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Spatial structure in soil chemical and microbiological properties in an upland grassland

K. Ritz [a,b,*], J.W. McNicol [c], N. Nunan [c], S. Grayston [d], P. Millard [d], D. Atkinson [e], A. Gollotte [e,1], D. Habeshaw [e], B. Boag [a], C.D. Clegg [a,2], B.S. Griffiths [a], R.E. Wheatley [a], L.A. Glover [f], A.E. McCaig [f,3], J.I. Prosser [f]

[a] *Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK*
[b] *National Soil Resources Institute, Cranfield University, Silsoe, Bedfordshire MK45 4DT, UK*
[c] *Biomathematics and Statistics Scotland, Invergowrie, Dundee DD2 5DA, UK*
[d] *Macaulay Institute, Craigiebuckler, Aberdeen AB9 2JQ, UK*
[e] *Scottish Agricultural College, West Mains Road, Edinburgh EH9 3JG, UK*
[f] *Department of Molecular and Cell Biology, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK*

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Abstract

We characterised the spatial structure of soil microbial communities in an unimproved grazed upland grassland in the Scottish Borders. A range of soil chemical parameters, cultivable microbes, protozoa, nematodes, phospholipid fatty acid (PLFA) profiles, community-level physiological profiles (CLPP), intra-radical arbuscular mycorrhizal community structure, and eubacterial, actinomycete, pseudomonad and ammonia-oxidiser 16S rRNA gene profiles, assessed by denaturing gradient gel electrophoresis (DGGE) were quantified. The botanical composition of the vegetation associated with each soil sample was also determined. Geostatistical analysis of the data revealed a gamut of spatial dependency with diverse semivariograms being apparent, ranging from pure nugget, linear and non-linear forms. Spatial autocorrelation generally accounted for 40–60% of the total variance of those properties where such autocorrelation was apparent, but accounted for 97% in the case of nitrate-N. Geostatistical ranges extending from approximately 0.6–6 m were detected, dispersed throughout both chemical and biological properties. CLPP data tended to be associated with ranges greater than 4.5 m. There was no relationship between physical distance in the field and genetic similarity based on DGGE profiles. However, analysis of samples taken as close as 1 cm apart within a subset of cores suggested some spatial dependency in community DNA-DGGE parameters below an 8 cm scale. Spatial correlation between the properties was generally weak, with some exceptions such as between microbial biomass C and total N and C. There was evidence for scale-dependence in the relationships between properties. PLFA and CLPP profiling showed some association with vegetation composition, but DGGE profiling did not. There was considerably stronger association between notional sheep urine patches, denoted by soil nutrient status, and many of the properties. These data demonstrate extreme spatial variation in community-level microbiological properties in upland grasslands, and that despite considerable numeric ranges in the majority of properties, overarching controlling factors were not apparent.

Keywords: Spatial variability; Microbial community; Grasslands; Geostatistics

1. Introduction

Specific instances of plant:microbe interactions are well known and thoroughly studied, but community-level associations between vegetation and microbial assemblages are less well understood. There is increasing evidence that plant community structure affects the

* Corresponding author. Tel.: +44-1525-863391; fax: +44-1525-863253.
E-mail address: k.ritz@cranfield.ac.uk (K. Ritz).
[1] Present address: UMR 1088 INRA/Université de Bourgogne, FRE/CNRS 2625 PME, INRA, BP86510, 21065 Dijon Cedex, France.
[2] Present address: Institute of Grassland and Environmental Research, North Wyke, Okehampton, Devon, EX20 2SB, UK.
[3] Present address: South West Regional Development Agency, Sterling House, Dix's Field, Exeter EX1 1QA, UK.

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density and composition of soil communities [1–7], and stronger evidence that there may be characteristic microbial communities associated with particular plant species, particularly in experimental circumstances where plants are grown in isolation [8–12]. The mechanisms by which such associations are generated are hypothesised to be related to the quality and nature of substrate deposited below ground by plants, which is known to regulate microbial community structure [13]. Implicit in such hypotheses is the notion that there will be a degree of *spatial* coupling between plant and microbial communities, but this aspect has been rarely studied. The concept of 'rhizosphere' essentially denotes a spatial relationship between plants and microbes focussed on the interface between root and soil, and studies indicate that there may be conditioning of microbial communities in the rhizosphere of specific plants [14,15]. At larger spatial scales, in ecosystems where plants tend to be sparse, there is evidence that soil organisms are also affected by proximity to individual plants [16,17]. In grasslands, however, plant density is very high and the spatial organisation of microbes relative to plant types is virtually unknown. Previous temporal studies on temperate upland grasslands have shown some evidence for community-level coupling [3,5,18]. However such relationships were obscured by high levels of spatial variation as the genetic composition of communities a few metres apart within a grassland can be as different as those separated by hundreds of kilometres [19]. Vegetation composition over these distances is less variable, and hence in the field other factors may be governing microbial community structure. One approach to exploring such drivers is to determine statistically natural variation associated with a wide range of potential factors, and analyse associations or trends in such data. The advantage of this approach is that multiple interactions may also be identified.

In the present study the spatial properties of genetic, phenotypic and functional aspects of microbial community structure, and associated vegetation, were rigorously measured in an area of unimproved upland grassland. A key aim was to determine the spatial scales over which various microbiological and chemical properties were structured. We chose this site since we knew from previous work that the microbial diversity was particularly high [20], and since the grassland was unimproved, plant growth would be entirely reliant upon natural processes of nutrient cycling. Since the underlying soil type and micrometeorology were effectively constant between samples, such factors would not confound the detection of any vegetation: microbe associations that may occur in multiple-site studies. It was also reasoned that at these scales, soil chemistry would vary significantly because this was grazed grassland and, therefore, subjected to patchy urine and dung deposition. We therefore hypothesised that there would be coupling between vegetation composition and soil microbial assemblages, and between soil chemical properties and microbial community structure.

2. Materials and methods

2.1. Site and soil

The study was carried out on Fasset Hill, Sourhope (55°28′30″ N; 2°14′ W) in the Scottish Borders. The site is a permanent *Festuca ovina – Agrostis capillaris – Galium saxatile* grassland, (unimproved grassland, National Vegetation Classification – U4a [21], at 370 m above sea level. The grassland has been freely grazed by sheep for at least 30 years. The underlying soil is a brown ranker (Haplumbrept; FAO/UNESCO, 1994) derived from old red sandstone [22]. The average rainfall for the site is 975 mm y^{-1} and the minimum and maximum temperatures are −10 and 27 °C, respectively.

2.2. Spatial sampling

A 12 × 12 m area of ostensibly uniform grassland was arbitrarily selected within the field site, avoiding any obvious major topological variation (emergent rock, erosion points, etc.). Within this region, nine origins were marked to form a uniform grid with 4 m between each origin; the overall grid was centred within the 144 m^2 region. These origins formed the foci of nine independent random-walk transects, each with nine subsequent steps (total $n = 90$). The steps in the walks, equating to sampling points, were defined by moving prescribed distances at a random angle, from each successive step (Fig. 1). The distances were defined as an incrementally increasing series starting at 0.1 m and increasing by a factor of 1.625 with each step; the order in which these distances were selected was also random. This design ensured that the frequency distribution of the entire set of inter-point distances was optimised, i.e. it guaranteed at least 10 instances of the closest distance and provided a comprehensive representation of inter-sample distances up to 12 m. The regular grid ensured that the entire area was covered, and the randomisation within each transect ensured unbiased sampling from each of the origins. At each sampling point, an intact sample of soil and associated vegetation (8 cm diameter, 8 cm deep) was taken using a core auger. The vegetation associated with each core was removed for analysis, and remaining soil sieved <2 mm and assayed as below. For the first two samples within each transect, an additional set of three 1 cm diameter sub-cores were taken from the 8 cm main cores. These were positioned centrally, 1 cm away from the centre, and at the periphery of the core (i.e. 3.5 cm from the centre) along a radial line. Field sampling was carried out on 2nd November 1999.

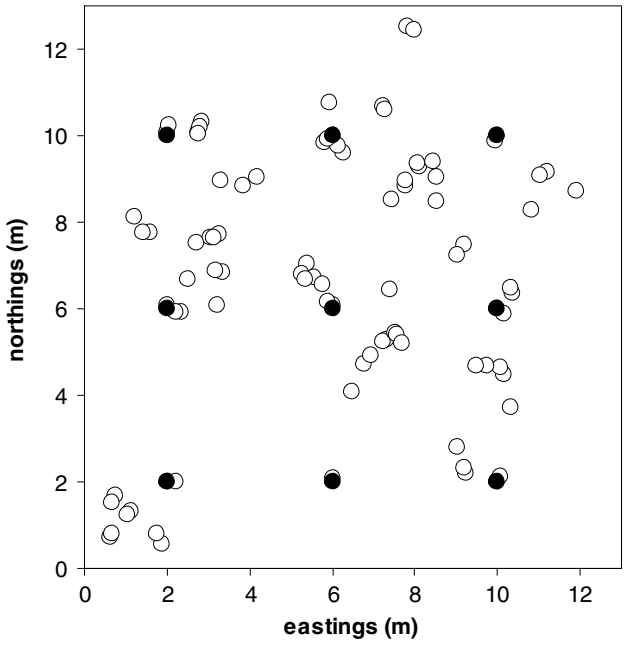

Fig. 1. Sampling design showing location of sampling points; filled circles represent origins of random-walk transects.

Time-sensitive parameters (e.g. microbial properties, mineral N) were determined within 72 h of sampling.

2.3. Vegetation analysis

Vegetation was removed from each core, the plants present identified to species level and scored on the basis of their presence or absence within individual cores.

2.4. Soil chemical properties

Organic matter was determined using loss on ignition. Soil samples were dried at 105 °C, then ashed at 900 °C. The total C and N content of the samples was determined by an automated Dumas combustion procedure [23] using a Carlo Erba NA1500 Elemental Analyser, (Carlo Erba Instruments, Milan, Italy). Total P was measured colorimetrically using the molybdate method [24], after extraction using NaOH. Soil pH was first measured in deionised water (1:3, soil:water) followed by $CaCl_2$, using a pH electrode. Extractable NO_2^--N and NO_3^--N (Perstorp Application Note AN 65/84, 65-31/84, 62/83), NH_4^+-N (Perstorp Application Note AN 65/84, ASN 65-32/84), and PO_4 [24] were measured colorimetrically using TRAACS segmented flow analysis, after extraction with 1 M KCl for 1 h, followed by filtration. Exchangeable cations (Ca^{2+}, Na^+, K^+, Mg^{2+}) were extracted using 1 M ammonium acetate and the extracts analysed using inductively coupled plasma optical emission spectroscopy (ICP-OES). Gravimetric soil moisture content was measured after drying at 80 °C overnight.

2.5. Soil microbial properties

2.5.1. Microbial biomass C

Microbial biomass C was measured by the fumigation-extraction technique [25,26] using 10 g d.w. equivalent of soil, fumigation for 24 h at 25 °C, extraction with 0.5 M K_2SO_4 (50 ml per sample for 30 min), and organic C estimated by the acid dichromate oxidation method [27]. A conversion factor (k_{EC}) of 0.35 was used to convert C-flush into biomass-C [28].

2.5.2. Bacterial 16S rRNA gene profiles

Total soil DNA was extracted from subsets of the 8 and 1 cm core samples using a bead beating method [29]. Purified DNA (10 ng per reaction) was used as the template in PCR amplifications using three sets of primers targeting 16S rRNA gene sequences. Amplifications for analysis of the total bacterial population were carried out using the general eubacterial primers p3 and p2 [30] incorporating a 40 bp GC-clamp at the 5′ end of amplified DNA to facilitate analysis by denaturing gradient gel electrophoresis (DGGE). The group-specific primer sets F243 and R1492 [31] and Ps-for and Ps-rev [32] and CTO189f and CTO654r [33] were used to amplify 16S rDNA gene fragments from actinomycetes, pseudomonads and ammonia oxidising bacteria (AOB), respectively. Group-specific amplification products were used as template DNA in a second round of reactions using the eubacterial DGGE primers described above in order to generate products of a suitable size for DGGE.

Quantitative comparison of samples using DGGE is limited by the number of lanes available on a single gel and inevitable differences between different gels run on different occasions. Analyses were, therefore, carried out on two subsets of approximately 30 samples. Set 1 comprised the first three cores in each of the 10 transects (tantamount to a random subset of the samples which covered the plot area); this was analysed using eubacterial primers only. Set 2 comprised all cores which contained one or more 'rare' plant species, defined as where the frequency of occurrence was seven or less out of the 90 cores, amounting to 25 cores, plus a random selection of five of the remaining cores; this was analysed using eubacterial, actinomycete and pseudomonad primers. In the case of the sub-core samples, a subset of 10 'parent' cores based on the first two cores from each of five randomly selected transects were analysed. The 30 subcore samples were analysed using eubacterial, actinomycete, pseudomonad and AOB primers. DGGE analyses were carried out using 100 ng of amplicons per lane, with denaturing gradients of 30–55% (total bacterial), 40–60% (actinomycetes and AOB) and 20–60% (pseudomonad), where 100% denaturant comprised 7 M urea and 40% (v/v) formamide, and 8% acrylamide gel. After electrophoresis, gels were fixed and silver-stained. For image analysis, gels were scanned on an Epson

GT-9600 scanner. The relative position of each band, compared to a composite lane of all sample lanes generated by the software, was determined using Phoretix 1D gel analysis software, Version 4.00 (Phoretix International, Newcastle-upon-Tyne, UK). Data were corrected for slight variations in the DNA loading between lanes and a simple binary matrix describing the presence or absence at each position in all lanes was produced for use in statistical analyses.

2.5.3. Phospholipid fatty acid profiling

Lipids were extracted from 1.5 g subsamples of soils using the procedure described previously [34]. The separated fatty acid methyl-esters were identified and quantified by chromatographic retention time and mass spectral comparison on a Hewlett Packard 5890 II gas chromatograph equipped with a 5972A mass selective detector (MSD II), using standard qualitative bacterial acid methyl ester mix (Supelco; Supelco UK, Poole, Dorset, UK) that ranged from C11 to C20. For each sample the abundance of individual fatty acid methyl-esters was expressed as μg PLFA g^{-1} dry soil. Fatty acid nomenclature used was that described by Frostegård et al. [35].

2.5.4. Community level physiological profiling

Community level physiological profiles (CLPP) were constructed using Biolog® GN microplates (Biolog Inc., Hayward, CA, USA), together with exudate profile microplates, prepared using Biolog® MT plates, containing an additional 30 ecologically relevant carbon sources identified mainly as plant root exudates [36]. Soil dilutions were adjusted to a similar inoculum density of approximately 10^4 CFU ml^{-1} (based on growth on 1/10th strength Oxoid tryptone-soy agar) and 150 μl inoculated into each of the microplate wells. The microplates were incubated at 15 °C for five days and colour development (carbon utilisation) was measured as absorbance at 590 nm (A_{590}) every 24 h using a microplate reader (Emax, Molecular Devices, Oxford, UK). Average well-colour development (AWCD) was calculated for aggregations of data relating to chemically similar C sources, viz. sugars, oligosugars, alcohols, carboxylic acids, acidic amino acids, basic amino acids, neutral amino acids, N heterocycles, amides/amines, phenolics, and aliphatics. Subsamples of soil from all main cores were analysed.

2.6. Statistical analyses

All statistical analyses were performed using Genstat [37] or Isatis (Geovariances, Fontainbleau, France), with appropriate transformations to normalise data where necessary. The dimensionality of the multivariate measurements (vegetation, DGGE, PLFA and CLPP) was reduced by multivariate analyses appropriate to the form of the data. Vegetation data were analysed by principal co-ordinate (PCO) analysis, deriving the PCO scores for the samples using simple matching among the plant species. PLFA profiles were analysed by principal component analysis (PCA) using correlative matching. DGGE profiles were analysed by PCO analysis of the presence/absence matrices for the banding profiles using the Jaccard coefficient of similarity, where a coefficient of 1 equates to identical profiles. CLPP profiles were analysed by PCA after first dividing by the value for the blank plate, log transforming, subtracting all means and finally subtracting means for each variable [3]. Plant species (presence/absence) data was analysed using indicator semivariograms. After checking for stationarity in the data, semivariograms were computed and plotted to a maximum distance of 6.5 m for all soil chemical parameters, individual PLFA concentrations plus scores from the first 10 principal components, AWCD of CLPP data and that grouped according to the different substrate groups, and scores from the first five principal components. Where robust semivariograms were apparent, chemical and microbiological parameters were estimated at unsampled locations by punctual kriging using the semivariograms and a weighted linear combination of 10 surrounding data points. Crossvariograms were used to determine whether two properties had common microscale variance, i.e. common variance below the minimum scale of measurement [38]. Where samples were categorised according to vegetation or urine-patch classes (see Section 4), one-way ANOVA was used to test the significance of differences between associated means of all basic parameters, PCAs and PCOs. All properties and multivariate summary parameters (first four PCAs, PCOs) were also cross-correlated.

3. Results

3.1. Characterisation of vegetation

Twelve species of plants were detected in the cores (Table 1(a)). Two grass species typical of upland grasslands, *Agrostis capillaris* and *Festuca rubra* were predominant, and a third graminaceous species, *Poa pratensis*, occurred in half the cores. The bryophyte *Rhytidiadelphus squarrosus* was also present in half the cores. The remaining eight species were relatively infrequent amongst the cores. Two cores contained monocultures of a single species (*A. capillaris* and *P. pratensis*); no core contained more than five species (Table 1(b)). PCO analysis of the vegetation profiles resulted in a very distinct separation of the cores into four categories, based on the combinatorial presence or absence of *P. pratensis* and *R. squarrosus* (Fig. 2). Hence, each core was strongly associated with one of

Table 1
Occurrence of plant species in sampled cores (based on presence only)

(a) Frequency of occurrence of species in cores

Species	Frequency of occurrence (% of 90 cores)
Agrostis capillaris L.	96
Festuca rubra L.	92
Poa pratensis L.	57
Rhytidiadelphus squarrosus (Hedw.) Warnst.	54
Nardus stricta L.	8
Galium saxatile L.	8
Cerastium holosteoides Fries.	6
Hypnum cupressiforme Hedw. (Moss)	3
Anthoxanthum odoratum L.	2
Deschampsia flexuosa L. Trin.	2
Luzula campestris (L.) DC.	1
Acrocladium cuspidatum (Hedw.) Lindb.	1

(b) Number of plant species co-occurring in cores

Number of species	Proportion (% of 90 cores)
1	2
2	13
3	47
4	28
5	10

these four vegetation categories; 15 cores contained neither species, with an approximately equal division of the other 75 cores among the other three classes. It was meaningless to test for spatial structure in the distributions of the two plant species that were ubiquitous, and the eight that were infrequent, because more than 90% of the samples had the same presence/absence score. Of the two remaining species, weak spatial structure was detected only for *R. squarrosus*, with a range of spatial autocorrelation of 2.56 m, with 26% of the total variance spatially autocorrelated.

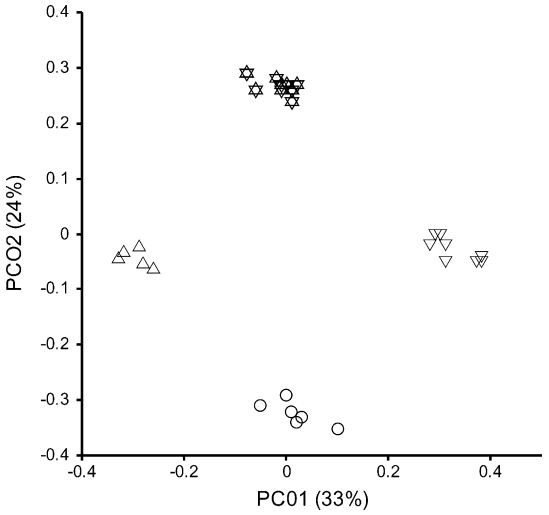

Fig. 2. Principal co-ordinate scores of vegetation community data, labelled according to the combinatorial presence or absence of the plants *Poa pratensis* (*Pp*) and *Rhytidiadelphus squarrosus* (*Rs*). (○) neither species present; (△) *Pp* present; *Rs* absent; (▽) *Pp* absent; *Rs* present; (✡) both species present.

3.2. Soil chemical properties

The basic statistical summary data for the soil chemical properties are shown in Table 2(a). All properties showed a wide range in magnitude, for example pH varied across 1.8 units, and extractable PO_4^{2-}-P over

Table 2
Basic statistical summary data for soil properties across all soil cores

Property	Mean	Median	Minimum	Maximum	c.v.
(a) Chemical properties					
Moisture loss (%)	32.0	32.6	17.0	47.0	22
pH_{H_2O}	4.29	4.26	3.85	5.28	6
pH_{CaCl_2}	3.74	3.69	3.32	5.15	8
Extractable NO_3^--N (μg g^{-1} dw)	70.2	59.5	13.3	338	71
Extractable NO_2^--N (μg g^{-1} dw)	0.23	0.20	0.20	0.63	40
Extractable NH_4^+-N (μg g^{-1} dw)	36.3	29.2	0.89	155	82
Extractable PO_4-P (μg g^{-1} dw)	4.87	1.93	0.17	179	385
Total C (%)	11.4	11.1	6.7	21.8	23
Total N (%)	0.91	0.88	0.61	1.54	21
Total P (mg g^{-1} dw)	2.73	2.64	1.84	4.01	15
Exchangeable Ca^{2+} (meq 100 g^{-1} dw)	2.8	2.3	0.7	11.1	70
Exchangeable Na^+ (meq 100 g^{-1} dw)	0.18	0.17	0.04	0.34	27
Exchangeable K^+ (meq 100 g^{-1} dw)	1.37	1.30	0.12	3.36	33
Exchangeable Mg^{2+} (meq 100 g^{-1} dw)	2.1	1.9	0.3	10.3	62
(b) Microbiological properties					
Microbial biomass (mg C g^{-1} dw)	2.13	2.07	1.07	3.99	28
Total PLFA (mg g^{-1} dw)	0.12	0.12	0.065	0.20	122
Cultivable bacteria (cfu g^{-1} dw)	4.92×10^7	2.33×10^7	1.69×10^6	6.85×10^8	178
CLPP[a] (average well colour development)	0.098	0.088	0.018	0.25	44

[a] Community-level physiological profiling see text.

three orders-of-magnitude. Mineral N also showed extensive variation between cores, with 25-fold and 175-fold variation in NO_3^--N and NH_4^+-N respectively. There was no evidence for spatial structure in pH_{H_2O}, K^+, PO_4-P and NO_2^--N, with the semivariance showing no correlation with distance. Correlated semivariograms were obtained for the remaining nine properties (Table 3(a); Fig. 3). In general, in excess of 50% of the total model variance was autocorrelated, the exceptions being Mg^{2+}, NH_4^+-N and moisture content. Geostatistical ranges were always shorter than the 6.5 m maximum lag, and varied between the properties (Table 3(a)).

3.3. Compositional and spatial characterisation of soil microbial communities

Microbial biomass properties showed an approximately 3-fold variation, whilst cultivable bacteria and CLPP data showed greater ranges in value between samples (Table 3(b)). Spatial structure was detected in many of the microbial properties (Table 3(b); Fig. 4). The semivariograms were varied, with linear and non-linear models being apparent. Model parameters showed extensive variation; geostatistical ranges from 0.7 to 6 m were apparent for PLFA data, but for CLPP data, ranges tended to be greater than 4 m (Table 3).

Banding patterns in the PCR-DGGE gels were generally complex, with 98 distinct bands apparent in the eubacterial profiles from the Set 1 cores and 124, 106 and 127 distinct bands in Set 2 cores for eubacterial, actinomycete and pseudomonad profiles respectively. For the sub-cores, there were 82, 146, 155 and 121 distinct bands for eubacterial, actinomycete, pseudomonad and AOB profiles respectively. There was no relationship between physical distance in the field between cores and the associated coefficient of similarity in community

Table 3
Semivariance analysis of spatial structure in soil properties where this was apparent

Property	Parameter				
	Range (m)	Total model variance	Nugget effect	Structural variance (%)	Model
(a) Chemical properties					
Moisture loss	1.07	37.4	25.1	33	Spherical
pH_{CaCl_2} [a]	1.56	0.061	0.027	56	Exponential
NO_3^--N	0.61	0.30	0.01	97	Spherical
NH_4^+-N	4.09	5.60	3.00	46	Spherical
Total C	3.21	0.15	0.05	67	Exponential
Total N	5.48	0.04	0.01	75	Exponential
Total P	3.83	0.015	0.005	67	Spherical
Ca^{2+} [a]	1.37	0.27	0.10	63	Spherical
Mg^{2+} [b]	1.08	0.157	0.081	48	Spherical
(b) Microbiological properties[c]					
Microbial biomass C	3.10	38.3	19.5	49	Spherical
PLFA					
C14:0i	4.71	0.53	0.21	61	Spherical
C16:0	0.73	0.044	0.016	64	Spherical
C16:1ω8	0.68	0.15	0.050	66	Spherical
C17:0br	1.19	0.11	0.054	49	Spherical
C17:0cy	1.64	0.096	0.060	38	Spherical
C17:0(10Me)	1.72	0.10	0.04	60	Exponential
C18:1ω9	0.73	16.4	5.44	67	Spherical
C19:0cy	1.36	25.6	17.11	33	Spherical
C19:1b	5.74	0.56	0.14	75	Spherical
Phthalate	0.87	0.88	0.39	56	Spherical
PC6[d]	5.61	1.01	0.32	68	Spherical
Community-level physiological profiling					
Acidic amino acid	5.92	0.0045	0.0018	60	Spherical
Basic amino acid	6.20	0.011	0.0041	62	Spherical
PC1	4.43	18.6	4.10	78	Spherical
PC2	4.98	13.9	2.10	85	Exponential
PC5	2.12	2.99	1.01	66	Spherical

[a] Two outliers (same sample) were removed prior to computation of semivariograms.
[b] Two other outliers removed.
[c] Properties showing a linear increase in semivariance (viz. PLFAs C12:0, C13:0, C15:0, C15:0ai, C20:0 and C20:4ω6, the fourth PC of the PLFA profiles and the average well colour development for neutral and basic amino acids, amides, sugars, oligosugars, alcohols, carboxylic acids and phenolics) are not presented.
[d] PC = principal component of combined PLFA data; 6 = sixth PC (same notation used for community-level physiological profiling data).

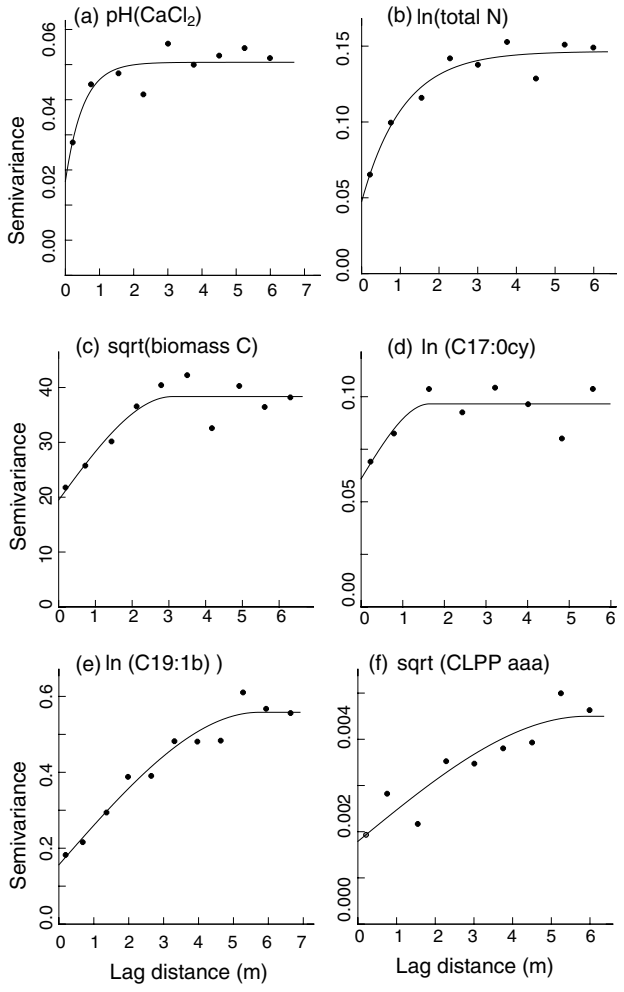

Fig. 3. Example semivariograms for soil chemical properties, with fitted models. (a) $pH_{(CaCl_2)}$, (b) ln(total N) and (c) sqrt(biomass C), (d) ln(C17:0cy), (e) ln(C19:1b) and (f) sqrt(CLPP acidic amino acids).

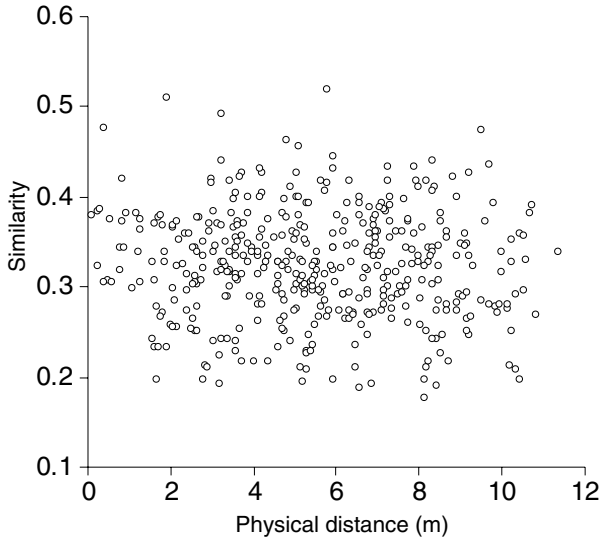

Fig. 4. Scatter plot of physical distance in the field between soil cores and genetic similarity based on principal co-ordinate analysis of PCR-DGGE profiles using eubacterial specific primers.

structure either for eubacteria in Set 1 (Fig. 4), or for eubacteria, actinomycete or pseudomonad DGGE profiles in Set 2 (data not shown). Profiles relating to sub-cores 1 cm apart showed similar ranges in similarity to those 3.5 cm apart for eubacteria, actinomycete and pseudomonad profiles (Table 4). However, for ammonia oxidiser profiles, cores 3.5 cm apart were generally less similar than those 1 cm apart (Table 4). Eubacterial DGGE profiles derived from sub-cores showed notably narrower ranges in similarity within cores than the overall range across all sub-cores (Table 4).

3.4. Kriged estimates of spatial distribution of properties

Kriged maps revealed a great variety in spatial patterns across the range of properties measured. There was no consistent or universal pattern, with some maps showing great heterogeneity (e.g. pH_{CaCl_2}, Fig. 5(a)), features such as lobes of high or low data values emanating from corners of the region (e.g. total N, biomass C, PLFA C17:0cy and C19:1b, Figs. 5(b)–(e)) and a general gradient across the area (e.g. CLPP for acidic amino acids, Fig. 5(f)). There were also examples of small-scale (e.g. pH_{CaCl_2}, Fig. 5(a)) and larger scale (e.g. PLFA C19:1b, Fig. 5(e)) patchiness.

3.5. Correlation between properties

A large number of properties were statistically significantly correlated with each other (df = 88; $P < 0.001$ if $r^2 > 0.1057$), however scatter diagrams showed that in the majority of such cases the data were in fact highly dispersed. Correlation tended to be stronger within the different classes of measurements (i.e. soil chemical properties, PLFA, CLPP, etc.) than between them. For example, many individual PLFAs and CLPP groups were strongly correlated. Exchangeable cations tended to show strong positive correlation with each other and with pH (r^2 up to 0.79). Total C and N were highly correlated ($r^2 = 0.90$). Biomass C correlated significantly with total C ($r^2 = 0.59$), total N ($r^2 = 0.52$), and total PLFA ($r^2 = 0.37$). Between classes of

Table 4
Range in similarity of PCR-DGGE profiles derived from 1 cm diameter subcores taken 1 or 3.5 cm from a central subcore, within 8 cm diameter soil cores. Data are based on 10 instances of such larger cores. A similarity value of 1 equates to identical profiles

Primers	Separation distance within main cores		Entire set of subcores
	1 cm	3.5 cm	
Eubacteria	0.31–0.49	0.31–0.45	0.11–0.59
Actinomycetes	0.11–0.48	0.15–0.46	0.09–0.58
Pseudomonads	0.23–0.47	0.23–0.48	0.09–0.49
Ammonia oxidisers	0.23–0.50	0.07–0.34	0.03–0.59

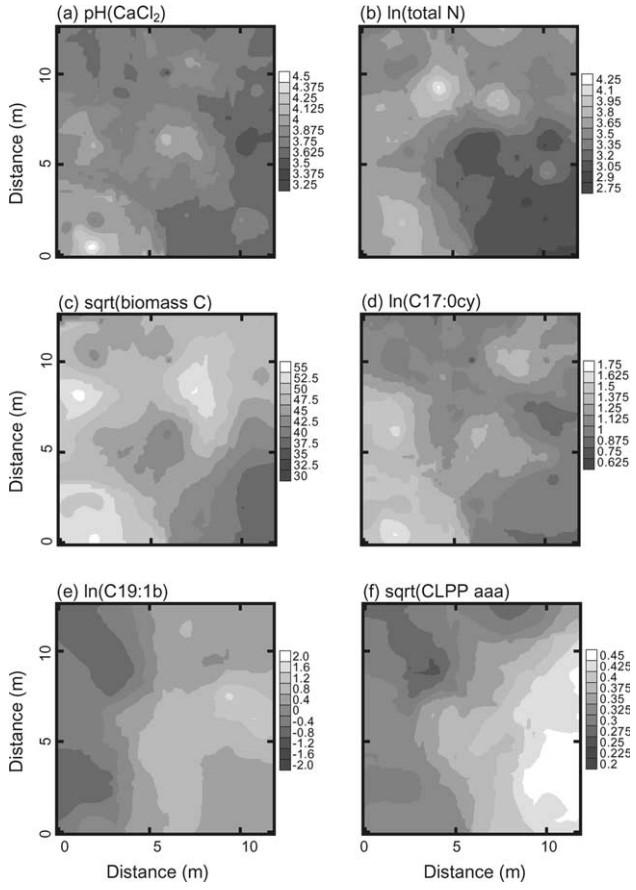

Fig. 5. Kriged maps for (a) pH_{CaCl_2}, (b) ln(total N), (c) sqrt(biomass C), (d) ln(C17:0cy), (e) ln(C19:1b) and (f) sqrt(CLPP acidic amino acids).

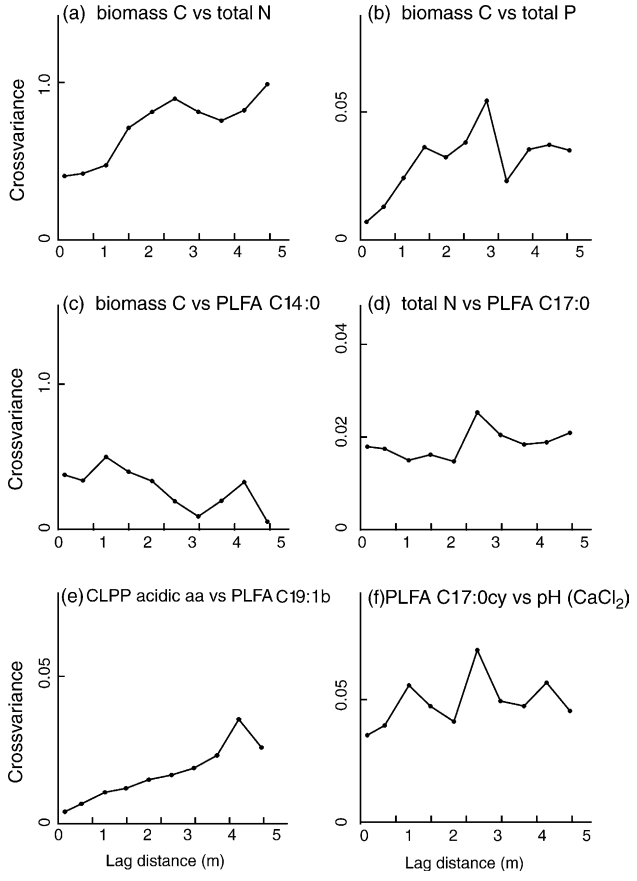

Fig. 6. Crossvariograms involving (a) biomass C vs. total N, (b) biomass C vs. total P, (c) biomass C vs. PLFA C14:0, (d) total N vs. PLFA C17:0, (e) CLPP acidic amino acids vs. PLFA C19:1b and (f) PLFA C17:0cy vs. pH_{CaCl_2}.

measurements, a number of properties had similar geostatistical ranges as well as being significantly correlated. Visual comparison of kriged maps suggests that there were similarities in the spatial distribution of these properties despite the dispersed nature of the scatter diagrams for these properties. For example, the kriged maps for pH_{CaCl_2} and PLFA C17:0cy (Figs. 5(a) and (d)); for total N and biomass C (Figs. 5(b) and (c)); and for PLFA C19:1b and CLPP acidic amino acids (Figs. 5(e) and (f)) indicated common causes of spatial variance between variables despite the relatively dispersed nature of the respective scatter diagrams ($r^2 = 0.54$ for pH_{CaCl_2} vs. PLFA C17:0cy; $r^2 = 0.52$ for biomass C vs. total N; $r^2 = 0.32$ for PLFA C19:1b vs. CLPP acidic amino acids). Crossvariograms (Fig. 6) showed a range of behaviours, from a relatively large nugget effect with increasing crossvariance as the lag distance increases (Fig. 6(a)), to a large nugget effect with decreasing crossvariance as the lag distance increases (Fig. 6(c)), through a more random fluctuation of the crossvariance with lag distance (Figs. 6(d) and (f)). Crossvariograms with small nugget effects and increasing crossvariance with lag distance were also observed (Figs. 6(b) and (e)).

4. Discussion

4.1. Spatial variation and structure in soil properties

A consistent feature of many of the soil properties measured in this study was a large range in the magnitude of the parameters, both primary and derived, demonstrating high levels of soil chemical and biological spatial heterogeneity in this grassland. There was no indication of general spatial trends across the sampled area, meaning that the data were 'stationary' in geostatistical terms. The most formal way to analyse spatial variation statistically is by means of semivariance analysis and here it revealed the presence of spatial structure in variance for many of the parameters measured. The ranges of spatial autocorrelation of the soil chemical properties were of the same order of magnitude with observations made in studies carried out at a similar scale in a mid-late successional field [39], and in a sagebrush steppe [16]. Other studies, carried out at a larger scale (i.e. field scale) in tillage fields, have reported ranges of spatial dependence one to two orders of magnitude greater for many soil properties than those

reported here [40,41]. Soil ecological variables are likely to be characterised by more than one scale of variability as factors influencing variability operate at different scales [42]. For example, the shorter range in spatial dependence observed here might be due to local factors such as the influence of vegetation, while the longer ranges observed in other studies might be due to agricultural practices or topography.

A number of microbial properties showed a linear increase in semivariance, with no indication of a sill; hence the geostatistical range for these properties remains unknown, but is in excess of 6.5 m. However, for the most part the degree and range of spatial dependence found here for microbial properties were consistent with results from other studies carried out at similar scales to the one we report here. In a Swedish forest soil, 20 (out of 32) PLFAs showed strongly correlated semivariograms, with ranges from 1 to 11.3 m and the first and second principal components showed structure at 4.6 and 1.5 m respectively [43]. Ranges of between 0.3 and 4.3 m were found for a variety of biological and chemical properties in Californian chaparral [17], where spatially structured properties included root biomass, cultivable bacteria and fungi, microbial biomass, nematodes and mites. Some of this variation was related to distances to specific shrubs or concentrations of rocks. In an arable soil supporting maize, spatial structure in many components of fatty acid methyl ester (FAME) profiles were found at scales of 4–30 cm [44], although these semivariograms contain few points.

Despite the relatively small sampling scale of the current study, a significant portion of the variance was present as nugget variance, suggesting the presence of microscale variability. The evidence from the DGGE analyses of the sub-cores supports this. The DGGE profiling of community DNA could not be analysed by means of robust semivariograms, but more basic analyses of this data also revealed extreme spatial variation in the genetic structure of the microbial communities. The maximum similarity between community structures in samples taken 1 cm apart was 60%, and the lowest similarity between any two samples was only 3% (Table 4). Samples 10 cm apart could be as similar or as dissimilar as those 12 m distant (Fig. 4). Analysis of eubacterial community structure by T-RFLP in a Wyoming grassland revealed weak spatial structure between 10 and 50 cm, expressed as an increase in dissimilarity as a function of distance. However, such structure was very weak with only of the order 6% of the variability accounted for [45]. The smallest sampling scale adopted in our study at the inter-core scale was 10 cm, which was operationally defined by the requirement for an adequate mass of soil to permit the range of analyses. Using a combination of DGGE and oligonucleotide hybridisation high degrees of heterogeneity in the ammonia oxidiser community has been detected within replicate 0.5 g sub-samples of homogenised unimproved Sourhope soil [46]; this was also reflected in heterogeneity in both ammonium concentration and pH. Studies on extremely small volumes of soil have also detected substantial genetic variation in ammonium and nitrite oxidiser community structure in arable soils at the mm and sub-mm scale [47,48]. Collectively these studies suggest that more pronounced spatial structure tends to be apparent as biological specificity is increased. However, the datasets available to date are too limited to generalise.

A distinct non-linear relationship between genetic distance, determined by PCR-RAPD analysis, and physical separation distance has been detected in an elevation gradient sampled across a salt-marsh sediment [49], which also showed directional dependence. Here, ranges of 0.35 and 0.17 m were apparent, depending on direction [49]. This clearer relationship than we observed in the pasture may have been manifest because there was a distinct elevation gradient away from a creek along the transect, whereas in the pasture studied here there was an absence of any distinct environmental gradients.

4.2. Coupling between vegetation and soil microbial community structure

The vegetation composition of the cores in this study was not particularly diverse, with 12 species represented, while approximately 120 plant species are recorded as occurring in UK upland grasslands [21]. We did not know a priori how the vegetation would vary between the samples. However, PCO analysis revealed a grouping of four distinct categories with sufficiently even representation to enable statistically robust testing of relationships between vegetation category and locally associated soil properties. Data categorised according to these classes that showed significant differences are summarised in Table 5. Such analyses showed that pH, total P, Ca^{+} and Mg^{2+} were significantly lower where *R. squarrosus* occurred in the absence of *P. pratensis*. Eight specific PLFA compounds were significantly different between vegetation classes. For example, cy17:0 (characteristic of Gram negative bacteria) was found in lowest concentrations where *R. squarrosus* was present, and in highest concentrations where neither *R. squarrosus* nor *P. pratensis* was present, whilst 18:2ω6 (characteristic of fungi) was present in significantly higher concentrations where *R. squarrosus* was present. The second principal component of the PLFA profiles showed highly significant differences between these vegetation classes. The PC loadings showed that this discrimination was based on a complex combination of PLFAs, with no clear dominance by particular types. The presence of *P. pratensis* alone was associated with lower utilisation of sugar and N-heterocycles in the CLPP assay. For CLPP, PC2 was significantly discriminated when neither *R. squarrosus* nor *P. pratensis* were

Table 5
Mean values for properties categorised according to vegetation class, where significant differences were apparent

Property	Vegetation category[a]				Residual m.s.	F	P_F[b]
	Neither ($n = 15$)	*Pp* ($n = 26$)	*Rs* ($n = 24$)	PpRs ($n = 25$)			
(a) Chemical properties							
pH_{H_2O}	4.33	4.35	4.15	4.33	0.068	3.13	*
pH_{CaCl_2}	3.80	3.82	3.55	3.79	0.085	4.68	**
Total P (mg g^{-1} dw)	2.85	2.87	2.56	2.66	0.17	3.15	*
Exchangeable Ca (meq 100 g^{-1} dw)	2.96	3.67	1.65	2.78	3.28	5.29	**
Exchangeable Mg (meq 100 g^{-1} dw)	2.31	2.58	1.5	2.17	1.60	3.21	*
(b) Microbiological properties							
PLFA: (μg g^{-1} dw)							
c12:0	0.022	0.039	0.0075	0.0112	0.0014	3.64	*
c13:0	0.15	0.23	0.11	0.14	0.015	5.44	**
c16:1w7c	5.68	5.35	6.56	6.58	3.39	2.75	*
c17:0cy	3.72	3.47	2.7	3.28	1.36	2.89	*
c18:2w6	2.32	2.02	2.89	2.65	1.56	2.23	*
c18:1w9	13.0	11.3	14.0	14.2	15.2	3.02	*
c18:w1	1.64	1.69	1.79	2.19	0.49	2.90	*
c20:4w6	0.32	0.41	0.22	0.29	0.031	4.66	**
PC2[c]	−0.44	−1.48	1.13	0.72	5.17	6.67	***
CLPP (WCD)							
Combined sugars	0.109	0.070	0.106	0.094	0.0026	2.84	*
Combined n-heterocycles	0.115	0.064	0.105	0.095	0.0034	3.23	*
PC2[c]	2.31	−1.39	0.39	−1.05	11.0	4.67	**

[a] Vegetation category according to combinatorial presence or absence of *Poa pratensis (Pp)* and *Rhytidiadelphus squarrosus (Rs)*. Values are means, n as shown in table.
[b] $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.
[c] PC = principal component of combined PLFA data; 2 = second PC (same notation used for community-level physiological profiling data).

present in the soil cores. There were no significant differences between any of the PCO scores of DGGE profiles based on eubacterial, actinomycete or pseudomonad primers applied to Set 2 between the four vegetation categories ($P > 0.05$ in all cases).

The plant community descriptions here were based on the presence/absence of plant species, and do not relate to the abundance or biomass of each component; casual observation suggested that the frequent grass species (Table 1) tended to dominate when present. Overall, there was evidence for vegetation-based associations in relation to both chemical and microbiological parameters. All four vegetation classes were discriminated by the multivariate syntheses of both PLFA and CLPP profiles. However, the bases of such associations were complex in relation to the components of the profiles and could not be ascribed to simple combinations of particular phospholipids or C substrates. These results, therefore, suggest a community-level coupling between vegetation and microbes, albeit in rather complex terms. This is in agreement with previous studies on temperate upland grasslands [3–5,50].

4.3. Impact of notional urine patches upon soil microbial community structure

Given that the grassland in this study was grazed by sheep, the very wide range of mineral nutrient concentrations in the soil samples are likely to be associated with urine patches. At any one time such patches will be of variable relative ages, with recent deposition most likely associated with relatively high concentrations of N and P. Consequently, the samples were categorised according to three notional urine classes by summing NO_3^--N, NH_4^+-N and PO_4^--P concentrations, ranking the samples according to these values and calculating the cumulative distribution of concentration. The 33-percentiles were then taken to denote notionally high, medium and low urine classes. Much of the data categorised according to these classes showed significant differences, as summarised in Table 6. Significant differences were apparent between mineral-N and PO_4-P, which would be expected by the definition of the urine classes. However, the majority of other soil chemical properties were also significantly different between the classes, with the consistent trend of greater values for the high urine categories, for example for microbial biomass and concentrations of PLFAs characteristic of bacteria and actinomycetes (18:0 10Me16), and there was a consistent and significant trajectory in PC1 (Table 6). AWCD showed a significant negative trend, and a consistent and significant trajectory in PC2 with increasing urine category. PCO data relating to DGGE profiles based on eubacterial, actinomycete or pseudomonad primers applied to Set 2 showed no significant difference between urine categories ($P > 0.05$; data not

Table 6
Mean values for properties categorised according to notional urine-patch classes, where significant differences were apparent

Property	Urine-patch category[a]			Residual m.s.	F	P_F[b]
	Low ($n = 52$)	*Medium* ($n = 25$)	*High* ($n = 13$)			
(a) Chemical properties						
pH_{H_2O}	4.19	4.43	4.42	0.06	10.6	***
pH_{CaCl_2}	3.63	3.87	3.88	0.08	7.89	***
Extractable NO_3-N ($\mu g\,g^{-1}$ dw)	52.5	80.8	120.7	1942	13.5	***
Extractable NO_2-N ($\mu g\,g^{-1}$ dw)	0.21	0.24	0.28	0.0081	3.27	*
Extractable NH_4^+-N ($\mu g\,g^{-1}$ dw)	18.0	46.6	89.5	259	109	***
Extractable PO_4-P ($\mu g\,g^{-1}$ dw)	1.7	4.5	18.1	328	4.25	*
Total C ($mg\,g^{-1}$ dw)	10.5	11.6	14.9	4.93	20.3	***
Total N ($mg\,g^{-1}$ dw)	0.83	0.94	1.17	0.022	28.3	***
Total P ($mg\,g^{-1}$ dw)	2.60	2.80	3.11	0.15	10.1	***
Exchangeable Ca (meq $100\,g^{-1}$ dw)	2.06	3.78	3.63	3.14	9.7	***
Exchangeable Na (meq $100\,g^{-1}$ dw)	0.16	0.18	0.22	0.0019	10.2	***
Exchangeable K (meq $100\,g^{-1}$ dw)	1.24	1.43	1.76	0.17	8.4	***
Exchangeable Mg (meq $100\,g^{-1}$ dw)	1.76	2.50	2.88	1.55	5.7	**
(b) Microbiological properties						
Microbial biomass C ($mg\,g^{-1}$ dw)	1.95	2.28	2.72	289	8.9	***
PLFA: ($\mu g\,g^{-1}$ dw)						
c15:0i	7.33	8.01	10.4	5.92	8.2	***
c15:0ai	2.45	2.93	3.72	1.18	7.5	***
c15:0	0.67	0.74	0.96	0.059	7.4	***
c16:0i	6.18	6.5	8.29	5.14	4.5	*
c16:0	14.8	15.8	19.6	12.5	9.6	***
c17:0br	0.33	0.43	0.54	0.016	15.4	***
c17:0i	1.67	1.91	2.44	0.22	14.5	***
c17:0ai	1.19	1.46	1.76	0.22	8.9	***
c17:1w8	0.47	0.57	0.73	0.032	11.7	***
c17:0cy	2.85	3.49	4.43	1.16	12.1	***
c17:0	1.37	1.56	1.89	0.10	14.3	***
c18:0br	0.02	0.10	0.09	0.0083	7.0	***
c17:0(10)	2.86	3.00	3.74	0.93	4.3	*
c18:0(10)	3.07	3.56	4.14	1.44	4.6	*
c19:0cy	15.5	16.1	20.1	21.3	6.4	**
c20:5	0.28	0.43	0.50	0.049	7.0	***
Total PLFA	115	123	148	770	7.3	***
PC1[c]	1.44	−0.76	−4.32	13.9	13.1	***
CLPP						
AWCD	0.109	0.083	0.075	0.0017	5.6	**
PC2[d]	0.78	−0.71	−1.77	12.5	3.4	*
DGGE						
Ammonia-oxidiser PCO1[e]	0.21 ($n = 13$)[f]	−0.21 ($n = 8$)	−0.34 ($n = 3$)	0.031	19.9	***

Values are means; n as in the table.
[a] See text for details of urine categorisation.
[b] $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.
[c] First principal component of combined PLFA data.
[d] Second principal component of combined CLPP (individual substrates) data.
[e] First principal co-ordinate of DGGE data.
[f] Data not available for all cores due to restriction of number of samples that can be fitted on one gel, hence n is as stated in this sub-body of the table).

shown). Ammonia oxidiser DGGE profiles derived from sub-cores showed highly significant discrimination between low urine category soils and medium or high, via PCO1 (Table 6).

Urine deposition directly elevates N in soils, in the form of urea which is rapidly transformed to ammonia and thence nitrate. Urine contains some C but little P [51], but in organic soils such as the one studied here, it induces a change in soil pH that results in the rapid release of large quantities of organic P into the soil solution which may then be mineralised [52]. In this study it appears such effects were largely confined to the biomass, PLFA and CLPP descriptors of the community; the 'background' genetic structure of the community did not appear to be affected. However, the genetic community structure of a subgroup of the microbial community associated with mineral-N transformations, the ammonia oxidisers, was significantly affected in high

urine-class regions. An increase in biomass is explained by the increase in substrate that must ensue following urine deposition. PLFA and CLPP profiles are essentially phenotypic and functional measures respectively, and as such are likely to be more responsive to changing environmental conditions. The elevation of nutrients following urine deposition is a transient event and over time concentrations decline; the evidence here is that the associated effects on microbes also decline concomitantly, in that the high urine class generally had a greater impact on microbial properties than the medium and low classes. In another study at the same site addition of urine stimulated bacterial and pseudomonad numbers immediately, but decreased overall carbon utilisation (though stimulated basic amino acid utilisation) by the microbial communities for the first week after urine addition. Thereafter, there was a stimulation of C utilisation from 2–5 weeks after urine addition [53]. This correlates with our findings and strongly suggests our 'high urine class' categories are sites of recent urination events. CLPP measures *potential* utilisation, albeit in vitro [54], and this suggests that the potential activity of the bacteria is lower immediately after a urine event, but then increases. Urine deposition will be spatially patchy in grasslands, but the scale of any patterning in deposition has not been explicitly measured before. The size of individual patches will depend upon the volume of urine deposited, and the location of patches will be related to the grazing behaviour of the sheep. We were unable to consider the potential impact of faecal deposits upon soil microbial properties in this study, but these are also likely to have an influence due to the high concentrations of potential microbial substrate, as well as the indigenous microbial communities in faeces. N fertilisation of grasslands has also been shown to influence genetic structure of eubacterial and actinomycete communities, and PLFA profiles [55]. Previous studies have shown that pH can have a strong influence on soil microbial communities, for example as measured by CLPP [56] or PLFA [57]. Whilst the pH varied in the sampled region across 1.8 log units, no such relationship was observed here.

4.4. Spatial coupling between soil chemical and microbiological properties

The degree and ranges of spatial dependence of a number of the microbiological measurements were similar to those of chemical soil properties, suggesting an association in the variance of these properties at the scale of measurement. The maps of kriged estimates confirmed this for a number of the properties measured. However, the linear correlation between properties was generally weak suggesting that other factors or, as is more likely, combinations of factors influence the variability of microbial measurements.

The semivariograms of the properties measured all displayed nugget variance of varying magnitude. Nugget variance is attributed to autocorrelated variance at scales below the scale of measurement (in this study the minimum *reliable* lag distance was 20 cm), and to measurement error. The nugget variance in crossvariograms is attributed to covariance between variables at scales below the shortest sampling interval and to covariance of measurement error. It can be assumed that the error associated with the measurement of two different variables (i.e. a chemical and a microbiological variable or two independently measured microbiological variables) are independent and that the measurement error covariance is therefore equal to zero [38]. Thus, the crossvariogram 'filters out' the uncorrelated part of the nugget variance and this property can be used to distinguish between experimental error and microscale autocorrelated variance [38]. A crossvariogram with a large nugget effect suggests the presence of microscale variation common to both variables (relatively large covariance), whilst a small nugget effect suggests little common variance between variables at smaller scales. Because this procedure assumes that experimental errors are independent, it cannot be used to explore common microscale variability of properties that might have common experimental errors (e.g. different PLFAs or different CLPPs).

The range of behaviours of the crossvariograms suggests that the effects of scale on relationships between variables is very variable. The nugget effects in the crossvariograms involving biomass C and total N and biomass C and total P suggest common origins of microscale variability for biomass C and total N (Fig. 6(a)) but less common microscale variability for biomass C and total P (Fig. 6(b)). The crossvariograms involving PLFAs and biomass C, total C and total N also suggest a common cause of microscale variability for these properties (e.g. Figs. 6(c) and (d)) even though the correlation between the PLFAs and biomass C, total C and total N was often weak at the scale of measurement (e.g. Fig. 6(c)). PLFAs also appeared to have common microscale variability with other soil properties such as pH (Fig. 6(f)). Microscale variability in the CLPPs did not appear to be associated with any of the other properties (microbial or otherwise), as the small nugget effects in all the cross-semivariograms would suggest. This was even true for the lipid C19:1b and the physiological response to acidic amino acids (Fig. 6(e)), despite the fact that these properties were correlated at the scale of measurement and displayed similarities in the spatial patterns at the scale of measurement (Figs. 5(e) and (f)). These data suggest some scale dependence in the relationships between microbial and chemical properties, and between microbial properties in soil. Because microbial communities respond simultaneously to multiple factors operating at different spatial scales and with subcomponents

of the communities displaying differential responses to a given factor, it is hardly surprising that microbial properties display such a complex set of interdependences amongst themselves and with other variables.

4.5. Comparison of methods in terms of their resolving power

In this study PLFA and CLPP were the most sensitive measures of microbially related effects of urine deposition and association with vegetation composition. DGGE profiling of DNA was insensitive in general, but there were indications that the more specifically targeted the primer sets were, in taxonomic or functional terms, the greater the apparent sensitivity. This may be due to the increased resolution of DGGE that is afforded when analysing less abundant groups. It is known that in these grasslands actinomycetes constitute about 8% of clones in 16S rDNA libraries from unimproved grasslands, pseudomonads were at 2% in improved, but not detected in unimproved, and AOB are likely to be $\ll$1% of the population [20]. This is intuitively reasonable; DNA composition is exceptionally diverse in soils [58,59] and hence lower resolution methods would only be expected to detect relatively large differences in overall composition.

5. Conclusions

This work has demonstrated the high levels of spatial complexity that prevail in unimproved upland pastures, and suggests that a complex set of interactions impact upon given soil microbial properties. Despite there being extensive numeric ranges in virtually all properties (i.e. the data were well-spread), there were no particularly clear associations between them, which could be used to infer governing factors. At the scale of one to a few metres, plant community composition had a weak but detectable effect on microbial assemblages. Nutrient status, principally mediated by urine deposition in this system, had a considerably stronger influence on microbial communities, but there was evidence that such effects are transient. Thus the mechanisms which have resulted in the spatial organisation of the pasture which have been mapped in this study do not appear to have been dominated by a few drivers but a complex accumulation of factors integrated over time. Given the evidence for plant community composition effects, future studies could focus on whether such associations are stronger at the rhizosphere scale. This study also focused on spatial variation at one time, outside of the primary growing season for the pasture. It is possible that spatial properties may also show a strong temporal dynamic, which would also warrant further study.

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