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Seasonal controls on grassland microbial biogeography: Are they governed by plants, abiotic properties or both?

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ABSTRACT

Temporal dynamics create unique and often ephemeral conditions that can influence soil microbial biogeography at different spatial scales. This study investigated the relation between decimeter to meter spatial variability of soil microbial community structure, plant diversity, and soil properties at six dates from April through November. We also explored the robustness of these interactions over time. An historically unfertilized, unplowed grassland in southwest Germany was selected to characterize how seasonal variability in the composition of plant communities and substrate quality changed the biogeography of soil microorganisms at the plot scale (10 m × 10 m). Microbial community spatial structure was positively correlated with the local environment, i.e. physical and chemical soil properties, in spring and autumn, while the density and diversity of plants had an additional effect in the summer period. Spatial relationships among plant and microbial communities were detected only in the early summer and autumn periods when aboveground biomass increase was most rapid and its influence on soil microbial communities was greatest due to increased demand by plants for nutrients. Individual properties exhibited varying degrees of spatial structure over the season. Differential responses of Gram positive and Gram negative bacterial communities to seasonal shifts in soil nutrients were detected. We concluded that spatial distribution patterns of soil microorganisms change over a season and that chemical soil properties are more important controlling factors than plant density and diversity. Finer spatial resolution, such as the mm to cm scale, as well as taxonomic resolution of microbial groups, could help determine the importance of plant species density, composition, and growth stage in shaping microbial community composition and spatial patterns.

1. Introduction

All natural systems are temporally and spatially bounded and the defined spatial organization observed in many ecosystems suggests that spatial organization is of functional importance (Legendre et al., 2005). In terrestrial systems many studies have shown that soil microbial communities are structured at several spatial scales (Franklin and Mills, 2003; Ritz et al., 2004; Bru et al., 2011; Keil et al., 2011), indicating effects of environmental drivers such as land use and abiotic conditions. For example, Franklin and Mills (2003) found multi-scale variations in microbial community spatial structure (from 30 cm to >6 m) with high spatial heterogeneity due to soil properties, in a wheat field study using DNA fingerprinting. Ritz et al. (2004), in an unimproved grassland study,

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observed a high degree of spatial variation in community-level microbiological properties, but were not able to characterize overarching controlling factors. Keil et al. (2011), in contrast, found that ammonia-oxidizing and denitrifying microorganisms were spatially structured in soils from 10 m × 10 m grassland plots. This was confirmed in a study by Berner et al. (2011), who found that spatial heterogeneity in grasslands at scales of 1–14 m was related to land use intensity; i.e., fertilization, mowing frequency, and grazing practices. Indeed, many studies indicate a close link between above and belowground components in terrestrial ecosystems (Reynolds et al., 2003; Zak et al., 2003; Wardle et al., 2004; van der Heijden et al., 2008). Plants may affect the soil microbial community directly via nutrient and water uptake, litter input, and root exudates, or indirectly, by changing composition or abundance of the decomposer community. Microbes may also have direct or indirect effects on plants; thus, understanding the patterns of interaction between plant and soil microbial communities is critical. However, the degree of coupling between plants and microbial communities has been hard to quantify in grasslands, probably due to the very high plant density (Ritz et al., 2004) and/or high plant species richness (Zak et al., 2003; Nunan et al., 2005). It is also possible that these interactions occur at scales that have not yet been identified.

The picture that emerges from the existing literature is that microbial communities are subjected to many external structuring influences and that the relative importance of these influences is both context and microbial group dependent (Martiny et al., 2006). Furthermore, many of the relationships are not particularly strong and it is therefore legitimate to ask whether they persist over time and through seasons. The vast majority of microbial spatial or biogeographic studies have been carried out at a single time point and those studies which have combined spatial and temporal approaches have yielded conflicting results. Zak et al. (2003), in a long term study, found that microbial composition and function were influenced by plant diversity, while Grayston et al. (2001) found plant productivity, temperature, and moisture to have the strongest effects on soil microbial community structure. However, Habekost et al. (2008) observed that distribution patterns of microbial communities in grassland soils changed with time, mainly in response to plant performance. Only a few studies have been carried out at the plot scale in grasslands or agricultural fields over multiple time points (Grayston et al., 2001; Habekost et al., 2008; Kulmatiski and Beard, 2011; Lauber et al., 2013). Coupled spatial characterization with temporal variability of soil microbial communities has been less often explored.

The goal of this study was to resolve some of this uncertainty by a detailed investigation of spatial patterns in microbial community structure to learn how the relationships between microbial communities and their local environment persist over time. Edaphic factors have been shown to exert the strongest influences on microbial community composition at regional and continental scales (Fierer and Jackson, 2006; Lauber et al., 2008; Dequiedt et al., 2011; Griffiths et al., 2011; Sayer et al., 2013). A physically homogeneous grassland plot was used for this study, however. This provided an opportunity to assess what other factors could be identified at specific dates as drivers of spatial relationships of the microbial community to both the local soil environment and to changes in the plant community. One 10 m × 10 m plot in a grassland characterized by low land use was intensively sampled over a complete growing season, from early April, before plants had begun to actively grow, until November of that year when plant growth had ceased after a hard frost. Sampling times were selected to coincide with stages of plant growth in the permanent grassland; replicate samples were separated by 50 cm. Using a combination of conventional and spatial statistical approaches, we characterized above- and below-ground communities both temporally and spatially for each date. Our aim was to learn whether or not changes in microbial abundance, in microbial community structure, or in distributions of plants and microorganisms could be temporally and spatially distinguished.

We hypothesized that (i) by a temporally and spatially intensive examination of an unimproved grassland at the plot scale (10 m × 10 m) we could distinguish spatial changes in microbial biogeography, and (ii) this sampling approach would clarify the degree to which the microbial spatial structures we observed could be correlated with stages of plant growth and soil abiotic properties. We expected also to gain insight into the persistence of microbial spatial structure and the relationships of microbial communities with their environment.

2. Materials & methods

2.1. Site description

The present study is part of a larger, interdisciplinary project of the German Biodiversity Exploratories (Fischer et al., 2010). Our study site is located near the village of Wittlingen, Baden-Württemberg, 48°25′0.01″ N, 9°30′0.00″ E, in the Swabian Alb, a limestone middle mountain range in southwest Germany. The study site is AEG31, within which a 10 m × 10 m grassland plot was established. Annual precipitation in 2011, the year in which this study was done, was 810 mm and average temperature was 8.1 °C (Appendix A: Fig. A1). The study site is managed at low intensity: no fertilizer is applied, it is mown once per year, and is briefly grazed by sheep for 1–2 weeks typically in late summer or early autumn. The soil type at the site is characterized as a Rendzic Leptosol (FAO classification), a calcareous, shallow AC-soil (typically 10 cm depth), with an average pH of 6.7, containing total 0.66 mg g^{-1} carbon (C) and 0.07 mg g^{-1} nitrogen (N). C/N ratios, pH, and soil texture were uniform over the sampling period.

2.2. Sample design

A 10 m × 10 m plot was established within this grassland and divided into 30 subplots (each 2 m × 1.67 m). Within each subplot six pairs of sample locations were randomly assigned, with one pair sampled at each of six dates over the growing season (Appendix A: Fig. A2). Each sample pair per subplot for a given date was separated by 50 cm to provide appropriate lag distances for later geostatistical analyses (Appendix A: Fig. A2). Sixty samples were collected at each date (two individual sample locations per subplot × 30 subplots). A total of 360 soil samples were collected over the season. Each sample location was assigned unique *x* and *y* coordinates with respect to the boundaries of the plot. Samples were collected in 2011: on April 5th at the beginning of the vegetation period, May 17th during the main growth phase, June 27th at around peak plant biomass, August 16th two weeks after the grassland was mown, October 5th, nine weeks after mowing and two weeks after it was lightly grazed, and November 21st after the first frost.

2.3. Sampling – aboveground

On each sampling date, before soil core samples were collected, 20 cm × 20 cm grids were centered over each of the sixty individual sampling points. Vegetation data and above ground biomass were collected from all grids. Above-ground biomass was harvested by cutting all plants at ground level. Biomass samples were sorted into litter (dead leaves and plant matter on the soil surface), grasses (Poaceae), legumes, forbs, bryophytes and *Rhinanthus minor*. The latter was separated because this species parasitizes other plants,

and thus may affect the productivity of grasslands (Stein et al., 2009). Plants that remained rooted but had senesced were included in living plant biomass. The biomass samples were dried for 48 h at 80 °C and weighed to the nearest 10 mg. From these data total above ground biomass as a measure of grassland productivity was calculated. Furthermore, in May, June, and October all vascular plant species were recorded and their percentage of total ground cover was estimated, following the nomenclature of Wißkirchen and Häupler (1998).

2.4. Sampling – belowground

Belowground samples were collected with core augers (diameter 58 mm) to a depth of 10 cm. Two cores, one for bulk density and one for biogeochemical analyses, were collected adjacent to each other at each sampling point (Appendix A: Fig. A2). The top one cm, consisting entirely of thatch, was removed from each soil core to avoid introducing surface plant residues into the soil. Stones, roots, and soil macrofauna were removed in the field. Soil samples were stored at 4 °C and sieved (<5 mm) within 24 h of collection, then subdivided for further analyses, with aliquots stored at 4 °C or frozen at −20 °C.

2.5. Physical, chemical, biological soil properties

Soil texture was determined by laser diffraction analysis (Beckman Coulter LS200 laser diffraction particle size analyzer, Beckman Coulter GmbH, Krefeld, Germany). To first determine the presence of carbonates in the samples they were tested using the Scheibler method (DIN ISO 10693:1997.05) for percent carbonate (CO_3^{2-}) determination in soil. Less than 0.1% CO_3^{2-} was detected; (Supplementary Material A: Methods). Soil pH was determined in 0.01 M $CaCl_2$ (soil to solution ratio w/v 1:2.5). Soil water content, reported as % soil dry weight, was determined gravimetrically after drying at 105 °C overnight. Bulk density cores were weighed, lengths were measured, cores were dried for 3 days at 105 °C, and re-weighed. Root biomass was determined in the bulk density cores; after flushing away the soil, roots were retrieved, dried at 60 °C for 3 days and weighed.

Ammonium (NH_4^+) and nitrate (NO_3^-) were extracted with 1 M KCl from soil samples (soil to extractant ratio of 1:4 w/v). Soil suspensions were placed on a horizontal shaker for 30 min at 250 rpm, then centrifuged (30 min at 4400 × g). Concentrations of NH_4^+ and NO_3^- in extracts were measured colorimetrically with a Bran & Luebbe autoanalyzer (Bran & Luebbe, Norderstedt, Germany). To determine the bioavailable phosphorus (P) fractions in soil, the second step of the sequential P fractionation was used (Hedley et al., 1982). Five hundred mg of each soil sample were extracted with 0.5 M $NaHCO_3$ (adjusted to pH 8.5) and shaken for 30 min before decantation and filtration (13 P Munktell & Filtrak GmbH, Bärenstein, Germany). Inorganic P concentrations in the extracts were determined colorimetrically with a continuous flow analyzer (Murphy and Riley, 1962). Elemental C and N were analyzed with a MACRO CNS Elemental Analyzer (Elementar-Analysensysteme, GmbH, Hanau, Germany). Because <0.1% carbonate was detected, total C was assumed to be organic C.

Microbial biomass carbon and nitrogen (C_{mic} and N_{mic}) were determined by chloroform fumigation extraction (Vance et al., 1987) with modifications (Keil et al., 2011). Extractable organic carbon and extractable organic nitrogen (EOC and EON) were calculated from the supernatants of the non-fumigated samples (Keil et al., 2011).

Two g of field moist soil were taken for lipid extraction and fractionation following the alkaline methylation method described in Frostegård et al. (1993a). Samples were measured by gas chromatography (AutoSystem XL. PerkinElmer Inc., Massachusetts, USA) using a flame ionization detector, an HP-5 capillary column and helium as the carrier gas. Fatty acid nomenclature used was described by Frostegård et al. (1993a, b). The following PLFA derived fatty acid methyl (FAMEs) were used as indicators for Gram positive bacteria (Gram+): a15:0, i15:0, i16:0 and i17:0; Gram negative bacteria (Gram−): cy17:0 and cy19:0 (Ruess and Chamberlain, 2010). Total bacterial PLFAs were calculated as the sum of Gram+ and Gram− plus the FAME 16:1ω7 which is widespread in bacteria in general. Fungal biomass was represented by the PLFA 18:2ω6.

Bacterial cell numbers were determined using a protocol modified after Lunau et al. (2005) and counted by epifluorescence microscopy under blue excitation (Zeiss Axio Imager M2, filter set 38 HE eGFP; Göttingen, Germany) at a magnification of 1,000×. A minimum of 20 microscopic fields were counted for each sample (for details Supplementary Material A: Methods).

2.6. Statistical analyses

All statistical analyses were carried out in the R environment, (R Development Core Team, 2012). Cell count data were log-transformed for all analyses to achieve homogeneity of variance. To test whether plant, microbial and abiotic variables exhibited seasonal changes, univariate, one-way ANOVAs with sampling date as a factor were calculated, followed by Tukey's HSD as post hoc test ($P < 0.05$).

To test whether variables exhibited spatial structure at a given date, semivariogram analyses were assessed using the gstat 2.4.0 Package (Pebesma, 2004). Where non-random spatial patterns prevail, spatial structure can be measured by plotting empirical geostatistical functions (i.e. semivariogram functions). Semivariances tend to increase with distance of the sampling points until a plateau (sill) is reached, after which values fluctuate randomly about the sill. In stationary data, the sill equals the total sample variance. The distance at which the sill is reached is called the range and represents the maximum distance of spatial autocorrelation. Semivariograms usually exhibit a discontinuity at the origin, called the nugget effect, which is due either to non-measurable variation below the minimum sampling distance or to measurement error. Structural variance is that part of the total sample variance which is spatially auto-correlated. Empirical semivariograms were calculated for each variable to a maximum distance of 8 m, and a spherical, exponential or linear model was fitted based on RMSE and visual control. Spherical and exponential models indicate that spatial structure occurs at the measured scale, whereas a linear model indicates spatial structure beyond that scale, i.e. a gradient through the plot. If only the nugget is apparent, no spatial structure can be detected at the sampled scale. The percent structural variance was calculated for spherical and exponential models by subtracting the nugget effect from the sill, and dividing the remaining, or partial, variance by the total sill. When a model could be fitted, a kriged map of the distribution of that property on the plot could be constructed. Maps were constructed using ArcGIS (ESRI 2010, Environmental Systems Research Institute, Redlands, CA, USA).

In order to determine how microbial communities were affected by spatial proximity and by environmental drivers, including root and litter mass, two approaches were used. In the first, principal components analysis (PCA) was used to reduce the dimensionality of the PLFA profiles, allowing the original samples to be scored on a small number of axes (principal components). Each of the principal components represents a distinct pattern of variation and can be considered to describe different aspects of the microbial community structure. Individual PLFAs were first normalized for every

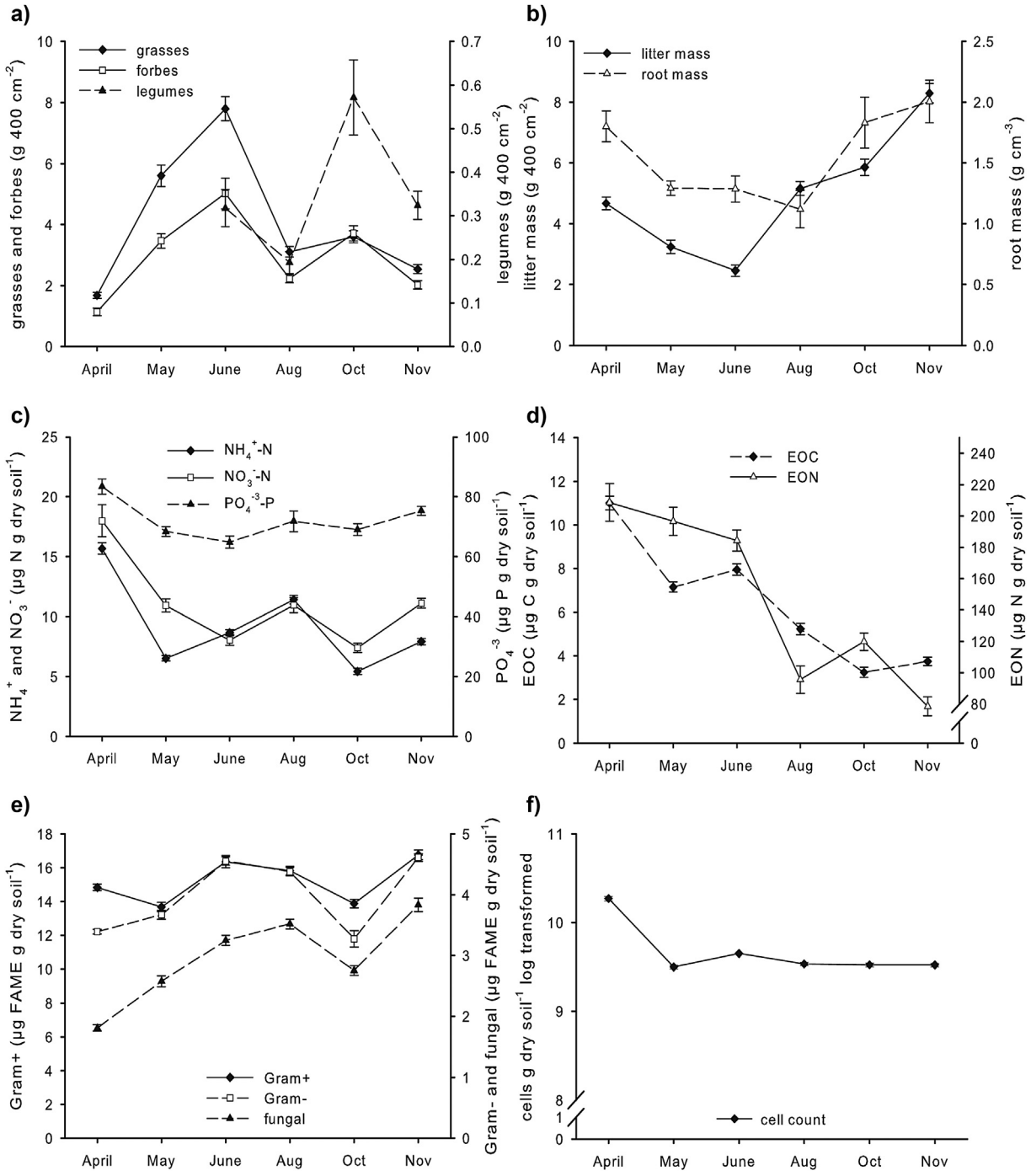

Fig. 1. a–f. Temporal changes in selected above- and below-ground properties for each sampling date. a) above ground plant biomass functional groups, b) litter mass and root mass, c) soil mineral nitrogen and phosphorus, d) extractable organic carbon and nitrogen, e) microbial fatty acids, and f) bacterial cell counts. Dotted lines indicate that the variable is scaled on the right *Y* axis. Error bars denote standard error. Cell count data were log-transformed for homogeneity of variance.

sampling date separately, then analyzed for each date with PCA. The PLFA loadings for the first three axes for each date were then examined to determine which PLFAs were most strongly associated with which axes, and whether these varied by date. Sample scores along each axis were then extracted and used as ordinary variables in semivariogram analysis to determine the extent to which each axis of variation was spatially structured on the plot, and, by extension, the extent to which the PLFAs associated with that axis were spatially structured. The spatial patterns and the relationships of the principal components with the abiotic or biotic environment were then examined as described above for the univariate data.

The second approach consisted in examining how the whole community data varied as a function of spatial separation and how the community data was related to multivariate descriptors of the local environment. This was achieved using Mantel tests (Franklin and Mills, 2009) with the package 'vegan' (Oksanen et al., 2013). The Mantel test tests for the association between distance matrices. Distance or similarity matrices were calculated between all pair-wise

combinations of samples for PLFA profiles, environmental conditions, plant biomass, plant species and geographic location. The distance matrices were constructed using the Euclidean distance. Euclidean distance was used because it is the metric that is usually used for PLFA and environmental data, as PLFA profiles generally have a linear response to environmental gradients. Four distance matrices were constructed for each sampling date: (i) spatial distances among pairs of sampling points using the x–y coordinates; (ii) distances in abiotic measures of the environment (soil moisture, bulk density, texture, pH, soil organic C, soil total N, EOC, EON, NH_4^+, NO_3^-, and mineral P); (iii) distances in biomass of different plant functional groups (grasses, forbs and legumes); and (iv) distances in the PLFA profiles of soil microorganisms. The environmental variables were scaled to unit variance and zero mean to account for the different units of measurement. Mantel statistics were then calculated for all pairs of distance matrices using the default setting of 999 permutations in the R package 'vegan' (Oksanen et al., 2013).

3. Results

3.1. Temporal patterns

3.1.1. Plants and litter

Total plant, grass and forb biomass was lowest in April and peaked in June, before it was harvested by mowing in early August. Legume biomass was too low to be measured in April and May, but showed a marked increase after mowing and a peak in October (Fig. 1a). By November, after the first hard frost, biomass of all plant functional groups declined as a result of senescence (Fig. 1a). In contrast, litter biomass decreased from April to June, and then steadily increased until November. Similarly, root biomass declined from April until August and increased to its highest level in November (Fig. 1b).

3.1.2. Abiotic soil properties

Bulk density changed slightly but significantly throughout the sampling period with highest bulk density in August (Table 1). Soil pH was relatively stable throughout the vegetation period, varying between 6.6 and 6.8 (Table 1).

Soil C and N content showed almost no differences over the sampling period (Table 1). Soil C/N ratios ranged from 10.0 to 10.3; with the lowest C/N ratios for the season recorded in June and the highest in August (Table 1). EOC differed significantly on most sampling dates with steadily decreasing values from April until October and a slight increase in November (Table 1, Fig. 1d). In contrast, EON was low in August, increased in October, and was lowest in November (Table 1, Fig. 1d).

Both mineral forms of nitrogen, NH_4^+ and NO_3^-, were highest in April and declined through May and June (Fig. 1c). Both increased after mowing in August, decreased in October, and increased again in November. Bioavailable P was also highest in April, lowest in June and increased slightly from August through November (Table 1, Fig. 1c).

3.1.3. Soil microbial community variables

Temporal patterns of both C_{mic} and N_{mic} were similar: mean values declined from April to May, increased slightly in June and August, declined again in October and were highest for both in November (Table 1). Bacterial PLFAs were highest in June, August, and November and lowest in October (Fig. 1e). Both Gram+ and Gram− bacteria exhibited a fluctuating pattern throughout the season, but Gram− bacteria did not decrease in May as did Gram+ (Fig. 1e). The fungal PLFA biomarker exhibited a different pattern from the bacterial markers. It was lowest in April, increased steadily to almost double its April value by August, declined in October and increased to its highest value of the season in November (Fig. 1e). This resulted in an increase in the fungal to bacterial ratio from August through November (Table 1). Total bacterial cell counts were significantly higher in April than at any other sampling date; from May through November there were no significant changes except in June, when cell counts increased slightly but significantly from all later sampling dates (log-transformed data) (Table 1, Fig. 1f).

3.2. Univariate spatial patterns over time

All measured variables showed spatial structuring on some of the sampling dates (Appendix A: Table A1). However, the spatial structure changed over the sampling period, with little or no

Table 1
Environmental, plant and microbial data measured at the six sampling dates in 2011. Means per sampling ($n = 60$) with standard deviation (SD). Different letters indicate significant differences at P-values ≤ 0.05 obtained from Tukey's HSD test.

	5 Apr	SD		17 May	SD		27 Jun	SD		16 Aug	SD		5 Oct	SD		21 Nov	SD	
Soil moist. (gravim. % H_2O)	57.56	±2.95	a	27.97	±1.71	e	36.81	±3.27	d	46.38	±2.36	b	26.79	±2.34	e	40.03	±2.35	c
Bulk density (g cm^{-3})	0.93	±0.12	b	0.83	±0.08	d	0.87	±0.07	cd	1.02	±0.07	a	0.91	±0.09	bc	0.86	±0.06	d
pH	6.65	±0.15	b	6.68	±0.15	b	6.78	±0.20	a	6.69	±0.24	ab	6.79	±0.19	a	6.78	±0.21	a
Soil organic C (μg g^{-1})	654	±41	a	658	±50	a	652	±38	a	654	±43	a	651	±45	a	667	±32	a
Soil total N (μg g^{-1})	65	±4	ab	65	±4	ab	65	±3	ab	64	±4	ab	64	±4	b	65	±3	a
C/N ratio	10.12	±0.20	bc	10.11	±0.25	c	9.97	±0.22	d	10.25	±0.26	a	10.24	±0.26	ab	10.20	±0.21	abc
EOC (μg g^{-1})	208.25	±33.60	a	154.57	±25.37	b	165.78	±28.52	b	127.83	±28.13	c	100.19	±24.38	d	107.19	±21.64	d
EON (μg g^{-1})l	11.04	±6.63	a	10.17	±5.09	a	9.28	±3.75	a	2.91	±4.87	bc	4.66	±3.08	b	1.69	±3.39	c
NH_4^+-N (μg g^{-1})	15.70	±3.66	a	6.52	±1.79	d	8.67	±1.83	c	11.41	±2.71	b	5.43	±2.03	d	7.91	±2.06	c
NO_3^--N (μg g^{-1})	17.99	±10.43	a	10.93	±4.31	b	8.04	±3.38	c	10.96	±4.86	b	7.40	±2.90	c	11.11	±3.21	b
PO_4^{3-}-P (μg g^{-1})	83.41	±19.50	a	68.39	±12.40	bc	64.88	±15.50	c	67.62	±15.20	bc	69.02	±15.30	bc	75.36	±11.40	ab
Plant biomass (g 400 cm^{-2})	2.81	±1.28	e	9.73	±2.99	b	13.54	±3.94	a	5.73	±1.58	d	8.27	±1.87	c	5.11	±1.24	d
Litter mass (g 400 cm^{-2})	116.71	±40.31	c	80.89	±42.96	d	61.46	±35.81	d	129.02	±44.43	bc	146.45	±52.47	b	207.33	±61.18	a
Root mass (g cm^{-3})	1.80	±0.98	ab	1.29	±0.46	bc	1.29	±0.84	bc	1.12	±1.19	c	1.83	±1.62	ab	2.01	±1.37	a
C_{mic} (μg g^{-1})	1714.7	±156.4	b	1545.4	±234.6	c	1633.6	±189.9	bc	1702.0	±191.1	b	1570.4	±163.3	c	2036.5	±181.2	a
N_{mic} (μg g^{-1})	269.2	±34.4	ab	215.9	±36.7	d	251.6	±40.0	bc	244.9	±35.6	c	213.4	±31.5	d	273.2	±34.6	a
Bacterial cell count (data log transformed)	10.27	±0.20	a	9.50	±0.17	c	9.65	±0.19	b	9.53	±0.14	c	9.52	±0.16	c	9.52	±0.15	c
Bacterial PLFAs μg g^{-1} dry soil	24.43	±2.34	c	22.93	±3.37	cd	27.42	±4.13	ab	26.49	±3.12	b	22.22	±3.17	d	28.38	±3.41	a
Gram+ PLFAs μg g^{-1} dry soil	14.84	±1.46	bc	13.69	±2.10	d	16.36	±2.75	ab	15.83	±1.96	ab	13.88	±1.88	cd	16.76	±2.16	a
Gram− PLFAs μg g^{-1} dry soil	3.40	±0.32	bc	3.67	±0.55	b	4.56	±0.56	a	4.38	±0.55	a	3.28	±1.05	c	4.61	±0.50	a
Fungal PLFA μg g^{-1} dry soil	1.81	±0.42	d	2.58	±0.71	c	3.25	±0.65	b	3.52	±0.63	ab	2.76	±0.62	c	3.84	±0.85	a
Fungal/bacterial ratio	0.07	±0.02	c	0.11	±0.03	b	0.12	±0.02	b	0.13	±0.02	a	0.12	±0.02	ab	0.14	±0.03	a

spatial structure in April, and more frequently detected spatial structure in both May and October. In addition, the ranges over which spatial structure was detected and the amount of variation explained varied considerably among properties, with spatial structures over distances of 2–8.6 m and explained variances between 11.4% and 94.1%. (Appendix A: Table A1). Ranges for abiotic soil properties with fitted spherical models varied from 2.0 to 8.6 m. (Appendix A: Table A1). Spatial structure in root biomass was evident only in November; with a range of 2.2 m. Plant functional groups exhibited no spatial structure until after the post-mowing sample collection. Forbs were first, in August, followed by grasses and legumes in October (Appendix A: Table A1). C_{mic} and N_{mic} were spatially structured from June to October and May to October, respectively, but their ranges and percent structural variance differed (Appendix A: Table A1). Total bacterial PLFAs exhibited spherical spatial structure in May, October, and November. Empirical variograms were also modeled for the individual PLFAs associated with either Gram+ or Gram− bacteria, as well as the single PLFA associated with fungi (Appendix A: Table A1). Although PLFA 18:2ω6 can also be associated with fresh litter, its value did not vary with litter so we think it accurately represented fungi in this plot. The Gram+ and Gram− PLFAs consistently exhibited spatial structure in May and October. Other dates were more variable among both groups and this variability displayed no pattern within or between groups (Appendix A: Table A1). Although measured values did not vary significantly among these dates, kriged maps of the distributions of exemplary PLFAs that were spatially structured indicated that their distribution on the plot shifted from spring to autumn (Fig. 2a–i). The distances over which bacterial PLFAs exhibited spatial autocorrelation also became shorter as the season progressed (Fig. 2c & f). Unlike the bacterial PLFAs, spherical spatial structure of the fungal PLFA was discernable only in June and August and its spatial autocorrelation increased; the model in October was linear and the variogram indicated that spatial autocorrelation extended past the limits of the plot (Appendix A: Table A1, Fig. 2h, i). The ranges in percent structural variance for the bacterial PLFAs were 23% in May and 42% in October, while for the fungal PLFA they were 47% in June. Percent structural variance for the fungal PLFA could not be calculated for October because the model was linear for that date (Appendix A: Table A1). Cell counts were spatially structured at our sampling scale at every date except April, at which date no model could be fitted. Their spatial structure began to emerge in May, and by June exhibited a spherical spatial structure which persisted through November (Appendix A: Table A1).

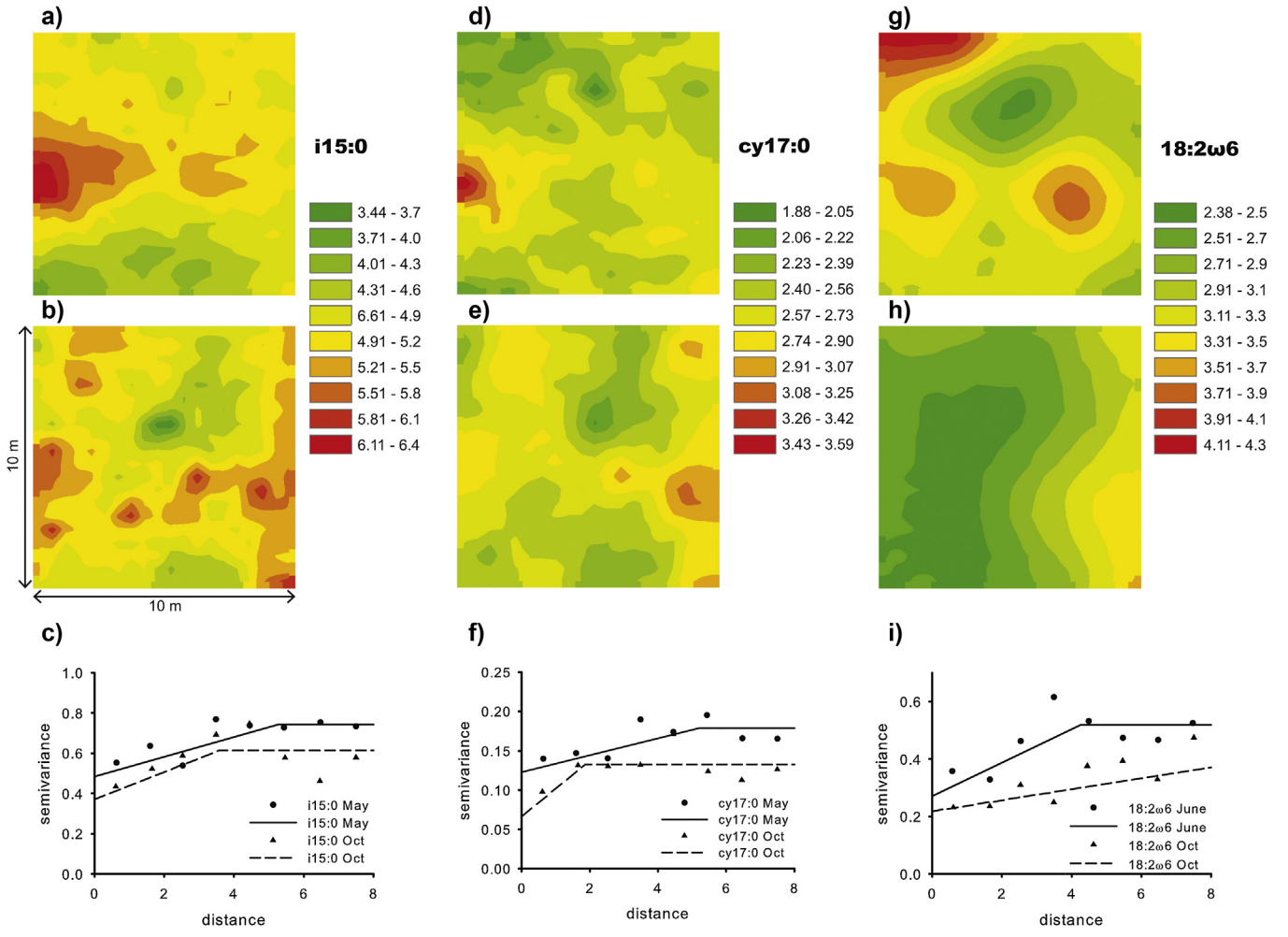

Fig. 2. a–i. Kriged maps of exemplary Gram+, Gram− bacterial PLFAs and fungal PLFA. a) Gram+ i15:0 PLFA in May, b) Gram+ i15:0 PLFA in October, c) semivariograms used to create maps a & b; d); Gram− cy17:0 PLFA in May, e) Gram− cy17:0 PLFA in October, f) semivariograms used to create maps d & e; f) fungal PLFA 18:2ω6 in June, g) fungal PLFA 18:2ω6 in October, i) semivariograms used to create maps g & h. Dimensions of all maps are 10 m × 10 m.

3.3. Changes in spatial patterns of microbial and plant community structure and environmental profiles over time

3.3.1. Mantel statistic – relationships among communities

Mantel statistics were calculated to characterize spatial relationships among soil abiotic properties, plant functional groups and the microbial community (Table 2). Overall, abiotic soil properties exhibited strong spatial correlation throughout the year, except in November. In contrast, plant functional groups showed significant spatial structure only in April and November, whereas microbial community structure (PLFA profiles) exhibited weak spatial structure in April, August, and November (Table 2). A weak relationship between plant functional groups and abiotic soil properties was observed only in April (Table 2). In contrast, microbial community spatial structure was significantly correlated with abiotic soil properties throughout the year, especially in spring and autumn but, though still significant, the correlation was weakest in June and August. At no sampling date was microbial community spatial structure significantly correlated to plant functional groups. Plant species composition, which was available for three of the six sampling dates, was not significantly related to microbial community spatial structure (results not shown).

3.3.2. PCA – distinctions within the microbial community

To take a closer look at the different groups of the microbial community, principal components analysis (PCA) was used to analyze individual PLFAs associated with the microbial community for each sampling date (Table 3). The first three principal components (PC) together accounted for 57–67% of total PLFA variance over the six sampling dates for all PLFAs. The PC scores of the first three PCs were also spatially modeled (Appendix A: Table A1). In April and May, there was little or no spatial structure to the variance in principal components. In June and August, there was more evidence of spatial structure in the second PC, but that structure was no longer evident in autumn (Appendix A: Table A1). Scores of the first three PCs were then correlated with soil environmental and abiotic properties to determine which were significantly correlated with each PC at each date (Table 3). In April and May there were few significant relationships (Table 3). Visual inspection of the PC loadings over the entire season indicated that PC1 was mainly associated with PLFAs indicative of Gram+ bacteria and fungi, while PC2 and PC3 were mainly associated with those of Gram− bacteria and this discrimination held throughout the season (Table 3). In June, of the seven PLFAs associated with particular subsets of the microbial community, two were more strongly related to measured soil properties. Gram− bacterial PLFAs were always associated with PCs that were strongly correlated to soil abiotic properties (Table 3). Furthermore, the correlations in PC2 were mainly negative, while those in PC3 were more often positive (Table 3). PLFA i16:0 was associated with PC2, and fungal PLFA 18:2ω6 with PC3 (Table 3). However, PC2 and PC3 together accounted for only 31% of the observed variance at this date. There were no apparent trends in the relationships between the microbial community and plants, although forbs were positively correlated with PC3 in August, and root biomass with PC1 in October.

Table 3
Correlations of scores of principal component analyses for microbial communities (using the microbial PLFA data) with abiotic properties, root and litter mass and plant functional groups at each of the six sampling dates in 2011. Only significant ($P < 0.01$) correlations of properties with each of the three PC-axes are shown. Properties in italics indicate negative correlations.

Sampling date	PLFAs			Abiotic parameters		
	PC1	PC2	PC3	PC1	PC2	PC3
April	a15.0		i17.0		PO_4^{3-}–P	
May	i15.0		cy17.0	*NO_3^-–N*		pH
	18.2ω6					NO_3^-–N
June	a15.0	i16.0			*pH*	*pH*
	i15.0	i17.0			*C*	
	18.2ω6	cy19.0			*Total N*	
			18.2ω6		*PO_4^{3-}–P*	
					EOC	
		cy17.0	i16.0		*EON*	
Aug	i15.0				*Soil moisture*	Forb mass
	18.2ω6				*N_{min}*	
	a15.0	i16.0	cy17.0		*PO_4^3–P*	
Oct	i15.0	i17.0		*Root mass*	Total N	NH_4^+–N
	18.2ω6	cy19.0			N_{min}	EOC
	a15.0		i16.0		EON	EON
Nov	i15.0		cy17.0	*PO_4^3–P*	*NO_3^-–N*	NH_4^+–N
			cy19.0		*PO_4^3–P*	EOC
						EON

4. Discussion

The overall structure of the microbial community was strongly related to the local abiotic environment throughout the sampling period, as indicated by the Mantel statistics (Table 2). Our results confirm the findings of others who have looked at soil microbial biogeography in croplands and grasslands at broader scales (Ettema and Wardle, 2002; Fierer and Jackson, 2006; Martiny et al., 2006; Lauber et al., 2008; Fierer et al., 2009; Ranjard et al., 2010; Dequiedt et al., 2011; Griffiths et al., 2011; Martiny et al., 2011). However, although the relationship between soil abiotic properties and the microbial community persisted throughout the year, the strength of that relationship varied, suggesting that it was not constant over time, and that other factors also influenced microbial community composition. The fact that subsets of the microbial community, as differentiated by PCA that discriminated PLFAs associated with Gram+ and Gram− bacteria and with saprotrophic fungi, were related to different environmental variables at specific times, tends to confirm this (Table 3). Gram+ bacteria exhibited little relationship to measured soil properties, and the sole correlation we could identify for Gram+ bacteria was only negatively related to root mass and only at one date. This suggests that Gram− bacterial PLFAs may have been more influenced by belowground processes than were Gram+ PLFAs (Table 3). This discrimination

Table 2
Results of the Mantel tests including data spatial structure (spatial), abiotic properties (abiotic), plant functional groups (plant) and microbial community data (microbial) from the six sampling dates in 2011. Pearson correlations (*r*-values) with significance assessed by permutation test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns = not significant.

Sampling time	Spatial/abiotic	Spatial/plant	Spatial/microbial	Abiotic/plant	Abiotic/microbial	Plant/microbial	
Apr	0.18^{***}	0.13^{**}	0.10^{*}	0.18^{*}	0.38^{***}	0.06^{ns}	675
May	0.16^{**}	-0.02^{ns}	0.05^{ns}	0.03^{ns}	0.38^{***}	0.05^{ns}	676
Jun	0.20^{***}	0.04^{ns}	-0.03^{ns}	0.04^{ns}	0.12^{*}	-0.08^{ns}	677
Aug	0.25^{***}	0.02^{ns}	0.09^{*}	-0.07^{ns}	0.27^{**}	-0.14^{ns}	678
Oct	0.12^{*}	-0.02^{ns}	0.01^{ns}	0.00^{ns}	0.35^{***}	-0.06^{ns}	679
Nov	0.06^{ns}	0.12^{**}	0.10^{*}	0.00^{ns}	0.27^{***}	-0.03^{ns}	

between Gram+ and Gram− bacterial responses to belowground processes, furthermore, persisted over time (Table 3).

Kriged maps of the changes in distributions of exemplary Gram+ and Gram− bacterial PLFAs showed that Gram+ bacteria varied more across the site than did Gram− bacteria at the two dates shown (Fig. 2a–d). One must use caution in interpreting changes in PLFAs; shifts can indicate changes in populations of microbes, in species composition, or in physiological adaptations of existing populations as a response to environmental stress (Wixon and Balser, 2013). However, the decline in Gram+ and the increase in Gram− bacterial numbers in May suggest differential responses to increased competition with plants for soil nutrients (Fig. 1e). In addition, Gram− bacteria may have been able to take advantage of root exudates more rapidly than Gram+ bacteria at this date. Increases in the PLFAs associated with Gram− bacteria under conditions of increasing environmental stress have been measured by Moore-Kucera and Dick (2008). Conversely, a slight increase in June in soil moisture and in EOC would have reduced the environmental stress on bacteria, resulting in our observed increases in all PLFAs at this date (Fig. 1d–f), as well as in the bacterial cell counts, which measure only the active portion of the bacterial community (Table 1, Fig. 1f). These observations support the findings of Lennon et al. (2012), who, using a taxonomic approach, linked functional traits of microbial groups to their responses to a moisture gradient. In their study, different members of the microbial community, characterized by the coarse taxonomic classifications of Gram+ or Gram−, demonstrated varying degrees of tolerance and resilience to small changes in environmental stresses over the season.

Spatial structure at this scale and at specific times suggests that extremely local processes were influencing the properties we modeled (Appendix A: Table A1). Exemplary Gram+ and Gram− PLFA maps in May and October indicated that, although the range of values did not differ much from one time point to the other, distributions of the bacteria shifted somewhat on the plot (Fig. 2a, b & d, e). The shift of Gram+ PLFA i15:0 from a cosmopolitan to a patchy distribution from May to October (Fig. 2a, b) was possibly due to competition with plants for soil nutrients. When nutrients are rate limiting, as may have been the case for Gram+ bacteria by late in the season, their growth could have been confined to “hotspots” in which nutrients were accessible (Nunan et al., 2003). In contrast, the overall pattern of distribution was more uniform for Gram− PLFA cy17:0 (Fig. 2d, e). Both Gram+ and Gram− PLFAs were low in the same regions in October, perhaps reflecting a process we were not able to capture at our sampling scale. The distribution of the fungal PLFA in October was almost uniformly low on the plot as compared to the more variable bacterial PLFAs, suggesting bacteria may have been able to take advantage either of different resources or of the same resources to a greater degree than fungi were at this time (Fig. 2b, e, h). The correspondence between our observed low fungal and higher bacterial distributions on the plot in October could have been due to competitive strategies for resources between bacteria and fungi described by de Boer et al. (2005). The ranges of spatial structuring in plant, abiotic, and microbial properties which we were able to characterize on the plot late in the season suggest that the local belowground environment had changed in tandem with seasonal aboveground processes, resulting in a much more structured microbial community at the scale of this study. This can be seen in the development of more spherical spatial models of most parameters toward the end of the vegetation period (October). The fact that much of the spatial structure at our sampling scale was no longer detectable by November also supports this claim (Appendix A: Table A1).

Our study in a low land use intensity grassland could not detect any effect of the biomass of plant functional groups on the structure of the microbial community (Table 2), even though plants have been shown to exert a strong effect on soil microbial communities when different plant communities such as deciduous or coniferous forests are being compared (Wardle et al., 2004). This could have been because plant functional groups exhibited no spatial structure themselves and were not correlated with abiotic soil conditions over most of the growing season (Table 2, Appendix A: Table A1). Our results are consistent with Fierer and Jackson (2006) and Sayer et al. (2013) who were not able to identify direct links between microbial and plant community composition or stage of plant growth. But they are in contrast to Reynolds et al. (2003) and Kulmatiski and Beard (2011), perhaps because many studies on plant-soil feedbacks concentrate on particular dominant species. Our studied grassland was a species-rich community with between 12 and 20 plant species per 20 cm × 20 cm, without a single dominant plant species. In grasslands roots are also very dense and enmeshed; microbial communities may therefore be affected by many plants at once, reinforcing the lack of dominance of individual species.

Despite the absence of spatial variability, plant biomass varied strongly over the season. Our plot was mowed in early August, two weeks before August sample collection. Biomass removal by cutting or mowing is known to increase root exudation (Kuzyakov et al., 2002) and several studies have shown positive effects of plant defoliation on microbial biomass and/or activity (Mawdsley and Bardgett, 1997; Macdonald et al., 2006). Therefore, we had expected to see an effect of mowing on bacterial PLFAs due to increased exudation of simple carbon compounds (Paterson and Sim, 2000). However, we saw a negative response; both groups of bacterial PLFAs declined in August. Exudates may have been depleted by the time of our sample collection; their turnover rate in soil can occur in hours to days (Bais et al., 2006; Drake et al., 2013). Therefore, two weeks after mowing may have been too late to see a positive response in the bacteria. EOC was also low in August, suggesting that available carbon might have been limiting at this date. EON was low as well, and there is evidence that nitrogen availability can be a rate-limiting step in microbial uptake of root exudates (Zhou et al., 2012; Drake et al., 2013). The fungal PLFA associated with saprotrophic fungi often increases after mowing in response to increased C input to the soil from exudation, and fungi can also take advantage of the recalcitrant carbon in litter (Bardgett et al., 1996; Denef et al., 2009). The fungal PLFA in our study did increase in August and so did litter (Fig. 1b,e). Increased litter could have contributed to the fungal PLFA increase we observed in August. Therefore changes we observed in both bacterial and fungal PLFAs at this date could not be clearly related to mowing. Although evidence of direct linkages between above- and below-ground processes could not be established in our study, indirect links were indicated by the relationship of changing substrate availability to changes in microbial PLFA abundances and distributions (Fig. 1c–e). The differential responses of Gram+ and Gram− bacteria suggest a need for a deeper look (for example, using pyrosequencing) into the members of these communities, to learn whether our observations hold at a finer scale of taxonomic resolution.

5. Conclusions

Over the season, the physical soil structure of this unplowed, unfertilized grassland was homogeneous. Dense root penetration throughout the soil meant that we could not identify individual plant effects at this site. This is in agreement with other studies of unfertilized grasslands, in which direct links between above- and belowground properties have proven elusive (Ritz et al., 2004). Nevertheless, it is clear that a complex combination of interactions was operative at the scale of our study. We identified variability in

microbial community composition through a close analysis of PLFA data, and showed that the controls on that variability differed over the season. Environmental properties were the main structuring agents of the microbial community, as they are at larger scales. However, although this relationship persisted over time, individual components of environmental properties varied with season, and those differences may be hypothetically related, albeit indirectly, to changes in plant growth. Changes in soil nutrient status, for example, were directly related to plant growth, and could have served to integrate a number of related processes, similar to the integrating effect of pH at the landscape scale. This in turn masked more ephemeral – but important – shifts in controls on microbial spatial distribution and community composition. We demonstrated not only evidence of the complexity of microbial communities in grassland soils but also the importance of a temporal component to the characterization of soil microbial biogeography.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2013.12.024.

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