental factor in explaining variation in bacterial profiles, while the angle between arrows indicated the degree to which they were correlated [37]. A Monte Carlo permutation test based on 199 random permutations was used to test the null hypothesis that bacterial profiles were unrelated to environmental variables.

3. Results

The pH of each grassland soil for each of the five sample times was determined (Table 1). Improved MG7b soil had a consistently higher pH than soil from the unimproved U4a grassland. ANOVA results revealed that the largest differences in soil pH were between soils from different grassland types, with season having a smaller but significant effect. The pH of both U4a and MG7b grassland soils was lowest in May and highest in October and December. The percentage of nitrogen in soil varied significantly only due to grassland type (Table 1), with nitrogen levels in U4a soil over one-third higher than those in MG7b soil. Phosphorus and potassium

levels varied widely across season and grassland type, with soils from MG7b grasslands having higher phosphorus and potassium contents than U4a grassland soils (Table 1). Season also had a significant effect on phosphorus and potassium levels, with the highest phosphorus levels seen in December for both grassland types, while potassium levels were affected by a season-grassland type interaction. Soil temperature readings were obtained from Met Eireann, in order to provide background information on seasonal temperature fluctuations (Table 1). Soil temperature was lowest in February and December, and highest in July. Monthly air temperature and rainfall means from 2001 were compared with 30-year averages (1973-2003) [30]. Air temperatures from 2001 varied from the 30-year averages by an average of only 0.8ºC, while rainfall from 2001 was on average 2 cm lower than the 30-year averages.

Bacterial ARISA detected a total of 788 unique ribotypes after analysis of all samples, ranging in size from 45 to 1236 bp. The majority of ribotypes found were between 300 and 600 bp in length. Although bacterial ribotype numbers were slightly higher in MG7b than in U4a grassland soils, bacterial ribotype number was not significantly affected by grassland type, season, or their interaction.

Bacterial ARISA profiles were compared using a randomization test to determine if season or grassland type caused significant differences in bacterial community structure. Significant differences between bacterial community structure in U4a and MG7b soils were found in every season except December. Bacterial community structure profiles from U4a soil varied between some seasons, with U4a soil sampled in December exhibiting a significantly different bacterial community structure than U4a soil sampled in February and May. MG7b soil also exhibited some significant differences in bacterial community structure due to season, with soil

sampled in February having a significantly different profile than soil sampled in October and December.

Bacterial ARISA profiles were further analysed by a multivariate analysis, canonical correspondence analysis, in order to determine which environmental factors most influenced bacterial community structure (Table 2 and Figure 1). Analysis was performed on the top 20 most abundant bacterial ARISA ribotypes, which totalled 29% of overall abundance. The first (eigenvalue 0.359) and second (eigenvalue 0.298) axes were found to explain a total of 9% of variation in bacterial ARISA ribotype profiles. Although this was a relatively small percentage, it was found that the axes explained a total of 58% of the species-environment relation, indicating that they accounted for the bulk of the variance in the ARISA profile data that could be attributed to environmental factors (grassland type and season). This was confirmed by species-environment correlation values, which were high for both axes (0.631 and 0.724 for Axes 1 and 2, respectively). Additionally, Monte-Carlo significance tests of the axes revealed that they explained a significant amount of variation within the data (p<0.005). In Figure 1, December appeared to have the longest arrow, indicating it explained the largest amount of variation between bacteial ribotype profiles, which was supported by its high intra-set correlations in Table 2. Axis 2 was most closely correlated with grassland type, with U4a and MG7b having opposite effects on the abundance of most bacterial ribotypes.

Results of CCA were confirmed by ANOVA analysis of the top twenty most abundant bacterial ribotypes (Table 3), which revealed season had a significant impact on the abundances of five of the top twenty ribotypes. Abundances of three of the ribotypes (Ribotypes 325, 326, and 362) were highest in December, indicating they were highly correlated with that season, as also indicated by their position in the CCA

diagram. Similarly, those ribotypes found by ANOVA to be significantly affected by grassland type (Ribotypes 361, 360, and 402) exhibited highest abundances in MG7b soil, and appeared to be correlated with the arrow representing MG7b in the CCA diagram.

4. Discussion

Both grassland type and season were found to impact upon soil chemical parameters and microbial community structure, but grassland type proved to have a more consistent effect, whereas seasonal effects varied. Soil pH was consistently higher in improved MG7b soil than in unimproved U4a soil, with only mild fluctuations due to season. The higher pH of the MG7b grassland was attributed to addition of lime. Higher %N levels in the U4a soil probably relate to higher levels of organic nitrogen in this grassland, as it has been demonstrated that organic forms of nitrogen, in particular proteins and amino acids, predominate in unimproved grasslands, whereas improved grasslands are richer in inorganic forms of nitrogen [11]. Concentrations of phosphorus and potassium were significantly greater in MG7b than U4a soils, which was most likely due to the treatment of the MG7b grassland with nitrogen-phosphorus-potassium (NPK) fertiliser.

The number of bacterial ribotypes found in MG7b grassland soil was higher than that found in U4a soil, indicating that improvement may result in an increase in bacteria, as many other studies of upland grasslands have found [14,17-19]. However, due to the high variability between sample replicates, the difference was not found to be statistically significant. Additionally, it must be noted that measures of species dominance or richness, such as ribotype number, tend to be poor indicators of

community structure or function [38], and therefore it was necessary to explore community profiling data further.

Both randomization test and CCA analyses indicated that both grassland type and season affected bacterial community structure profiles. The randomization test showed grassland type had a clear effect on bacterial community structure, while the effect of season was less pronounced. CCA found samples from December to be most strongly correlated with variation in bacterial community structure. Each sampling time (February, May, July, October and December) was depicted in the CCA diagram as a separate arrow, and it was seen that samplings from colder months (December, February, October) appeared to be more highly correlated with changes in bacterial community structure than samplings from warmer months (May, July). This observation was supported by ANOVA results for each of the top twenty most abundant ribotypes, which revealed that those ribotypes significantly affected by season were most abundant in samples from colder months (December, February, October). CCA also revealed that grassland type strongly affected bacterial community structure, with U4a and MG7b appearing to have equal but opposite effects. This indicates that the bacterial communities of each grassland type are quite different, a finding also noted by Brodie et al. [19], who found that bacterial community structure, as measured by terminal restriction fragment length polymorphism (TRFLP), differed between the U4a and MG7b grasslands at this site. It must be noted that our samples were taken over one year only, and the possibility of variation in seasonality from one year to the next exists; however, comparison of climatic data from this year with averages from the past 30 years indicated that it was representative of typical seasonal changes in this climate.

Many studies have noted shifts in microbial community structure due to improvement, with indications that improvement of grasslands may result in increased bacterial:fungal microbial biomass ratios, while unimproved grasslands may be richer in fungal biomass and diversity [8,10,14,17-20]. Differences in microbial genetic complexity and diversity between soils from unimproved and improved grasslands have also been noted, with both the complexity of %G+C DNA profiles [12-13] and the diversity of bacterial clones [15-16] found to be lower in improved soils. These reductions suggest that improvement results in selective pressure on microbial populations, possibly selecting for particular bacterial groups. It must be noted that the studies mentioned all used fairly broad-scale approaches to determining microbial community structure, with many relying on methods such as culturing, community level physiological profiling, or fatty acid profiles, which do not give a full view of microbial community structure [39]. In contrast, our study represents the first time an ARISA approach has been used to elucidate microbial community structure of grassland soils, which provides a more discriminatory view of changes between populations. DNA-based community fingerprinting approaches can be difficult to interpret because of species differences in rRNA gene copy number [40,41], biases resulting from PCR amplification [42,43], and the difficulty in standardizing the amount of DNA analyzed in each replicate [44,45]. However, as all samples were subject to the same biases, it was still possible to compare between them on a relative basis, especially after standardization of ribotype peak heights into proportions per sample. Our findings are in agreement with the trend seen in other studies of upland grassland improvement, with MG7b soil found to have a greater number of bacterial ribotypes than U4a soil, while CCA analysis of bacterial community structure found grassland type to have a major influence.

Although it is clear that improvement results in changes in microbial community structure, the precise cause of these changes is less clear. Several factors have been postulated, including changes in floristic composition, grazing, differences in pH, and effects of fertiliser application. The dominant plant species of unimproved and improved grasslands differ in growth rates and exudation patterns [46-47], possibly causing differences in substrate availability between grasslands, resulting in differing microbial community structures [9,17]. Plant composition can also affect soil microbial communities through changes in litter deposition [48-49]. Increased pH in improved grasslands resulting from liming may encourage the growth of bacteria, with bacterial numbers known to be positively correlated with increasing pH [50-51]. Nitrogen addition has also been shown to alter the microbial community structure of grassland soil both in the field and in microcosms [21-22,52-53]. Soil microbes may be affected directly by chemical interactions with nitrogen, which can repress enzyme activity and inhibit decomposition [54], or indirectly, with fertilisation resulting in changes to plant species composition and growth rates, which in turn affect microbial community composition [14,48].

In our study, season was found to impact upon bacterial community structure, with samplings from colder months (December, October, February) showing a greater correlation with change in bacterial commmunity structure than those from warmer months. It is possible that lower temperatures associated with these sampling times result in changes to bacterial community structure. Populations of ammonia-oxidising bacteria in soil have been shown to be affected by changes in temperature, with differences in 16S rRNA gene DGGE profiles noted at different temperatures over a range of 4-37°C [55], and other field-based studies have found seasonal fluctuations in bacterial community parameters [7,11,17,25-27]. It has been hypothesised that

seasonal effects on bacterial community structures result from soil bacteria responding to seasonal changes in plant growth, with rhizodeposition of carbon compounds by plants changing throughout the year and resulting in temporal differences in substrate availability [17]. There is some evidence from other agroecosystems to support this view; bacterial DNA profiles have been found to change according to the growth stage of potato and maize plants, indicating root growth caused changes in substrate availability and altered bacterial community structure [56-57]. In addition to root exudation, variations in the quantity and composition of plant litter occur seasonally [24], and can affect soil microbial community structure [48-49].

Physical characteristics that change according to season, such as soil moisture and temperature, may be important controllers of microbial community composition [25,58]. It is possible that cold months may see a reduction in microbial biomass [59], thereby decreasing competition and allowing less dominant species to be detected in profiles of community structure. This is supported by a review of microbial biomass studies [58], which revealed that no study found a winter maximum in microbial biomass. A decrease in biomass as soil temperatures decreased could account for the impact of colder months on soil bacterial community structure seen in our study, even though other studies have indicated that warmer months have more of an impact on microbial biomass [17,25-26]. Agricultural inputs may also vary seasonally; for example, at our field site, lime and fertiliser are applied to the improved grassland annually in April, which could contribute to seasonal variation in bacterial community structure in soil from this grassland.

Overall, it is probable that seasonal variations in soil bacterial community structure, while present, are dependent upon local soil type and conditions.

Importantly, it seems that the effect of grassland type is more consistent, and is detectable in samples from the majority of seasons. This is supported by the work of Grayston et al. [17], who found that differences in microbial community structure from unimproved and improved grassland soils, as measured by PLFA, were "robust over time", and that of Buckley and Schmidt [60], who found changes in soil microbial community structure caused by management practices were not obscured by temporal variation, even when such variation was significant. Further work needs to be done to elucidate how seasonality and management impact upon the functioning of microbial populations in grassland soils, and to determine the critical factors controlling seasonal responses in grasslands.

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Table 1. Soil physicochemical characteristics and bacterial ribotype number, as affected by season and grassland type (U4a, unimproved; MG7b, improved). Means and standard error of differences (SED) are shown (n=5 for means of grassland type at each season, n=25 for average of grassland types over all seasons). Same letter denotes no significant difference. ANOVA p-values shown as *, p<0.05; **, p<0.01; ***, p<0.001.

*Also included are 2001 mean soil temperatures for Casement Aerodrome, Co. Kildare, as published on the Met Eireann website (http://www.meteireann.ie) in January 2002.

	pH		**% Nitrogen**		**Phosphorus**		**Potassium**		**Soil Temperature**	**Bacterial Ribotype Number**	
					(mg P kg^{-1})		(mg K kg^{-1})		(ºC)	(mean ribotypes per replicate)	
	U4a	MG7b	U4a	MG7b	U4a	MG7b	U4a	MG7b		U4a	MG7b
SEASON											
February	5.09	7.02	0.77	0.63	12.8	13.6	89	253	2.5	99	58
May	4.93	6.64	0.73	0.57	13.2	14.0	104	275	14.0	46	74
July	5.03	6.87	0.78	0.62	9.9	16.8	84	267	17.4	90	103
October	5.10	7.25	0.79	0.59	12.2	12.7	106	213	11.8	80	131
December	5.26	6.95	0.90	0.56	13.9	21.8	93	189	3.7	60	39
Average	**5.08**	**6.95**	**0.79**	**0.59**	**12.4**	**15.8**	**95**	**239**		**75**	**81**
SED	0.17		0.06		0.51		18.2			32.7	
***p* values**											
Season	*		NS		*		**			NS	
Grassland Type	***		***		***		***			NS	
Season*Grassland Type	NS		NS		NS		**			NS	

Table 2. Results of canonical correspondence analysis (CCA) of bacterial community structure as determined from bacterial ARISA data. Values are for Axes 1 and 2 plotted in the CCA diagram in Figure 1. The highest intra-set correlations are highlighted in bold. U4a, unimproved; MG7b, improved.

Axis	**1**	**2**
Eigenvalue	0.359	0.298
Cumulative percentage variance:		
Of species data	5.1	9.0
Of species-environment relation	23.6	57.6
Species-environment correlations	0.631	0.724
Monte-Carlo significance test:		*For all axes:*
F-ratio	4.321	2.6
p-value	0.005	0.005
Correlations (100 x *r*):		
U4a	-6	**-48**
MG7b	6	**48**
February	-19	44
May	-14	6
July	1	7
October	11	34
December	**60**	2

Table 3. Abundance rankings and ANOVA results for the top twenty most abundant bacterial ARISA fragments, as ranked by average abundance over all samples. U4a, unimproved; MG7b, improved.

	Abundance			***p*-values**	
Bacterial ARISA Fragment	Abundance Rank	% Abundance	Cumulative % Abundance	Season	Grassland Type
325	1	4.5	4.5	**<0.001**(Dec)	0.493
326	2	3.2	7.7	**0.045**(Dec)	0.322
321	3	2.2	10.0	0.125	0.581
327	4	2.2	12.2	**0.023**(Oct)	0.795
324	5	2.0	14.2	0.880	0.629
361	6	1.9	16.1	0.588	**0.007**(MG7b)
360	7	1.5	17.6	**0.002***(Feb)	**0.004***(MG7b)
313	8	1.5	19.1	0.128	0.256
354	9	1.0	20.1	0.760	0.056
362	10	1.0	21.1	**0.049**(Dec)	0.417
401	11	0.9	22.1	0.231	0.453
441	12	0.9	23.0	0.128	0.579
328	13	0.9	23.9	0.064	0.927
339	14	0.9	24.8	0.438	0.482
402	15	0.8	25.6	0.0154	**<0.001**(MG7b)
443	16	0.7	26.3	0.365	0.791
455	17	0.7	27.0	0.641	0.096
353	18	0.7	27.8	0.051	0.090
397	19	0.7	28.5	0.639	0.118
351	20	0.7	29.1	0.284	0.266

Bold indicates a significant effect ($p<0.05$)
Season/grassland type with highest abundance of fragment is indicated next to significant effect
*Significant interaction with each other

Figure captions

Figure 1. Effect of grassland type and season on bacterial community structure, as measured by bacterial ARISA ribotype profiles. Canonical correspondence analysis (CCA) ordination diagram of bacterial ARISA data, with grassland types (U4a, unimproved; MG7b, improved) (dashed lines) and seasons (unbroken lines) represented as large arrows and ribotypes represented as black dots. Ribotypes are labelled according to size (base pairs).