Microbial Ecology

Soil Bacterial and Fungal Community Structure Across a Range of Unimproved and Semi-Improved Upland Grasslands

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Abstract

Changes in soil microbial community structure due to improvement are often attributed to concurrent shifts in floristic community composition. The bacterial and fungal communities of unimproved and semi-improved (as determined by floristic classification) grassland soils were studied at five upland sites on similar geological substrata using both broad-scale (microbial activity and fungal biomass) and molecular [terminal restriction fragment length polymorphism (TRFLP), automated ribosomal intergenic spacer analysis (ARISA)] approaches. It was hypothesized that microbial community structure would be similar in soils from the same grassland type, and that grassland vegetation classifications could thus be used as predictors of microbial community structure. Microbial community measurements varied widely according to both site and grassland type, and trends in the effect of grassland improvement differed between sites. These results were consistent with those from similar studies, and indicated that floristic community composition was not a stable predictor of microbial community structure across sites. This may indicate a lack of correlation between grassland plant composition and soil microbial community structure, or that differences in soil chemistry between sites had larger impacts on soil microbial populations than plant-related effects.

Introduction

Grassland plant communities can often be described in terms of the characteristic species that make up their composition. However, the extent of coupling between grassland plant composition and soil microbial structure is poorly understood. It is thought that plants can influence microbial populations in soil through rhizodeposition, with changes in the rate and quality of substrate input occurring due to alterations in plant species composition and diversity [40, 50]. Plant species typical of upland grasslands have been shown to exhibit a selective effect on the microbial populations of their rhizospheres, with *Agrostis capillaris*, *Lolium perenne*, and *Trifolium repens* stimulating the growth of *Proteobacteria* [22, 41], whereas specific associations between dominant species of unimproved and improved upland grasslands and mycorrhizal fungi have been discovered [21, 44, 52, 57, 64]. Shifts in overall microbial community structure have also been linked to plant species traits, with these changes usually attributed to differences in substrate deposition of different plants [3, 53, 61].

Exploration of links between plant and microbial community structure in grasslands is desirable because, although vegetation composition is relatively easy to qualify and quantify, soil microbial communities are much more difficult to characterize. Full characterization of soil fungal and bacterial populations requires multiple samplings and the use of expensive and time-consuming analyses, such as DNA-based community fingerprinting [28]. These approaches provide a detailed view of microbial community structure, but require much more time and effort than the vegetational surveys required to classify grasslands. If consistent links could be found between plant composition and soil microbial community structure, grassland vegetational classifications could be used as predictors of associated soil microbial structure.

In recent years, the National Vegetation Classification (NVC) system [49] has been successfully used to characterize plant communities in the UK and Ireland. Although broad classification systems have previously been used for grasslands [46], the NVC allows classifications into much more precise categories. Its applica-

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tion to upland grasslands allows differentiation between unimproved grasslands (classified as U4a), and semi-improved grasslands (classified as U4b) [49]. Unimproved U4a grasslands are dominated by the plant species *Festuca ovina*, *A. capillaris*, and *Galium saxatile*. Semi-improved grasslands, although dominated by the same species as unimproved grasslands, also contain subcommunities of *L. perenne* and *T. repens*, indicating a limited degree of improvement [49].

This study attempts to determine if links between plant community composition and microbial community structure are consistent across a range of geographically separated sites with similar physical and chemical characteristics. The microbial community structure of unimproved (U4a) and semi-improved (U4b) grassland soils was studied at five upland sites on similar bedrock substrata in the Wicklow Mountains, Ireland, using both broad-scale (microbial activity and fungal biomass) and molecular [terminal restriction fragment length polymorphism (TRFLP), automated ribosomal intergenic spacer analysis (ARISA)] approaches. The central hypothesis was that microbial community structure would be similar in soils from the same grassland type, and that NVC grassland vegetation classifications could thus be used as predictors of microbial community structure.

Methods

Field Sites. Soil was taken from areas of unimproved (U4a) and semi-improved (U4b) grassland at field sites (Long Hill, Sally Gap, Lough Tay, Kings River, and Annagh Hill) in upland regions of counties Wicklow and Wexford in June 2000 (see Table 1 for grid references). The maximum longitudinal distance between sites was 45 km, and the maximum latitudinal distance between sites was 22 km. All sites were located on granite/quartzite bedrock substrata (as determined by reference to Geological Survey of Ireland map [20]), had similar peaty podzol soil composition (determined in consultation with soil survey bulletin map [17]), and contained areas of unimproved (U4a) and semi-improved (U4b) grassland. Floristic composition was analyzed according to the UK National Vegetation Classification (NVC) system [49]. Briefly, 1 m quadrats were placed randomly five times around the grassland area and plant species composition and estimates of abundance as percent cover were determined visually [49].

Sampling Regime. Three sampling points within each grassland type at each site were randomly selected, and soil samples were taken using a corer to remove three replicate cores (4 cm diameter, 10 cm depth) at each sampling point. As extensive grass roots were visible throughout all cores, all soil was assumed to have been in contact with plant roots and was considered rhizosphere. Soil was sieved to <4 mm to remove plant and root material, and stored at 4°C for less than 7 days for chemical analyses, microbial activity, and biomass analysis, and at −20°C for molecular analyses.

Soil Physical and Chemical Analysis. Soil samples were analyzed for pH_{water} by an electrometric method [55], using a single junction reference electrode (Orion Instruments, Boston, MA, USA). Total nitrogen was determined by the Kjeldahl method [7], using a Kjeltec system 2000 Digestion apparatus and 2100 Distillation unit (Foss Tecator, Höganäs, Sweden). Soil was digested at 420°C for 1 h with a mixture of potassium sulfate, copper sulfate, and sulfuric acid, before undergoing steam

Table 1. Map grid references [Ordnance Survey of Ireland (OSI) grid coordinates] and chemical composition of soils from U4a and U4b grassland types at each field site ($n = 3$), and the average value for each soil type over all sites ($n = 15$)

		pH		*% Nitrogen*		*Phosphorus (mg P kg^{-1})*		*Potassium (mg K kg^{-1})*	
	OSI coordinates	*U4a*	*U4b*	*U4a*	*U4b*	*U4a*	*U4b*	*U4a*	*U4b*
Site									
Long Hill	O 218 124	3.82	4.57	0.97	0.72	12.8	13.7	241	366
Sally Gap	O 116 124	4.23	4.12	0.44	0.47	8.2	12.4	42	43
Lough Tay	O 160 087	3.46	3.95	0.68	0.71	11.5	11.6	167	241
Kings River	O 008 014	4.36	3.92	0.61	0.72	11.1	12.2	157	173
Annagh Hill	T 107 672	3.46	3.68	1.27	1.18	12.7	12.3	283	220
Average		3.87	4.05	0.80	0.76	11.3	12.4	178	209
SED (Site*Grassland type)		0.159		0.048		0.73		4.9	
p Values									
Site		***		***		***		***	
Grassland type		*		NS		**		***	
Site*Grassland type		***		***		**		***	

Means and standard error of differences (SED) are shown. ANOVA *p* values for site, grassland type and their interaction are shown as NS, not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

distillation. Nitrogen concentrations were determined after colorimetric titration with 0.1 M hydrochloric acid. Using ammonium sulfate as a standard, the recovery of nitrogen from these soil samples was estimated at 82.8 ± 2.6%.

Extractable phosphorus levels in soil samples were determined using the Morgan extraction [45]. Phosphorus was measured using the colorimetric ammonium molybdate–ascorbic acid method [36]. Absorbance was measured on a Jenway 6300 Spectrophotometer (Essex, UK) and compared to that of standard phosphorus solutions to determine extractable phosphorus concentration. Soil potassium concentrations were measured using the ammonium acetate centrifuge method [27]. Phosphorus was extracted with ammonium acetate and analyzed by atomic emission spectrometry on a Corning 410 flame photometer (High Wycombe, Bucks, UK), with sample readings compared to those of standard potassium solutions to determine potassium content.

Total Microbial Activity. Total microbial activity was assessed as triphenylformazan dehydrogenase activity and was determined based on a modification of the method of Thalmann [54], as previously described [8].

Fungal Biomass. Total soil ergosterol was quantified as described previously [9]. Briefly, 5 g soil was vortexed and sonicated with methanol and potassium hydroxide solution at 0°C. Subsequently, after incubation at 85°C for 30 min, followed by cooling at 4°C for 20 min, high-performance liquid chromatography (HPLC)-grade pentane was used to extract ergosterol from the soil mixture. Three pentane extracts were combined and dried under N_2 gas before being redissolved in methanol and filtered through 0.2-μm Teflon filters, then analyzed on a Waters Sugar Analyzer I HPLC system (Elstree, Hertfordshire, UK). Incorporation of an internal ergosterol standard allowed the extraction efficiency of ergosterol from soil in these experiments to be estimated at 87 ± 4.7%.

Total Soil DNA Extraction and Purification. Total soil DNA was extracted as previously described [8]. Briefly, soil (0.5 g) was added to tubes containing glass and zirconia beads, to which hexadecyltrimethylammonium bromide (CTAB) extraction buffer was added. After incubation at 70°C for 10 min, phenol/chloroform/isoamylalcohol (25:24:1) was added and tubes were then shaken in a Hybaid Ribolyser (Ashford, UK) at 5.5 m/s for 30 s. Following bead beating, tubes were centrifuged and the aqueous layer was removed and extracted twice with chloroform/isoamlyalcohol (24:1). A further purification procedure was performed involving incubation with lysozyme solution (100 mg/mL) for 30 min at 37°C. Tubes were again centrifuged and the aqueous layer removed and further purified using a High Pure PCR Product Clean Up Kit (Roche Diagnostics GmbH, Penzberg, Germany) according to manufacturer's instructions. DNA was eluted in 50 μl of the manufacturer's proprietary elution buffer solution and was consistently suitable for polymerase chain reaction (PCR) amplification without further treatment.

Bacterial Community Fingerprinting by T-RFLP Analysis. Terminal restriction ribotype lengths were determined using a modification of the method of Liu *et al.* [38]. After extraction and purification of total DNA from soil, the bacterial 16S small subunit rRNA gene was amplified using primer set F27 (5′-AGAGTTTGATCMT GGCTCAG-3′) and R1492 (5′-TACGGYTACCTTGT TACGACT-3′) [37]. The forward primer F27 was labeled with Beckman Coulter fluorescent dye D4 (Invitrogen, Paisley, Scotland, UK). PCR reactions were performed in 50-μl volumes containing 5 μl of 10× Mg-free PCR buffer, 1.25 mM $MgCl_2$, 15 pmol of each primer, 200 μM of each dNTP, 25 μg BSA, ~10 ng extracted total soil DNA, and 2.5 U *Taq* DNA polymerase (Promega, Southampton, UK). The thermocycling conditions were as follows: a hot start at 94°C for 3 min (1 cycle); 94°C for 1 min, 53°C for 2 min, 72°C for 2 min (26 cycles); 72°C for 7 min. PCR products were first visualized on a 1% agarose gel and purified using a High Pure PCR product purification kit (Roche Diagnostics) according to the manufacturer's instructions. The purified PCR product was quantified on a 1.2% agarose gel then digested enzymatically as follows: approximately 50 ng of PCR product was added to a reaction mixture containing sterile Millipore water, 20 U of restriction endonuclease *Msp*I, and 2 μl of corresponding enzyme buffer. Digests were performed in a final volume of 20 μl and incubated in a water bath at 37°C for 4 h. Digests were desalted and aliquots (1 μl) were mixed with 38.75 μl of deionized formamide and 0.25 μl of Beckman Coulter size standard 600 (High Wycombe, Bucks, UK).

Fungal Community Fingerprinting by ARISA (Automated Ribosomal Intergenic Spacer Analysis). The fungal genetic region containing the two internal transcribed spacers (ITS) and the 5.8S rRNA gene (ITS1–5.8S–ITS2) was amplified using primer set ITS1-F (CTTGGTCATTTAGAGGAAGTAA) [16] and ITS4 (TCCTCCGCTTATTGATATGC) [62]. Amplified sequences contained the two ITS regions and the 5.8S gene plus 22 bp from the forward primer. The forward primer ITS1-F was labeled with Beckman Coulter fluorescent dye D4 (Invitrogen). PCR reactions were performed in 50-μl volumes of 25 μl master mix [containing 50 units/mL *Taq* DNA polymerase in a proprietary reaction buffer (pH 8.5), 400 μM each dNTP, and 3 mM $MgCl_2$; Promega], 15 pmol each primer, 25 μg BSA, and ~10 ng extracted total soil DNA. The

thermocycling conditions were as follows: a hot start at 95°C for 4 min (1 cycle); 95°C for 1 min, 53°C for 30 s, 72°C for 1 min (35 cycles); 72°C for 7 min (1 cycle). PCR products were first visualized on a 1.2% agarose gel, then purified using a High Pure PCR product purification kit (Roche Diagnostics) and eluted in 50 μl of sterile water heated to 50°C. The purified PCR products were quantified on a 1.2% agarose gel before mixing aliquots (0.5–1 μl, equivalent to ~2 ng DNA) 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 to 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).

TRFLP and ARISA Ribotype Analysis. Bacterial terminal restriction and fungal ARISA ribotype lengths were determined by electrophoresis using a Beckman Coulter (CEQ8000) automated sequencer, version 6.0.2 (High Wycombe, Bucks, UK) (resolution ±1 bp up to 400 bp ribotype length, ±2 bp thereafter). Run conditions were 60°C separation temperature, 4 kV voltage, and 120 min separation time to allow for separation of the larger ribotypes. Analysis of ribotype profiles was performed using the Beckman Coulter ribotype analysis package 8000, version 8.0.52, ribotype analysis algorithm version 2.2.1 (High Wycombe, Bucks, UK). 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 ribotype sizes, particularly for ribotypes in the range 400–1200 bp. Ribotypes with peak heights less than 1% of the total peak height for all ribotypes in a sample were regarded as background noise and excluded from analysis. Ribotypes that differed by less than 0.5 bp in different profiles were considered identical. The peak heights of individual ribotypes were relativized as a percentage of their abundance within a sample to account for DNA quantity differences between replicate profiles, resulting in profiles containing data on ribotypes present and their relative abundances.

Statistical Analysis. Results for chemical analyses, microbial activity, fungal biomass, ribotype number, and individual abundances of the top 20 overall most abundant bacterial and fungal ribotypes were analyzed by one-way factorial analysis of variance (ANOVA) using Genstat v6 (VSN International, Oxford, UK). The significance level was set at $p < 0.05$. In addition, Genstat was used to calculate the overall abundance of each individual ribotype for all samples. Ribotypes were then ranked according to overall abundance and these rankings were used to select individual ribotypes for ANOVA. Bacterial TRFLP and fungal ARISA profiles were analyzed using Canoco for Windows, v4.02 (Centre for Biometry, Wageningen, The Netherlands). Initial analysis by detrended correspondence analysis (DCA) revealed that bacterial TRFLP data exhibited a linear response to the environmental variables (grassland type and site), thereby indicating redundancy analysis (RDA) as the most appropriate multivariate approach, whereas fungal ARISA data exhibited a unimodal response, indicating canonical correspondence analysis (CCA) was most appropriate. Initially, RDA or CCA of all ribotypes with all environmental factors, including all of their interactions, was carried out. This resulted in overly complex analysis results and diagrams, necessitating a reduction in the amount of data analyzed. The environmental factors most important in explaining variation in ribotype profiles were selected by eliminating those with low canonical coefficients and *t*-values [33]. Next, analysis was limited to the top 20 ribotypes, as ranked by abundance. As there was little or no change in analysis results after limiting the environmental variables and ribotype set, it was concluded that the variables and ribotypes accounting for the majority of variance in the data had been selected. The resulting ordination biplots 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 ribotype profiles, whereas the angle between arrows indicated the degree to which they were correlated [33]. A Monte Carlo permutation test based on 199 random permutations was used to test the null hypothesis that ribotype profiles were unrelated to environmental variables.

Results

Grassland Floristic Composition. Vegetational analysis revealed that unimproved U4a grasslands were dominated by *A. capillaris*, *Anthoxanthum odoratum*, and *F. ovina*, with high frequencies of *Potentilla erecta* and *G. saxatile*. Semi-improved U4b grasslands, although still dominated by *A. capillaris*, showed reduced abundances of *F. ovina* and increased occurrences of *F. rubra*. Additionally, more mesophytic species such as *Holcus lanatus* and *T. repens* were evident.

Soil Chemical Composition. Analysis of soil pH, nitrogen, phosphorus, and potassium (Table 1) indicated soil chemical composition varied due to both grassland type and site. On average, over all sites, U4a grassland soils had a slightly lower pH, higher nitrogen content, and lower phosphorus and potassium content than U4b grassland soils. However, these trends varied by site.

Microbial Activity and Fungal Biomass. To determine broad-scale trends in soil microbial community

structure, microbial activity and fungal biomass were determined (Table 2). Microbial activity varied widely between sites, with soils from Kings River having the highest values, whereas soils from Annagh Hill and Sally Gap had the lowest. Grassland type had a smaller but significant impact, with microbial activity higher in U4b grassland soils than in U4a grassland soils at every site. Fungal biomass, measured as ergosterol, also experienced wide variation due to site, with soils from Long Hill highest in fungal biomass, while those from Sally Gap were lowest (Table 2). Grassland type and site interacted significantly, with soil from U4b grasslands lower in fungal biomass than those from U4a grassland, at every site except Kings River.

Bacterial and Fungal Ribotype Number. Bacterial TRFLP detected a total of 89 unique terminal fragments (ribotypes) after analysis of all samples, ranging in size from 60 to 640 bp. A total of 453 fungal ribotypes was detected by fungal ARISA, ranging in size from 47 to 1100 bp. Bacterial ribotype number (Table 2) was affected by a significant interaction between grassland type and site, with numbers higher in soils from U4b grasslands than in U4a grassland soil at three sites, but the reverse true at the other two sites. Fungal ribotype number (Table 2) was significantly affected only by grassland type, with fungal ribotype numbers higher in U4a grassland soil than in U4b soil at every site.

Bacterial Community Structure. The effects of grassland type and site on bacterial community structure were further explored using redundancy analysis (RDA), with results shown in the ordination plot in Fig. 1. The top 20 most abundant bacterial ribotypes, accounting for 86.4% of total abundance, were included in the analysis. Axes 1 and 2 together accounted for 30.8% of total variation within bacterial ribotype profiles, and 72.6% of variation in profiles that could be attributed to the environmental variables of grassland type and site. Species–environment correlations for both axes were quite high (over 0.81), indicating that changes in bacterial ribotype profiles were closely correlated with changes in grassland type and site. Both the first axis on its own, then all axes together, were analyzed by the Monte Carlo test and found to explain a significant ($p < 0.05$) amount of variation within the data.

Intraset correlations revealed that Axis 1 was most closely correlated with grassland type (U4a and U4b), indicating that grassland type had the biggest impact on community structure, while Axis 2 was most closely correlated with the Sally Gap site. These observations were supported by noting the length of the arrows for U4a and U4b grassland types and the Sally Gap site in the RDA plot in Fig. 1, which appeared longer than arrows for other sites, indicating that these factors had a large impact on bacterial community structure. The arrow for the Kings River site was also long, and values for its intraset correlations confirmed that it also had a large effect on bacterial community profiles.

Correlations shown in the RDA plot were supported by ANOVA results (Table 3); for example, the arrow for the second most abundant ribotype, TRF 150, appeared close to the arrow for U4b grassland type on the RDA plot, and this relationship was confirmed by ANOVA, which found that the abundance of TRF 150 was significantly affected by grassland type, and that its abundance was highest in U4b grassland soil. Grassland type was found to have a significant effect on many (50%) of the top 20 most abundant ribotypes. Site also had a strong effect on abundances of the top 20

Table 2. Microbial activity, fungal biomass, and bacterial and fungal ribotype numbers of soils from U4a and U4b grassland types at each field site ($n = 3$), and the average value for each grassland type over all sites ($n = 15$)

	Microbial activity (μg TPF g^{-1} dry soil)		*Fungal Biomass (μg ergosterol g^{-1} soil)*		*Bacterial ribotype number (mean TRFs per replicate)*		*Fungal ribotype number (mean fragments per replicate)*	
	U4a	*U4b*	*U4a*	*U4b*	*U4a*	*U4b*	*U4a*	*U4b*
Site								
Long Hill	5	190	3.27	1.43	14	31	38	26
Sally Gap	9	52	0.05	0.01	19	31	28	26
Lough Tay	31	87	2.01	0.25	6	10	40	18
Kings River	388	520	0.44	2.24	17	9	29	25
Annagh Hill	2	20	1.92	1.10	27	9	35	16
Average	86	174	1.53	1.00	17	18	34	22
SED	69.4		0.842		8.3		12.1	
(Site*Grassland type)								
p Values								
Site	***		*		*		NS	
Grassland type	*		NS		NS		*	
Site*Grassland type	NS		*		*		NS	

Means and standard error of differences (SED) are shown. ANOVA *p* values for site, grassland type and their interaction are shown as NS, not significant; $^{*}p < 0.05$; $^{***}p < 0.001$.

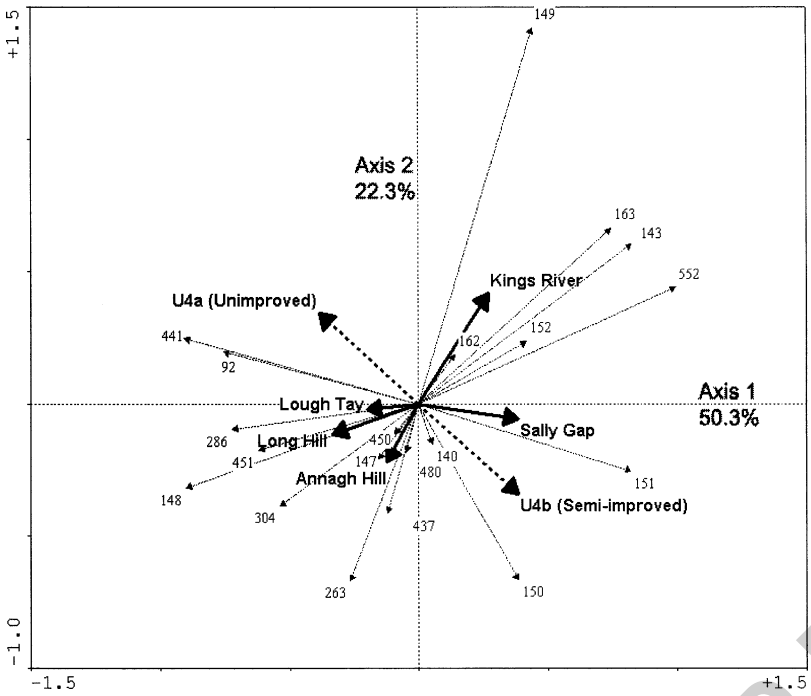

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Figure 1. Redundancy analysis (RDA) ordination diagram of bacterial TRFLP data, with grassland type (*dashed lines*) and sites (*bold solid lines*) represented as *large arrows* and bacterial TRF ribotypes represented as *smaller arrows*. TRFs are labeled according to ribotype size (bp). Axis 1 explains 50.3% of the TRFLP–environment variance, whereas Axis 2 explains a further 22.3% of the TRFLP–environment variance.

ribotypes, having a significant effect on 30% of them. Ribotypes that were significantly affected by site often had highest abundances at Sally Gap or Kings River, indicating these sites significantly affected the abundances of dominant ribotypes. In addition, significant interactions between grassland type and site were also noted.

Fungal Community Structure. Fungal ARISA ribotype profiles were investigated using canonical correspondence analysis (CCA) after initial data exploration revealed fungal community profiles responded to environmental variables (grassland type and site) in a unimodal fashion. The top 20 most abundant fungal

t3.1 **Table 3. Abundance rankings and ANOVA results for the top 20 most abundant bacterial TRFLP fragments, as ranked by average abundance over all samples**

t3.2		*Abundance*			*p Values*	
t3.3	*TRFLP fragment (bp)*	*Rank*	*% Abundance*	*Cumulative % abundance*	*Grassland type*	*Site*
t3.4	92	1	23.4	23.4	0.084	**0.015**(LT)
t3.5	150	2	12.6	36.0	**0.007**(U4b)	0.273
t3.6	151	3	11.8	47.8	0.074	**0.011**(SG)
t3.7	148	4	7.8	55.6	**0.039**(U4a)	0.100
t3.8	441	5	4.2	59.8	**0.004**(U4a)[a]	0.051[a]
t3.9	286	6	3.3	63.1	**0.043**(U4a)	0.209
t3.10	143	7	2.8	65.9	0.705	<**0.001**(SG)
t3.11	552	8	2.6	68.5	**0.011**(U4b)[a]	<**0.001**(KR)[a]
t3.12	152	9	2.3	70.8	0.604	0.341
t3.13	149	10	2.3	73.1	**0.003**(U4a)[a]	<**0.001**(KR)[a]
t3.14	263	11	2.2	75.3	**0.038**(U4a)	0.339(AH)
t3.15	304	12	1.8	77.1	**0.007**(U4a)	0.654
t3.16	163	13	1.4	78.5	**0.035**(U4b)	0.654
t3.17	480	14	1.4	80.0	0.809	0.916
t3.18	451	15	1.4	81.3	**0.028**(U4a)	0.158
t3.19	147	16	1.1	82.4	0.265	0.670
t3.20	437	17	1.1	83.5	0.358	0.499
t3.21	140	18	1.1	84.5	0.917	0.657
t3.22	450	19	0.9	85.5	0.058[a]	0.823[a]
t3.23	162	20	0.9	86.4	0.492	0.431

t3.24 Data in bold indicate a significant effect ($p < 0.05$).
Grassland type/site with highest abundance of fragment is indicated next to significant effect (LT: Lough Tay; SG: Sally Gap; KR: Kings River; AH: Annagh Hill).
[a]Significant interaction with each other.

ribotypes, accounting for 38.4% of total abundance, were included in the analysis. Results of CCA (Fig. 2) showed both Axes 1 and 2 had high eigenvalues (0.556 and 0.303), indicating that grassland type and site accounted for a large percentage of variation between fungal profiles. This was confirmed by calculations that indicated Axes 1 and 2 together accounted for 17.0% of the total variation within the data, and 63.6% of the variation that could be attributed to grassland type and site. Species–environment correlations were also very high (above 0.76), indicating that changes in fungal community profiles corresponded strongly with changes in grassland type and site. Monte Carlo tests of significance for the first axis alone, and all axes combined, indicated the analysis accounted for a significant percentage of variation within fungal ARISA data ($p < 0.05$).

The longest arrows in the CCA ordination diagram (Fig. 2) were for the sites Annagh Hill and Kings River, indicating these had the greatest impact on community structure. This was confirmed by intraset correlations, which revealed that Axis 1 was most strongly correlated with Annagh Hill, while Axis 2 was associated with Kings River. Grassland type (U4a and U4b) also had long arrows and high intraset correlations, suggesting that grassland type was also an important factor affecting fungal community structure. A third site, Sally Gap, also had a long arrow in the CCA plot and high correlation values, indicating it affected fungal community structure. In contrast, arrows for the Lough Tay and Long Hill sites were notably shorter than those for the other sites and grassland type, signifying that these had a relatively smaller impact on fungal communities.

These observations were supported by ANOVA of the top 20 most abundant ribotypes (Table 4), which revealed that grassland type had a significant effect on abundances of four of the top 20 ribotypes. Interestingly, all of the four affected had highest abundances in U4b grassland soil. The effect of site was also evident, with those ribotypes significantly affected by site having the highest abundances in Annagh Hill, Kings River, and Sally Gap. In addition, significant interactions between grassland type and site affected the abundances of several fungal ribotypes.

Discussion

The effect of a moderate change in grassland floristic composition (from unimproved U4a to semi-improved U4b) on microbial community structure was investigated at five geographically separate field sites with similar underlying geological substrata. Both grassland type and site were found to impact microbial community structure, but these effects varied and neither influence had a consistently larger impact than the other. The original

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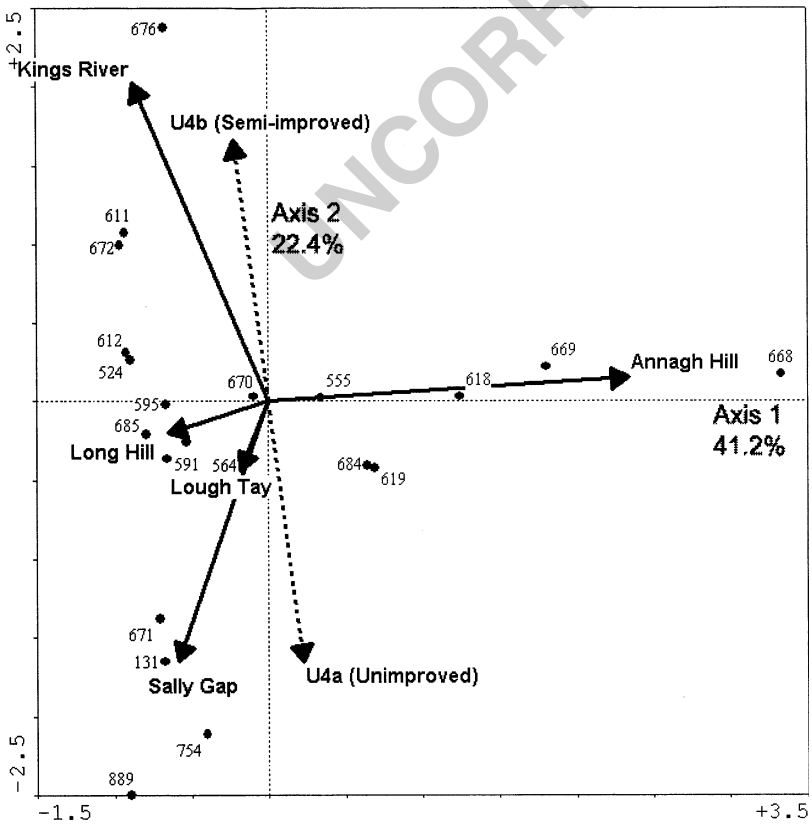

Figure 2. Canonical correspondence analysis (CCA) ordination diagram of fungal ARISA data, with grassland type (*dashed lines*) and sites (*bold solid lines*) represented as *large arrows* and fungal ARISA ribotypes represented as *black dots*. Ribotypes are labeled according to ribotype size (bp). Axis 1 explains 41.2% of the FARISA–environment variance, whereas Axis 2 explains a further 22.4% of the FARISA–environment variance.

t4.1 **Table 4. Abundance rankings and ANOVA results for the top 20 most abundant fungal ARISA fragments, as ranked by average abundance over all samples**

t4.2		*Abundance*			*p values*	
t4.3	*ARISA fragment (bp)*	*Abundance rank*	*% Abundance*	*Cumulative % abundance*	*Grassland type*	*Site*
t4.4	668	1	3.6	3.6	0.754	0.073
t4.5	670	2	3.0	6.6	0.624[a]	0.196[a]
t4.6	524	3	2.5	9.1	**0.049**(U4b)[a]	**0.047**(SG)[a]
t4.7	555	4	2.5	11.6	0.485	0.458
t4.8	684	5	2.4	14.0	0.527	0.326
t4.9	671	6	2.3	16.3	0.859	0.358
t4.10	595	7	2.2	18.5	0.848	0.417
t4.11	672	8	2.1	20.7	**0.015**(U4b)[a]	**0.001**(KR)[a]
t4.12	611	9	2.0	22.7	**0.005**(U4b)	**0.025**(KR)
t4.13	612	10	1.9	24.6	0.137	0.131
t4.14	669	11	1.7	26.3	0.264	0.163
t4.15	131	12	1.6	27.9	0.556	0.771
t4.16	564	13	1.4	29.4	0.060	0.601
t4.17	685	14	1.4	30.8	**0.033**(U4b)	0.099
t4.18	619	15	1.4	32.2	0.072	0.826
t4.19	618	16	1.3	33.5	0.081	**0.049**(AH)
t4.20	889	17	1.3	34.9	0.431	0.329
t4.21	676	18	1.3	36.2	0.193	0.063
t4.22	754	19	1.1	37.3	0.498	0.492
t4.23	591	20	1.1	38.4	0.533	0.061

t4.24 Data in bold indicate a significant effect ($p < 0.05$).
Grassland type/site with highest abundance of fragment is indicated next to significant effect (SG: Sally Gap; KR: Kings River; AH: Annagh Hill).
[a]Significant interaction with each other.

question posed in this study was whether a grassland classification system such as the UK National Vegetation Classification (NVC) could be used as a predictor of microbial community structures at geographically separated sites with similar geological origins. Our results indicate that this hypothesis must be rejected, as microbial community measurements varied widely according to site, and trends in the effect of grassland type were inconsistent.

Strong variation in soil physicochemical properties between sites was noted, indicating that grassland type did not correspond with soil properties. Changes in pH due to semi-improvement were evident, with U4a grasslands having a slightly lower pH overall, although this effect varied between sites. Soil nitrogen levels also varied between sites, as has been noted elsewhere in geographically separated sites with similar grassland types [23]. Although some site variation was noted in soil phosphorus and potassium, at most sites U4b grassland soil had higher phosphorus and potassium contents than U4a, possibly due either to movement of these elements from improved areas, or from increased nutrient mobilization due to increased pH [6].

Although microbial activity was strongly affected by site, there was a consistent influence of grassland type, with semi-improved U4b soils having higher microbial activity than unimproved U4a soils from the same site. This increase in activity from unimproved to semi-improved soils was also noted by Brodie *et al.* [8] and Williams *et al.* [63]. The decrease seen in fungal biomass in semi-improved soils at most sites indicates that that there may be more fungi in unimproved soils, which has been a common finding in studies of upland grasslands [1, 2, 4, 9, 23, 24].

Bacterial ribotype numbers were significantly affected by site, but increased from unimproved U4a to semi-improved U4b grassland soil at most sites, mirroring trends in activity. An increase in bacterial ribotype number from unimproved to semi-improved soils was also noted by Brodie *et al.* [8]. Bacterial community structure was explored by multivariate analysis, which indicated that although grassland type had the largest influence, the Sally Gap and Kings River sites also had a strong impact. The large impact of unimproved and semi-improved grassland types on bacterial community structure indicated that semi-improvement resulted in differing bacterial community structures. Other studies have also observed changes in bacterial community structure after semi-improvement [8, 43]. The strong impact of the Kings River and Sally Gap sites may be related to high microbial activity and low soil nutrient levels, respectively.

Fungal ribotype number was affected significantly by grassland type, with fungal ribotypes decreasing in semi-improved U4b soil as compared to unimproved U4a soil at all sites, which corresponded with the decrease in

fungal biomass noticed at most sites. Although site did not significantly affect fungal ribotype number, canonical correspondence analysis (CCA) of fungal ARISA profiles revealed that certain sites (Annagh Hill, Kings River, and Sally Gap) had a large influence on fungal community structure. Grassland type also had an important influence on soil fungal populations, with unimproved U4a and semi-improved U4b soils having differing fungal community structures.

Studies of geographically separated upland acidic grasslands have been conducted previously. Clegg *et al.* [12], studying unimproved (U4a), semi-improved (U4b), and improved (MG6) grassland soils at three sites, found that complexity rankings of DNA from different grassland types varied between sites, based upon percent G + C content. Grayston *et al.* [23, 24], working on similar sites and grasslands, found differences between grassland types at each site, with variation between sites and seasons, using community-level physiological profiling (CLPP), phospholipid fatty acid contents (PLFA), and percent G + C profiling. These studies, in concurrence with the results presented here, indicate a large impact of site on microbial community structure, often obscuring differences between grassland types. However, they have relied upon broad-scale analyses such as percent G + C content, PLFA, and CLPP. The results presented in this work mark the first time that DNA-based profiling approaches (TRFLP and ARISA) have been applied together to a geographic survey of upland acidic grasslands. DNA-based community fingerprinting approaches can be difficult to interpret because of species differences in rRNA gene copy number [13, 15], biases resulting from PCR amplification [34, 47, 56], and the difficulty in standardizing the amount of DNA analyzed in each replicate [14, 39]. However, as all samples were subject to the same biases, it was still possible to compare them on a relative basis, especially after standardization of ribotype peak heights into proportions per sample.

There are several possible reasons for the lack of grouping in microbial communities in accordance with grassland type. It may be that changes in floristic composition due to semi-improvement did not significantly affect microbial community structure, or were too small to have consistent significant effects. There is evidence suggesting that certain plant species impact microbial community structure more than others [32, 42]. Other studies on the impact of plant community composition on soil microbial communities [10, 18, 25, 26, 29, 31, 59] indicate that changes in functional groups of plants may have stronger impacts than plant diversity or composition *per se*. In our study, floristic composition changed from *Festuca–Agrostis–Galium* domination in unimproved (U4a) grasslands, to *Festuca–Agrostis–Galium* with *Holcus–Trifolium* subcommunities in semi-improved (U4b) grasslands. Although *Trifolium* is a legume, both grassland types were dominated by grasses. Therefore, there was little change in plant functional group composition, which may have contributed to the inconsistency of grassland type effects on microbial community structure.

Site can have a considerable impact on microbial community structure [12]. Several studies have found strong links between soil physicochemical properties such as soil texture and type, and microbial community structure [5, 19, 25, 32, 51, 58]. Correlations between soil physicochemistry and microbial community structure have been found both in field and microcosm studies on acidic upland grassland soils [8, 9, 24, 30, 35]. In our study, site affected all soil physicochemical characteristics (pH, percent N, phosphorus, potassium) significantly, and effects of semi-improvement on soil chemistry also varied significantly between sites. These inconsistent trends between sites may mean that minor changes in soil chemistry within a site could be sufficient to support a change in floristic composition, but are not enough to strongly affect soil microbial populations. Often, changes in soil chemical parameters were greater between sites than between grassland types at a site, which may contribute to the impact of site on microbial community structure. For example, the same floristic composition can occur at different sites at a range of pH values (published values for U4a grassland soils range from 3.3 to 6.3, and for U4b grassland soils range from 3.98 to 6.4 [8, 11, 12, 23, 24, 60]); such large differences in pH between sites with the same floristic grassland classification could strongly affect soil microbial communities.

In practice, the real determinants of microbial community structure between sites and grassland types are likely to be complex interactions between plant composition effects, and individual site characteristics, including soil physicochemical composition. A recent study of an unimproved (U4a) grassland in Scotland reported that CLPP, DGGE, and PLFA profiles were affected by both vegetation class and soil physical and chemical factors, but no single factor or factors stood out [48]; rather, it appeared that these interacted in complex mechanisms to influence soil microbial communities. It is likely that a similar situation exists in relation to the sites and grassland types investigated in our study.

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