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Impact of lime, nitrogen and plant species on bacterial community structure in grassland microcosms

Nabla Kennedy,[1] Eoin Brodie,[1†] John Connolly,[2] and Nicholas Clipson[1*]

[1]*Microbial Ecology Group, Department of Industrial Microbiology, University College Dublin, Belfield, Dublin 4, Ireland.*

[2]*Department of Statistics, University College Dublin, Belfield, Dublin 4, Ireland.*

Summary

A microcosm-based approach was used to study impacts of plant and chemical factors on the bacterial community structure of an upland acidic grassland soil. Seven perennial plant species typical of both natural, unimproved (*Nardus stricta*, *Agrostis capillaris*, *Festuca ovina* and *F. rubra*) and fertilised, improved (*Holcus lanatus, Lolium perenne* and *Trifolium repens*) grasslands were either left unamended or treated with lime, nitrogen, or lime plus nitrogen in a 75-day glasshouse experiment. Lime and nitrogen amendment were shown to have a greater effect on microbial activity, biomass and bacterial ribotype number than plant species. Liming increased soil pH, microbial activity and biomass, while decreasing ribotype number. Nitrogen addition decreased soil pH, microbial activity and ribotype number. Addition of lime plus nitrogen had intermediate effects, which appeared to be driven more by lime than nitrogen. Terminal restriction fragment length polymorphism (TRFLP) analysis revealed that lime and nitrogen addition altered soil bacterial community structure, while plant species had little effect. These results were further confirmed by multivariate redundancy analysis, and suggest that soil lime and nitrogen status are more important controllers of bacterial community structure than plant rhizosphere effects.

Introduction

Increased intensification of grasslands is common in upland areas, with fields previously used for rough grazing being fertilised and limed to increase plant and animal productivity (Blackstock *et al*., 1999). Intensification processes have led to diminished floristic diversity (Newbould, 1985; Green, 1990), with a shift from seminatural, unimproved species-rich grasslands to species-poor but agriculturally high-yielding (improved) plant communities. In Ireland and the UK, the predominant grass species of upland acidic grasslands is *Agrostis capillaris*, with *Nardus stricta*, *Festuca ovina*, *F. rubra* and *Holcus lanatus* occurring at lower abundances as part of a highly diverse plant assemblage (Rodwell, 1992). Such seminatural acidic grasslands classify as U4 types within the UK National Vegetation Classification (Rodwell, 1992), and are converted under improvement to much less diverse mesotrophic forms, principally MG6 or MG7, dominated by *Lolium perenne* and *Trifolium repens*. Loss in floristic diversity resulting from intensification has been a cause for concern (Grime, 1998; Hooper and Vitousek, 1997), particularly as little is known about the possible resulting impacts on below-ground diversity including soil microbial communities. Changes in soil biodiversity may affect important soil ecosystem processes such as biogeochemical cycling and decomposition (Stoate *et al*., 2001).

At present, there is no clear view of the factors determining below-ground microbial diversity, with data indicating both plant-species-related influences and the influences of changes in soil chemical status. Several possible factors driving shifts in microbial community composition during intensification have been postulated, including soil physico-chemical variables such as pH and nitrogen concentration, the influence of shifts in floristic composition during improvement, physical factors such as changes in soil structure and tillage (Sessitsch *et al*., 2001; Ibekwe *et al*., 2002), and grazing (Bardgett *et al*., 1997). pH has been found to have a significant impact on microbial diversity in soils (Frostegård *et al*., 1993; Bååth, 1996; Blagodatskaya and Anderson, 1998; Bååth and Anderson, 2003; Steenwerth *et al*., 2003), whereas addition of nitrogen also affects the structure of soil microbial communities (Steer and Harris, 2000; Clegg *et al*., 2003; Gray *et al*., 2003). Soil type, encompassing mineral composition (percentage silt, sand, clay), chemical characteristics (pH, carbon, nitrogen, phosphorus), and management practices (tilling) can also be dominant factors affecting soil microbial community structure (Groffman *et al*., 1996; Latour *et al*., 1996; Marschner *et al*.,

Received 16 December, 2003; accepted 15 March, 2004. *For correspondence. E-mail nicholas.clipson@ucd.ie; Tel. (+353) 17161365; Fax (+ 353) 17161183. †Present address: Department of Environmental Science, Policy and Management, Division of Ecosystem Sciences, University of California, Berkeley, CA 94720, USA.

2001; Schutter *et al*., 2001; Girvan *et al*., 2003). Another significant factor may be the change in plant species that occurs during grassland improvement. Grayston *et al*. (1998) found that microbial communities from the rhizospheres of different plant species produced characteristic C-source utilization patterns, and in a later study Grayston *et al*. (2001) using phospholipid fatty acid analysis (PLFA) determined that plant species was an important factor in the composition of rhizosphere microbial populations. Other studies (Miethling *et al*., 2000; Marschner *et al*., 2001; Smalla *et al*., 2001) using denaturing or temperature gradient gel electrophoresis (D/TGGE) have also found rhizosphere community composition to be plant-species specific.

Relatively little is known specifically about the factors determining soil bacterial community structures in acidic grasslands. Some previous studies based upon analysis of soil PLFAs have suggested that changes in plant composition may be associated with changes in soil microbial communities (Grayston *et al*., 2001). Brodie *et al*. (2002, 2003), using molecular fingerprinting approaches, observed that numbers of bacterial ribotypes increased in soil along a vegetational gradient between U4a and MG7 grassland, whereas fungal biomass decreased and fungal ribotype number was unaffected. These changes in microbial community structure were coincident with a reduction in plant species diversity over the vegetational gradient. Brodie *et al*. (2002) suggested that this inverse relationship between bacterial and plant diversity might indicate that the influence of changes in environmental parameters between vegetational types outweighs the influence of plant rhizosphere effects on bacterial community structure. However, as these were field studies, it was not possible to separate abiotic and biotic influences. In our study, the effects of individual plant species and of three chemical treatment regimes were isolated through the use of monoculture microcosms, which have been widely used for studying mechanisms controlling microbial communities and nutrient availability in upland soils (e.g. Bardgett *et al*., 1998; Duineveld *et al*., 1998). It was hypothesized that soil physicochemical changes, resulting from addition of lime and nitrogen, would impose a stronger selective pressure on bacterial communities than plant species effects alone.

Results

The pH of microcosm soil from each plant species was assessed at harvest (Table 1). ANOVA revealed that the biggest effects resulted from the addition of lime ($P < 0.001$) and nitrogen ($P < 0.001$), and their interaction ($P < 0.001$). Responses to the chemical treatments were fairly consistent across all plant species, with lime addition (with or without nitrogen) resulting in an average increase of 0.6 pH units over untreated soil, whereas nitrogen addition alone acidified the soil by an average of 0.4 pH units compared to untreated soil. A smaller but significant effect ($P = 0.002$) of plant species on pH could be attributed to *A. capillaris*, which had a significant acidifying effect on the soil. A significant ($P = 0.007$) interaction between plant species and nitrogen was also observed, with the addition of nitrogen lowering the pH of the soil by an average of 0.2 units for all species except *A. capillaris*, where nitrogen addition caused a reduction of 0.7 units.

Microbial activity and biomass-C of rhizosphere soil communities were assessed by measurements of dehy-

Table 1. Soil and microbial community measurements as affected by chemical treatments and plant species.

	pH	Microbial activity (μg TPF g^{-1} dry soil)	Microbial biomass (μg biomass-C g^{-1} dry soil)	Ribotype number (mean TRFs per replicate)
Chemical treatments				
Plant Only	4.23b	64.5b	25.9a	36.0b
+ Lime	4.84c	176.3d	32.8b	14.8a
+ Nitrogen	3.80a	13.3a	23.2a	16.5a
+ Lime + N	4.83c	120.5c	34.1b	23.7a
SED	0.069	8.11	1.64	4.18
Plant species				
N. stricta	4.52b	110.2bc	33.5b	26.6
A. capillaris	4.13a	38.3a	32.1b	21.1
F. ovina	4.40b	88.0b	25.8ab	24.9
F. rubra	4.36b	75.7ab	24.1a	26.6
H. lanatus	4.51b	96.1bc	27.2ab	22.7
L. perenne	4.48b	104.2bc	29.0ab	20.1
T. repens	4.51b	134.7c	27.7ab	19.1
Bare soil	4.50b	101.9bc	32.6b	20.9
P-value (Plant species)	**	***	**	NS
SED	0.098	11.47	2.32	5.91

Means and standard error of differences (SED) are shown ($n = 32$ for chemical treatments, $n = 16$ for plant species). Same letter denotes no significant difference between values in each category (chemical treatments or plant species). ANOVA *P*-values for plant species shown as **, $P < 0.01$; ***, $P < 0.001$; NS, not significant. ANOVA results for chemical treatments and interactions are discussed in the text.

drogenase and substrate-induced respiration respectively. Addition of lime ($P < 0.001$) and nitrogen ($P < 0.001$) had the biggest effect on microbial activity, with plant species accounting for a smaller but significant ($P < 0.001$) effect. The means for chemical treatments displayed in Table 1 show a large increase in activity as a result of lime addition, with addition of nitrogen causing a sharp decrease in activity, as compared to unamended control soil. Addition of lime plus nitrogen increased microbial activity compared to unamended soil, although not to as great an extent as lime alone. Differences in means for plant species were mainly caused by *A. capillaris*, which displayed significantly lower activity than every other plant species except *F. rubra*, whereas soil from *N. stricta* and *T. repens* had the highest activity readings. There was also a small but significant ($P = 0.002$) interaction between plant species and lime, which could be attributed to large increases in activity in soil from *N. stricta* and *T. repens* after addition of lime.

Addition of lime ($P < 0.001$) and plant species ($P = 0.002$) had the biggest effects on microbial biomass, with less significant effects from the interaction between plant species and lime ($P = 0.04$), and nitrogen having no significant effect. Means for the chemical treatments (Table 1) revealed a marked effect of lime, with addition of lime alone and lime plus nitrogen increasing microbial biomass significantly as compared to unamended control soil. Plant species means showed that soil from *F. rubra* had a significantly lower microbial biomass than soils from *N. stricta*, bare soil and *A. capillaris*. Analysis of the interaction between plant species and lime revealed that soil from *N. stricta* and bare soil exhibited larger increases after lime addition than the other plant species, as compared to unamended soil.

Terminal restriction fragment length polymorphism (TRFLP) was used to generate a profile for each sample, consisting of the terminal restriction fragments (TRFs) present and their relative abundances. Each TRF indicated one sequence polymorphism or ribotype. A total of 216 unique TRFs were detected after analysis of all soils, with the number of ribotypes varying between treatments (Table 1). The lime–nitrogen interaction was found to have the biggest impact on ribotype number (ANOVA, $P < 0.001$), with lime having a smaller effect ($P = 0.02$). Chemical treatment means indicated that the highest ribotype numbers were found in unamended soils, with addition of lime and nitrogen decreasing ribotype number significantly as compared to unamended soil. Addition of lime plus nitrogen decreased ribotype number significantly in relation to unamended soils, but to a lesser extent than lime or nitrogen alone. There was no significant effect of plant species on ribotype number.

Analysis of bacterial TRFLP profiles by a randomization test approach revealed that most differences occurred between chemical treatments, rather than among plant species. Table 2A details the effect of chemical treatments on individual plant species. A large effect of chemical treatment could be seen, with addition of lime, nitrogen, and lime plus nitrogen causing a significant change in TRFLP profiles of soils from all plant species. The only exceptions were *F. ovina* and *F. rubra*, which were not significantly affected by the addition of lime plus nitrogen. Comparisons between plant species without chemical amendments are shown in Table 2B. In contrast to the differences seen between chemical treatments, there were no significant differences between TRFLP profiles from soils of different plant species in the plant-only treatments. Table 2C compares different plant species within a chemical treatment. Addition of lime resulted in significant differences between the TRFLP profiles of soil from *A. capillaris* and every other plant except *N. stricta*, while bare soil was found to have a different TRFLP profile to every other species except *F. ovina* and *T. repens*, and *T. repens* was significantly different to *F. rubra*. Addition of nitrogen also caused differences in the TRFLP profiles of different plant species, with soil from *N. stricta* significantly different to all plant species except *T. repens*. *A. capillaris* and *F. rubra* also exhibited several significant differences from other plant species after addition of nitrogen. In soils amended with nitrogen plus lime, the plant species exhibiting significant differences were *H. lanatus*, *A. capillaris* and *L. perenne*.

Multivariate statistical analysis was carried out in order to determine the main factors affecting TRFLP profiles in microcosm soils. First, redundancy analysis (RDA) of all TRFs with all environmental factors including plant species, lime level, nitrogen level and all of their interactions was carried out. Then, analysis was limited to sets of the top 100, 50 and 20 TRFs, as ranked by abundance. Comparison of the results from these analyses revealed only minor changes in axes scores after limiting the data set, indicating that the top 20 most abundant TRFs (totaling 85% of the overall abundance) accounted for the majority of the variance in the data; therefore only the top 20 TRFs were used in subsequent analysis. Table 3 details the redundancy analysis. Axes 1 ($P < 0.005$) and 2 were found to explain 28.0% and 14.6% of the overall variance within the TRF data, respectively, accounting for a total of 42.7% of the total variance. The cumulative species-environment relation for Axes 1 and 2 was 81%, indicating that these axes accounted for the bulk of the variance in the TRF data that could be attributed to environmental factors (lime, nitrogen and plant species). Species-environment correlations for both axes were above 0.77, indicating that the TRF data were strongly correlated with environmental parameters. Monte-Carlo significance tests revealed that both the first axis, and all axes combined, explained a significant amount of the variation within the data.

Table 2. Results of randomization test analysis of TRFLP profiles. *, $P < 0.05$; NS = non-significant; $n = 4$.(A) Pairwise comparisons between plant only and chemical treatments for the same plant species.

	Effect of chemical treatments							
Chemical treatment	*N. stricta*	*A. capillaris*	*F. ovina*	*F. rubra*	*H. lanatus*	*L. perenne*	*T. repens*	Bare soil
+ Lime	*	*	*	*	*	*	*	*
+ Nitrogen	*	*	*	*	*	*	*	*
+ Lime + N	*	*	NS	NS	*	*	*	*

(B) Pairwise comparisons between plant species from the plant-only treatment.

		Effect of plant species						
Chemical treatment	Plant species	*N. stricta*	*A. capillaris*	*F. ovina*	*F. rubra*	*H. lanatus*	*L. perenne*	*T. repens*
Plant only	*A. capillaris*	NS						
Plant only	*F. ovina*	NS	NS					
Plant only	*F. rubra*	NS	NS	NS				
Plant only	*H. lanatus*	NS	NS	NS	NS			
Plant only	*L. perenne*	NS	NS	NS	NS	NS		
Plant only	*T. repens*	NS	NS	NS	NS	NS	NS	
Plant only	Bare soil	NS	NS	NS	NS	NS	NS	NS

(C) Pairwise comparisons between different plant species within a chemical treatment.

		Interactions between chemical treatments and plant species						
Chemical treatment	Plant species	*N. stricta*	*A. capillaris*	*F. ovina*	*F. rubra*	*H. lanatus*	*L. perenne*	*T. repens*
+ Lime	*A. capillaris*	NS						
+ Lime	*F. ovina*	NS	*					
+ Lime	*F. rubra*	NS	*	NS				
+ Lime	*H. lanatus*	NS	*	NS	NS			
+ Lime	*L. perenne*	*	*	NS	*	*		
+ Lime	*T. repens*	NS	*	NS	*	NS	NS	
+ Lime	Bare soil	*	*	NS	*	*	*	NS
+ Nitrogen	*A. capillaris*	*						
+ Nitrogen	*F. ovina*	*	NS					
+ Nitrogen	*F. rubra*	*	*	NS				
+ Nitrogen	*H. lanatus*	*	NS	NS	*			
+ Nitrogen	*L. perenne*	*	*	NS	NS	NS		
+ Nitrogen	*T. repens*	NS	*	NS	NS	NS	NS	
+ Nitrogen	Bare soil	*	*	NS	*	NS	NS	NS
+ Lime + N	*A. capillaris*	NS						
+ Lime + N	*F. ovina*	NS	*					
+ Lime + N	*F. rubra*	NS	*	NS				
+ Lime + N	*H. lanatus*	NS	*	NS	*			
+ Lime + N	*L. perenne*	NS	NS	NS	NS	*		
+ Lime + N	*T. repens*	NS	*	NS	NS	*	*	
+ Lime + N	Bare soil	NS	NS	NS	NS	*	NS	NS

Figure 1 shows a plot of the top 10 terminal restriction fragments in relation to the lime, nitrogen, and lime and nitrogen interaction axes (although the top 20 most abundant TRFs were analysed, only the top 10 are displayed for clarity). Canonical coefficients and intraset correlations for the environmental factors for each axis indicated that Axis 1 was primarily a nitrogen gradient and Axis 2 a lime gradient. The plot can be interpreted qualitatively by noting that the length of an arrow indicates how much variance is explained by that factor. The direction of arrows for individual environmental factors indicates an increasing concentration of that factor. Arrows for nitrogen, lime, and their interaction were at least twice those for plant species, indicating that these chemical factors accounted for a much greater proportion of variance in the TRFLP profiles than plant species. The top 10 most abundant TRFs are shown on the plot as arrows. Terminal restriction fragment arrows pointing in approximately the same direction as environmental factors indicate a high positive correlation (the longer the TRF arrow, the stronger the relationship). For example, the position of TRF 92, pointing in the opposite direction to the arrow for nitrogen, indicated that the bacterial species represented by that fragment was negatively correlated with nitrogen. These

Table 3. Results of redundancy analysis (RDA). Values are for Axes 1 and 2 plotted in the RDA diagram in Fig. 1. The highest canonical coefficients and correlations are highlighted in bold.

Axis	1	2
Eigenvalue	0.280	0.146
Cumulative percentage variance:		
Of species data	28.0	42.7
Of species-environment relation	53.2	81.0
Species-environment correlations	0.93	0.77
Monte-Carlo significance test:		For all axes:
F-ratio	45.6	13.0
P-value	0.005	0.005
Coefficients ($100 \times c$):		
Lime	15	84
Nitrogen	62	36
Lime*Nitrogen	12	–76
Correlations ($100 \times r$):		
Lime	28	50
Nitrogen	88	–10
Lime*Nitrogen	72	–8

observations were supported by ANOVA results for TRF 92 (Table 4), which revealed that nitrogen had a significant, negative effect on its relative abundance. ANOVA results also indicated which environmental factors significantly contributed to the variance in the TRFLP profile for each particular fragment. The overall trend for the 10 TRFs analysed by ANOVA agreed with that of the multivariate analysis, with lime, nitrogen and their interaction appearing most frequently as significant environmental factors. Occasionally, plant species or the interaction of plant species with a chemical factor was significant, but lime, nitrogen, and/or their interaction were always significant at a higher level.

Discussion

The primary findings of this microcosm-based study indicated that addition of lime or nitrogen had a much greater influence on soil bacterial community structure than the

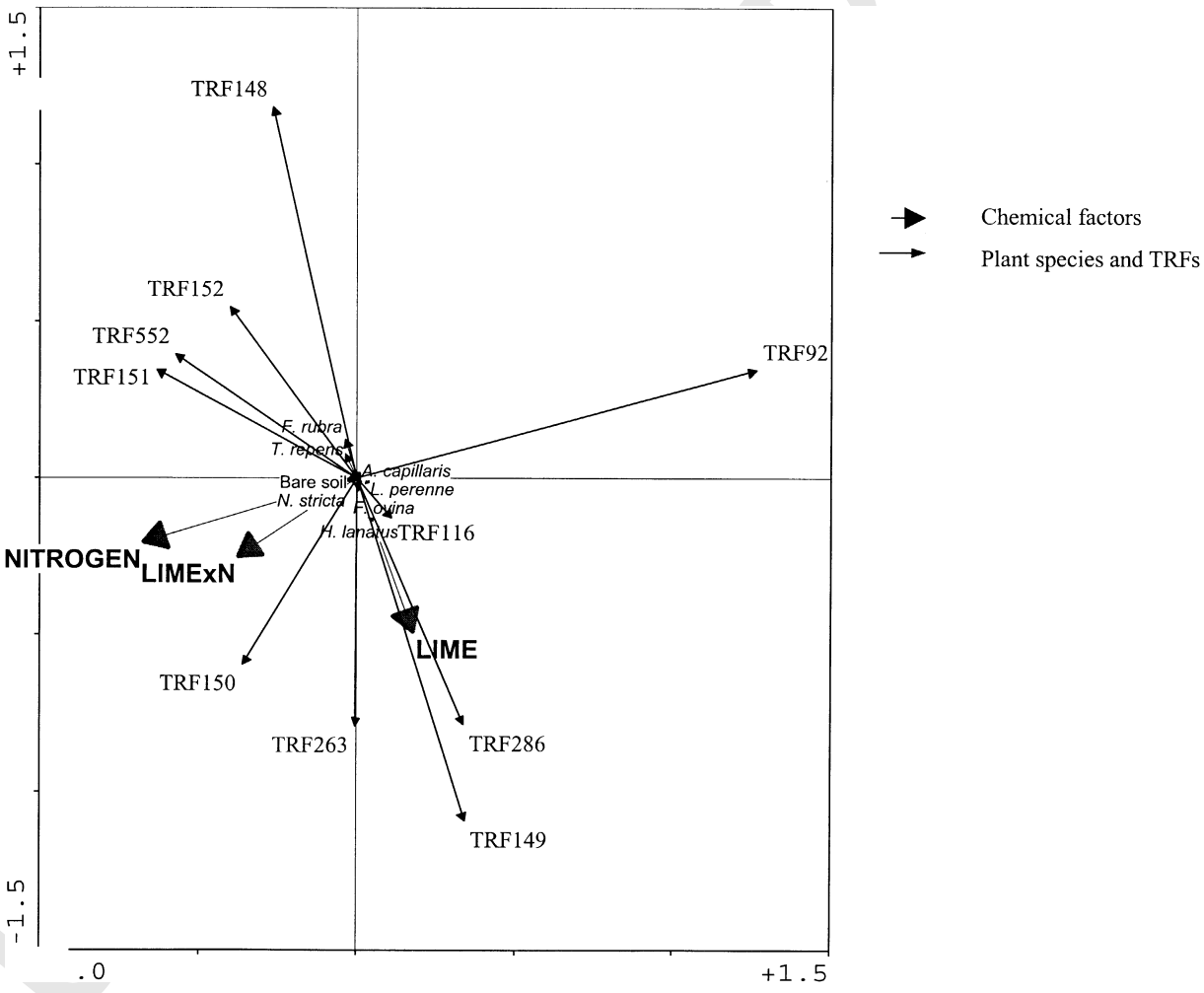

Fig. 1. Redundancy analysis (RDA) ordination diagram of TRFLP data, with chemical variables represented as large arrows and plant species and TRFs represented as smaller arrows. TRFs are labelled according to fragment size.

Table 4. ANOVA results for the top 10 terminal restriction fragments (TRFs) in the TRFLP data, as ranked by abundance *P*-values.

TRF	Abundance rank	% Abundance	Cumulative % abundance	*P*-values		
				Plant species	Lime	Nitrogen
150	1	18.0	18.0	0.046	0.140	**<0.001**$^{+}$
92	2	11.8	29.8	0.516	0.009	**<0.001**$^{-b}$
151	3	11.6	41.4	0.005	**<0.001**$^{-}$	**<0.001**$^{+a}$
149	4	5.8	47.2	0.025	**<0.001**$^{+c}$	0.089
116	5	4.7	51.9	0.262	0.049	0.016
148	6	4.3	56.2	0.130	**<0.001**$^{-c}$	0.320
552	7	4.0	60.2	0.028	0.084	**<0.001**$^{+}$
286	8	3.9	64.1	0.568	**<0.001**$^{+c}$	0.002
263	9	3.0	67.1	0.088	**<0.001**$^{+c}$	0.731
152	10	2.9	70.0	0.014	**<0.001**$^{-c}$	0.006

a. Significant interaction with species.
b. Significant interaction with lime.
c. Significant interaction with nitrogen**.**
Bold indicates a significant effect ($P < 0.001$); $^{+}$positive effect on TRF abundance; $^{-}$negative effect on TRF abundance.

rhizosphere effect of individual grassland plant species. The addition of lime (alone or in conjunction with nitrogen) increased pH, microbial activity and microbial biomass. Although these measurements were not able to discriminate between bacterial and fungal contributions, fungal biomass is known to be higher in acidic upland grassland soils in comparison to mesotrophic soils (Bardgett *et al*., 1996; Brodie *et al*., 2003), which might suggest that changes in microbial biomass observed here may largely be the result of bacteria. This theory is supported by the work of Neale *et al*. (1997), who found that increases in soil respiration and microbial biomass after addition of lime to acid soils could be attributed to proliferation of indigenous acid-intolerant bacteria. Although increasing microbial activity and biomass, lime was found to reduce bacterial ribotype number. This would suggest that liming, probably through raising soil pH, acts as a selective agent on the soil bacterial community, increasing the biomass and dominance of neutrophilic species.

Amendment of soils with nitrogen reduced pH, microbial activity and ribotype number, significantly, but had no effect on microbial biomass. Addition of nitrogen typically reduces soil pH (Fenton and Helyar, 2000), as was observed during these experiments, and it remains unclear whether nitrogen-induced changes in bacterial community structure are a product of soil pH change or are due to the effects of increased concentrations of ammonium or nitrate ions (Fog, 1988). Amendment with lime plus nitrogen raised pH to nearly the same level as lime alone, indicating that the neutralizing effect of lime overrode the acidifying effect of nitrogen. Microbial activity exhibited an increase in response to addition of lime plus nitrogen, however, it was not as large as the increase in activity observed after addition of lime alone. The increase in microbial biomass occurring after addition of lime plus nitrogen could be attributed to the effect of lime, as ANOVA analysis revealed that there was no significant effect of nitrogen or the lime–nitrogen interaction. In general, changes caused by lime plus nitrogen seemed to be driven more by lime than nitrogen, which might suggest that changes in soil pH may be more influential in determining soil microbial community structure than nitrogen. Interestingly, ribotype number was strongly affected by the lime–nitrogen interaction, with addition of lime plus nitrogen causing a significantly smaller decrease in ribotype number than addition of either lime or nitrogen alone.

In contrast to lime and nitrogen, the effect of plant species on soil microbial community measurements was small. *Agrostis capillaris* was found to have an negative effect on both soil pH and microbial activity, whereas soil from *N. stricta* and *T. repens* exhibited high microbial activity, especially after addition of lime. *Nardus stricta* also experienced a positive interaction with lime in relation to microbial biomass, as did bare soil. However, for most measurements the effects of the chemical treatments outweighed those due to plant species.

Further analysis was necessary to reduce the complexity of community fingerprinting data so that the underlying factors responsible for changes in bacterial community structure could be elucidated. Terminal restriction fragment length polymorphism is a powerful tool in that it provides information about bacterial dominance (number of ribotypes), whereas the fragment peak height indicates the relative abundance of each ribotype within a soil sample. A randomization test procedure was devised in order to determine which changes between communities were statistically significant. Community fingerprinting approaches can be difficult to interpret because of species differences in rRNA gene copy number, biases resulting from PCR amplification, and the difficulty in standardizing the amount of DNA analysed in each replicate (Crosby and Criddle, 2003; Egert and Friedrich, 2003; Lueders and Friedrich, 2003). However, as all samples were subject to the same biases, it is still possible to compare

between them on a relative basis, especially after standardization of TRF peak heights into proportions per sample. The randomization tests revealed significant changes in bacterial community structure as a result of the chemical treatments, with very few changes seen due to plant species. There did appear to be some significant interactions between plant species and chemical treatments, with a few significant changes occurring between plant species in chemically amended soils. However, the overall trend revealed a large impact of lime and nitrogen on soil bacterial community structure, with plant species only having an effect in conjunction with chemical factors. This trend could also clearly be seen in the multivariate redundancy analysis (RDA). The randomization test procedure gave a view of the individual differences between samples, but required multiple comparisons. In contrast, RDA is a multivariate approach that is useful for revealing large-scale trends. The RDA relates each individual TRF profile to linear combinations of the environmental variables (plant species, lime, nitrogen, and their interactions) in order to reveal which environmental factors are correlated with changes in the TRF data. Redundancy analysis of TRF profiles revealed that a large percentage (42.7%) of the variation within the TRF data was attributable to two factors, nitrogen and lime. The effect of individual plant species was very small in comparison, reinforcing the view that lime and nitrogen concentrations affected bacterial community structure far more than plant species.

With both lime and nitrogen amendment affecting bacterial community structure, a key question is how community shifts are mediated. Bacterial community structure is driven by changes in both species diversity and abundance, often termed species richness and evenness. In this study, ribotype number was used as a measure of the diversity of the dominant bacterial species, and TRF peak height as a relative indicator of evenness, although this must be treated with caution due to the problems of variable rRNA gene copy number, as discussed above. Presumably, environmental parameters act as selective factors (McCaig *et al*., 2001), with liming perhaps selecting for neutrophilic species. Nitrogen addition (as ammonium nitrate) influences bacterially mediated nitrogen cycling processes such as nitrification and nitrogen fixation, with higher nitrogen concentrations promoting bacterial decomposition and lower concentrations promoting decomposition by fungi (Bardgett *et al*., 1999). Addition of ammonium nitrate in this study was also found to lower soil pH, an interaction that was found to be important, indicating that chemical factors can influence each other and thus affect the soil bacterial community both directly and indirectly. Additionally, time must be considered as a factor when discussing influences on bacterial community structure. In a microcosm study, Bardgett *et al*. (1999) found significant differences in the PLFA profiles from rhizospheres of different grassland soils after 107 days, there being no significant difference at earlier sampling dates. Although direct comparison is difficult because of the different profiling techniques used, it is possible that plant species effects may take longer than the 75-day time span of our experiment to appear. In contrast, in Bardgett and co-workers' study, nitrogen addition affected PLFA profiles from earlier sampling dates, indicating that nitrogen may affect microbial communities more in the short term.

Our study attempted to isolate the factors responsible for the differences in bacterial community structure of unimproved upland acidic grasslands and improved mesotrophic areas seen in field studies. Brodie *et al*. (2002) examined changes in bacterial communities over a floristic gradient between an acidic and a mesotrophic community, which was coincident with a gradient of increasing pH and nitrogen. Bacterial ribotype numbers were higher than those found in our study, which may indicate that monoculture microcosms limit bacterial numbers as compared to field situations, where plant species grown together can interact synergistically (Wardle and Nicholson, 1996). It was found that bacterial ribotype number increased with improvement, but interpretation of this effect is problematic as other parameters such as soil nitrogen status and floristic composition were changing in parallel. However, definite changes in TRFLP profiles from unimproved and improved soils were seen, which concur with the changes found in our study after addition of nitrogen and lime. Similar changes in bacterial community structure between acidic and mesotrophic soils were found in other studies using DGGE (McCaig *et al*., 2001), and PLFA (Grayston *et al*., 2001; 2003). In another study of an upland grassland soil, Gray *et al*. (2003) found that eubacterial community structure, assessed using temporal temperature gradient electrophoresis, changed when lime was added to field plots. Although caution must be used when extrapolating controlled laboratory experiments to field conditions (Drake *et al*., 1996; Ibekwe and Kennedy, 1998), the changes caused by lime and nitrogen in our experiment appear to be similar to the changes in bacterial community structure seen in field-based studies after upland acidic grassland improvement.

The most surprising findings in this data were the lack of effect of plant species on bacterial community structure. It is generally thought that plants influence their rhizospheres by exudation of organic compounds and root senescence (Tyler and Ström, 1995; Kapulnik and Okon, 2002), and that these influences can also affect bacterial community structure. The plant species used in this microcosm study included those characteristic of unimproved acidic upland grasslands (*A. capillaris*, *N. stricta*, *F. ovina*, *F. rubra* and *H. lanatus*), and those typical of improved mesotrophic grasslands (*L. perenne* and *T. repens*). The

microcosms were performed using unimproved acidic grassland soil, representing native habitat to the upland grass species studied but not to the mesotrophic species, which normally grow on more neutral, nutrient-rich soils. However, analysis of TRFLP profiles showed that the mesotrophic species *L. perenne* and *T. repens* had no significant rhizosphere effect on bacterial community structure in the absence of chemical treatments. This would suggest that under acidic conditions, at least in terms of how they influence their rhizospheres, all the acidic and mesotrophic plant species studied act similarly. This changed when chemical treatments were applied, particularly for *A. capillaris* after addition of lime and lime plus nitrogen, and *N. stricta* on the application of nitrogen. These species are typical of acidic, low nutrient grasslands, and the addition of chemical treatments may have caused changes in rhizodeposition as the plants adapted to nutrients. Thus, it would appear that plant-mediated effects only significantly impact upon bacterial community structure after interaction with chemical factors. In other studies of grassland microbial community structure, plant species effects have often been found to be influential in determining bacterial communities (Grayston *et al*., 1998; Bardgett *et al*., 1999; Johnson *et al*., 2003; Steenwerth *et al*., 2003), although several recent studies have found plant diversity to have inconsistent effects on soil microbial measurements (Gastine *et al*., 2003; Hedlund *et al*., 2003). The results reported in this study represent the first data combining a microcosm based approach with molecular community fingerprinting for an acidic grassland soil. The TRFLP-based approaches are able to give a fuller breakdown of populations present than other commonly used techniques such as CLPP and PFLA (Hill *et al*., 2000), and support the hypothesis that plant species is not a major determinant of bacterial community structure for this acidic grassland soil.

In conclusion, this study demonstrates that soil physicochemical factors (lime and nitrogen) affect bacterial community structure in upland acidic grassland soil more than plant species. Although the experiment was laboratory-based, these findings are consistent with those from previous field-based studies (Brodie *et al*., 2002; 2003). Changes in bacterial community structure are likely to have consequences for nutrient cycling and related processes, and therefore understanding the factors influencing microbial communities in these swards is essential for proper ecological management of upland acidic grassland areas.

Experimental procedures

Soil

Soil was collected in June 2000 from an area of unimproved Nardo-Galion grassland at Longhill, Kilmacanogue, County Wicklow, Ireland (National Grid Reference O 218 124). The site is 300 m above sea level and consists of a peaty podzolic soil formed over granite/quartzite bedrock (Geological Survey of Ireland, 2000), with an annual rainfall between 2000 and 2800 mm (Met Eireann, 2003). Approximately 20 kg of soil (pH 4.5) were taken from the Ah horizon (5–15 cm depth), sieved to <6 mm, and used for microcosms.

Microcosms

Microcosms were prepared by weighing 80 g (dry mass) soil into opaque cylindrical PVC pots (40 mm diameter, 110 mm height), pierced to allow free drainage of water. Water content was adjusted to 30% (w/w) with distilled water and pots were planted with 15 surface-sterilized (2% sodium hypochlorite) seeds (Emorsgate Seeds, King's Lynn, UK) of the required grassland plant species (*Nardus stricta*, *Agrostis capillaris*, *Festuca ovina*, *Festuca rubra*, *Holcus lanatus*, *Lolium perenne*, or *Trifolium repens*). A set of control pots was left unplanted (bare soil). Sixteen pots of each of the seven plant species and the bare soil were prepared, and microcosms were incubated in a greenhouse in a randomized block design for 75 days from seed germination, from 31 July to 16 October 2000. Water content was maintained at 30% by addition of distilled water as necessary. At day 25, both planted and unplanted soils were treated as follows: treatment 1 – no addition; treatment 2 – addition of lime equivalent to 5 tons ha^{-1}; treatment 3 – addition of nitrogen (as ammonium nitrate) equivalent to 150 kg ha^{-1}; treatment 4 – addition of both lime and nitrogen as in treatments 2 and 3. Microcosms were destructively sampled on Day 75. Plant root and shoot material were removed, dried at 70°C for 7 days, and weighed. Because of the small size of the microcosms used and the high root density in pots, all soil was assumed to have been in contact with plant roots and was considered rhizosphere. Soil was sieved to <4 mm and stored at 4°C for less than 7 days for pH, microbial activity and biomass analysis, and at −20°C for molecular analyses.

pH

Microcosm soil samples were analysed for pH_{water} by standard methods according to Sparks *et al*. (1996).

Total microbial biomass

Soil microbial biomass carbon was measured using a modification of the substrate induced respiration methods of Anderson and Domsch (1978) and West and Starling, 1986), as described previously (Brodie *et al*., 2002).

Total microbial activity

Total microbial activity was assessed as triphenylformazan dehydrogenase activity and was determined based on a modification of the method of Thalmann (1968), as previously described by Brodie *et al*. (2002).

Total soil DNA extraction and purification

Total soil DNA was extracted as described in Brodie *et al*. (2002). Briefly, soil (0.5 g) was added to tubes containing

glass and zirconia beads, to which CTAB (hexadecyltrimethylammonium bromide) 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 at 5.5 m s^{-1} for 30 s. Following beadbeating, 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^{-1}) 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 a final volume of 50 μl and was consistently suitable for PCR amplification without further treatment.

Bacterial community fingerprinting by T-RFLP analysis

Terminal restriction fragment lengths were determined using a modification of the method of Liu *et al*. (1997). After extraction and purification of total DNA from soil, the bacterial 16S small subunit rRNA gene was amplified using primer set F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and R1492 (5'-TACG GYTACCTTGTTACGACT-3') (Lane, 1991). The forward primer F27 was labelled with Beckman Coulter fluorescent dye D4. 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, WI, USA). 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 according to 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. Terminal restriction fragment lengths were determined by electrophoresis using a Beckman Coulter (CEQ2000) automated sequencer, and analysis of fragment profiles was performed using the Beckman Coulter fragment analysis package v.4.0. Only fragments with a fluorescence greater than 1% of the total fluorescence were included in the analyses. 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 fragments in the range 400–600 bp. Terminal restriction fragments (TRFs) that differed by less than 0.5 bp in different profiles were considered identical. Each individual TRF was considered a ribotype, and the mean TRF or ribotype number in each sample was determined.

Statistical analysis

pH, microbial biomass, microbial activity, ribotype number and top 10 TRF results were analysed by one-way factorial analysis of variance (ANOVA) using Genstat version 6. The significance level was set at $P < 0.05$. Means for chemical treatments and plant species were compared using Bonferroni-corrected *t*-tests. Multivariate statistical analysis was performed on TRFLP profiles using Genstat version 6 and Canoco for Windows version 4.02. To determine which differences between plant species and chemical treatments were significant, peak heights for each terminal restriction fragment (TRF) were first converted into proportions of the total peak height of all TRFs for each replicate. Plant species and treatments were then tested in pairwise comparisons using a variation of the city-block (Manhattan) randomization test procedure. An index was derived based on the average TRF profile similarity among the four replicates within each of two treatment combinations compared with the average TRF profile similarity between replicates of the two treatment combinations. All possible permutations of the eight replicates were calculated to produce a reference population of 35 similarity values. The actual similarity value was compared with the reference population to test for significance, using a critical level of $P < 0.05$. In addition, Genstat was used to calculate the overall abundance of each individual TRF for all samples. The TRFs were then ranked according to overall abundance and these rankings were used to select TRFs for inclusion in multivariate analysis. Redundancy analysis (RDA) using the Canoco program was chosen to analyse the TRFLP data after initial analysis by detrended correspondence analysis (DCA) revealed that the data exhibited a linear, rather than unimodal, response to the environmental variables (plant species, lime addition, and nitrogen addition). Redundancy analysis related each TRF to the environmental variables by selecting the linear combination of environmental variables that gave the smallest residual sum of squares.

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