RESEARCH ARTICLE

Over 150 Years of Long-Term Fertilization Alters Spatial Scaling of Microbial Biodiversity

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ABSTRACT **Spatial scaling is a critical issue in ecology, but how anthropogenic activities like fertilization affect spatial scaling is poorly understood, especially for microbial communities. Here, we determined the effects of long-term fertilization on the spatial scaling of microbial functional diversity and its relationships to plant diversity in the 150-year-old Park Grass Experiment, the oldest continuous grassland experiment in the world. Nested samples were taken from plots with contrasting inorganic fertilization regimes, and community DNAs were analyzed using the GeoChip-based functional gene array. The slopes of microbial gene-area relationships (GARs) and plant species-area relationships (SARs) were estimated in a plot receiving nitrogen (N), phosphorus (P), and potassium (K) and a control plot without fertilization. Our results indicated that long-term inorganic fertilization significantly increased both microbial GARs and plant SARs. Microbial spatial turnover rates (i.e., *z* values) were less than 0.1 and were significantly higher in the fertilized plot (0.0583) than in the control plot (0.0449) ($P < 0.0001$). The *z* values also varied significantly with different functional genes involved in carbon (C), N, P, and sulfur (S) cycling and with various phylogenetic groups (archaea, bacteria, and fungi). Similarly, the plant SARs increased significantly ($P < 0.0001$), from 0.225 in the control plot to 0.419 in the fertilized plot. Soil fertilization, plant diversity, and spatial distance had roughly equal contributions in shaping the microbial functional community structure, while soil geochemical variables contributed less. These results indicated that long-term agricultural practice could alter the spatial scaling of microbial biodiversity.**

IMPORTANCE **Determining the spatial scaling of microbial biodiversity and its response to human activities is important but challenging in microbial ecology. Most studies to date are based on different sites that may not be truly comparable or on short-term perturbations, and hence, the results observed could represent transient responses. This study examined the spatial patterns of microbial communities in response to different fertilization regimes at the Rothamsted Research Experimental Station, which has become an invaluable resource for ecologists, environmentalists, and soil scientists. The current study is the first showing that long-term fertilization has dramatic impacts on the spatial scaling of microbial communities. By identifying the spatial patterns in response to long-term fertilization and their underlying mechanisms, this study makes fundamental contributions to predictive understanding of microbial biogeography.**

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Microbial communities constitute a large portion of the Earth's biosphere and play important roles in maintaining various biogeochemical processes that mediate ecosystem functioning. They inhabit almost all natural environments, with populations undergoing dynamic changes in composition, structure, and function over space and time. Understanding the geographic patterns of microbial diversity and their relationships to plant diversity is critical for understanding the mechanisms controlling microbial biodiversity (1, 2). In recent years, the spatial distribution patterns of microbial diversity have attracted substantial attention (3–6). Taxon-area relationships (TARs), species-area relationships (SARs), and gene-area relationships (GARs) are among the best known spatial patterns. The power law equation, $S = cA^z$, where S is species richness, A is area, c is the intercept in log-log space, and the species-area exponent, z, is a measure of the rate of species change with space, is typically used to describe SARs. Despite their ecological and biogeographical importance, the TARs of microbial communities have only been studied recently. This is due to their complexity, especially under natural settings, and their unique features that differ considerably from those of macroorganisms, such as high dispersal rates, high functional redundancy, massive population sizes, rapid asexual reproduction, resistance to extinction, and horizontal gene transfer (3, 7–9). The slopes of TARs for microorganisms also vary substan-

tially among different studies (z = 0.019 to 0.470), especially in soils, which could be due to variations in environmental conditions, experimental design, spatial scales, and the analytical approaches used (9). However, it has been generally recognized that the slopes of the microbial TARs are considerably lower than the slopes of TARs associated with plants and animals (7).

In the past centuries, human activity has greatly affected the biosphere and global biogeochemical processes. For example, fertilization, changes in land use, and fuel combustion have substantially altered the structures of communities and their ecological functions (10). Although microbial TARs have been documented in natural habitats of microorganisms, such as natural forest soils (9, 11), grassland soils (12, 13), marsh sediments (3, 4), lakes (14, 15), and marine environments (16), it is not yet clear how they are affected by anthropogenic activities. Thus, there is an urgent need to determine how microbial communities respond to anthropogenic activities at different spatial scales.

The use of nitrogen (N) and other fertilizers (e.g., phosphorus [P] and potassium [K]) has long been an agricultural practice to increase the net primary productivity of plants. While fertilization increases plant productivity, it generally decreases plant species diversity (17). It is also known that N fertilization has significant impacts on microbial diversity and activity (18–20), but the impacts of other inorganic fertilizers, such as P and K, are less clear. Also, most of those studies focused on short-term responses, which are expected to differ considerably from those in the long term. Generally, long-term fertilization can have more persistent impacts on soil characteristics (21, 22), plant growth (23), and fungal diversity by decreasing root exudates (24). A meta-analysis of 82 published field studies indicated that microbial biomass declines with N fertilization and that longer periods of fertilization resulted in stronger decreases in microbial biomass (20), indicating the significant influence of the duration of fertilization on microbes. However, one potential pitfall in examining spatial dynamics in the context of human interference is the lack of studies addressing long-term response to fertilization. To date, little is known about the effect of fertilization on microbial TARs, especially over long time periods.

The Park Grass Experiment (PGE) at Rothamsted Research, United Kingdom, conducted on a permanent grassland, is the oldest grassland experiment in the world (25). Since 1856, its plots have received different amounts and combinations of inorganic N, P, and K fertilizers and lime, while control plots have gone without fertilization. During such long-term fertilization, soil geochemical characteristics and soil biota can represent a long-term response (26–28). As such, the PGE provides a unique opportunity to study spatial patterns of microbial and plant biodiversity. Here, we examined microbial GARs under long-term inorganic fertilization, using GeoChip-based metagenomics technologies to address the following questions. (i) Does long-term inorganic fertilization affect microbial GARs and plant SARs? If yes, does the influence on GARs vary across different functional and phylogenetic groups in a community? (ii) How do environmental variables contribute to the spatial scaling of microbial and plant diversity? (iii) How do microbial GARs relate to plant SARs? Our results indicate that long-term fertilization has significant impacts on spatial scaling of microbial communities. This study is important for predictive understanding of microbial biogeography.

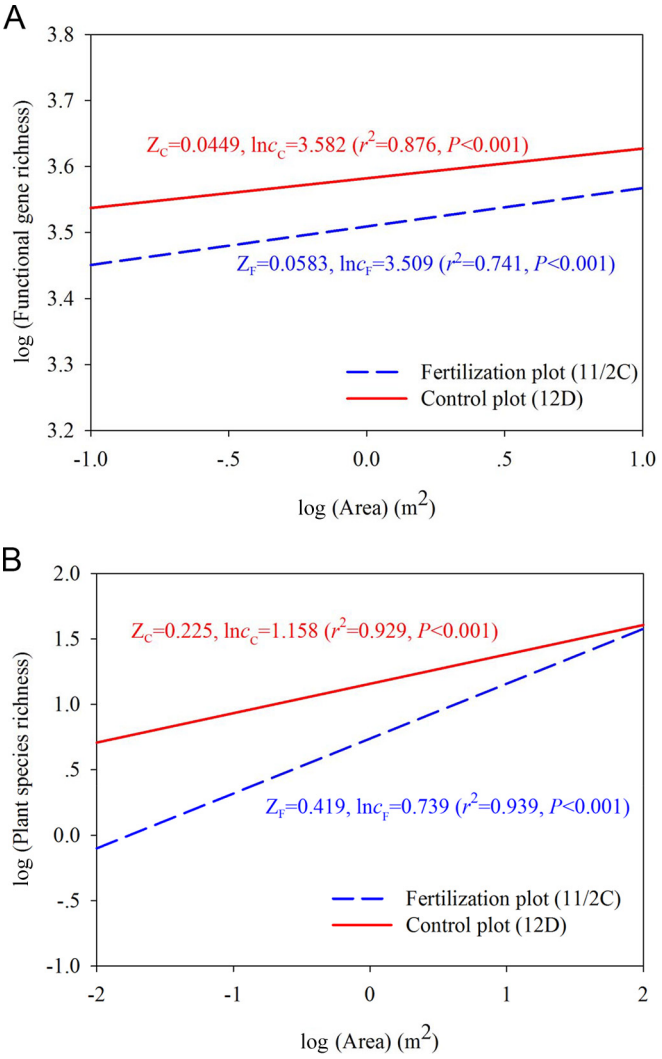

FIG 1 Microbial gene-area relationship (A) and plant species-area relationship (B) in a long-term fertilized plot and a control plot of the Park Grass Experiment.

RESULTS

Gene-area relationship under long-term inorganic fertilization. A total of 42 soil samples (21 samples each from the fertilized plot and the control plot; see Fig. S1 in the supplemental material) were analyzed with GeoChip 3.0. Altogether, 2,126 (±459) (mean ± standard deviation) functional genes were detected in the control plot and 1,670 (±434) in the fertilized plot. The average percentages of genes common to pairwise samples in the 12D plot (control), 11/2C plot (fertilization), and between these two plots were 53.5%, 48.5%, and 47.8%, respectively. The fertilized plot had fewer genes in common than the control plot, based on t test results (t = 8.603, $P < 0.0001$). Microbial z values (the exponent of GAR) for all functional genes were estimated by linear regression of log-transformed raw data, and they were 0.0583 in the fertilized plot and 0.0449 in the control plot (Fig. 1A). The differences of the z values were statistically significant as determined by the pairwise t test based on bootstrapping ($P < 0.0001$) (Table 1).

Plant species and abundance were also surveyed in nested squares with 0.025 m^2 for each quadrant in the same experimental

TABLE 1 The slopes of gene-area relationships for various functional and phylogenetic groups under control or long-term fertilization

	Control (12D plot)					N fertilization (11/2C plot)					*t* test[c]	
	z value	95% C.I.	*n*	*t*	*P*	*z* value	95% C.I.	*n*	*t*	*P*	*t*	*P*
All functional genes	0.0449	1.47×10^{-3}	4006	−60.21	<0.001	0.0583	2.10×10^{-3}	3405	−54.59	<0.001	10.22	0.0001
Functional groups												
C degradation	0.0588[a]	1.90×10^{-3}	432	−59.87	<0.001	0.0630[b]	2.26×10^{-3}	414	−53.5	<0.001	2.78	0.0053
C fixation	0.0421	1.36×10^{-3}	141	−58.7	<0.001	0.0754[b]	2.52×10^{-3}	129	−57.77	<0.001	22.80	0.0001
N fixation	0.410[a]	1.38×10^{-3}	174	−58.95	<0.001	0.0365[b]	1.36×10^{-3}	151	−52.5	<0.001	4.54	0.0001
Assimilatory N reduction	0.0660[a]	2.18×10^{-3}	34	−58.73	<0.001	0.0582	2.23×10^{-3}	29	−51.71	<0.001	4.90	0.0001
Dissimilatory N reduction	0.0599[a]	1.94×10^{-3}	28	−57.59	<0.001	0.0592	2.29×10^{-3}	25	−51.08	<0.001	0.45	0.6479
Nitrification	0.0671[a]	2.91×10^{-3}	14	−45.42	<0.001	0.0842[b]	2.86×10^{-3}	7	−56.99	<0.001	8.21	0.0001
Denitrification	0.0543[a]	1.81×10^{-3}	190	−58.93	<0.001	0.0613	2.21×10^{-3}	148	−54.73	<0.001	4.81	0.0001
Phosphorus	0.0234[a]	8.02×10^{-4}	76	−55.08	<0.001	0.0609	2.18×10^{-3}	56	−57.55	<0.001	31.71	0.0001
Sulfur	0.0367[a]	1.22×10^{-3}	193	−58.82	<0.001	0.0724[b]	2.56×10^{-3}	176	−54.44	<0.001	24.65	0.0001
Phylogenetic groups												
Archaea	0.0428	1.42×10^{-3}	94	−58.68	<0.001	0.0492[b]	1.72×10^{-3}	79	−55.57	<0.001	5.60	0.0001
Fungi	0.0604[a]	2.02×10^{-3}	182	−58.93	<0.001	0.0704[b]	2.61×10^{-3}	153	−54.13	<0.001	5.93	0.0001
Bacteria	0.0444	1.47×10^{-3}	3191	−58.03	<0.001	0.0565[b]	2.04×10^{-3}	2705	−53.7	<0.001	9.43	0.0001
Gram-positive	0.0263[a]	8.79×10^{-4}	539	−59.6	<0.001	0.0347[b]	1.25×10^{-3}	496	−53.28	<0.001	10.75	0.0001
Gram-negative	0.0254[a]	8.54×10^{-4}	1998	−59.3	<0.001	0.0312[b]	1.12×10^{-3}	1656	−54.93	<0.001	8.05	0.0001
α-Proteobacteria	0.0356[a]	1.20×10^{-3}	763	−59.71	<0.001	0.0567	2.02×10^{-3}	661	−54.92	<0.001	17.58	0.0001
β-Proteobacteria	0.0437	1.45×10^{-3}	429	−60.84	<0.001	0.0557	2.07×10^{-3}	356	−54.61	<0.001	9.30	0.0001
γ-Proteobacteria	0.0496[a]	1.65×10^{-3}	522	−58.56	<0.001	0.0528[b]	1.89×10^{-3}	399	−53.76	<0.001	2.50	0.0123
δ-Proteobacteria	0.0619[a]	2.03×10^{-3}	144	−59.35	<0.001	0.0583	2.13×10^{-3}	126	−54.88	<0.001	2.39	0.0165

[a] The *z* values of functional groups and phylogenetic groups are significantly different from all functional genes ($P < 0.05$) in control plot.
[b] The *z* values of functional groups and phylogenetic groups are significantly different from all functional genes ($P < 0.05$) in fertilized plot.
[c] The right-most columns test whether the *z* values for the two treatments were significantly different.

plots to determine the plant species-area relationships (SARs). The *z* values were 0.419 in the fertilized plot and 0.225 in the control plot (Fig. 1B), respectively. Statistical analysis using the pairwise *t* test with bootstrapping indicated that the plot with long-term fertilization had significantly ($P < 0.0001$) higher *z* values than the control plot.

Variations in gene-area relationships among different functional genes and phylogenetic groups. Gene-area relationships were observed for different microbial functional groups (carbon [C], N, P, and sulfur [S] cycling), and the influence of fertilization on the *z* values of each functional group was examined (see Table S1 and Fig. S2 in the supplemental material). For all of the functional groups, the mean *z* value was 0.0617 (±0.0131) in the fertilized plot and 0.0512 (±0.014) in the control plot. For C cycling genes, the fertilized plot had much higher *z* values (z_f) than the control plot (z_c) for both C fixation ($z_c = 0.0588$, $z_f = 0.063$) and degradation ($z_c = 0.0421$, $z_f = 0.0754$; $P < 0.01$). For N cycling genes, long-term fertilization resulted in higher *z* values for nitrification ($z_c = 0.0671$, $z_f = 0.0842$; $P = 0.0001$) and denitrification ($z_c = 0.0543$, $z_f = 0.0613$; $P = 0.0001$) but lower *z* values in assimilatory N reduction ($P = 0.0001$) and N fixation ($P = 0.0001$). No significant influence of fertilization on the *z* value of dissimilatory N reduction was observed ($P = 0.648$). In addition, fertilization resulted in higher *z* values for P cycling genes ($z_c = 0.0234$, $z_f = 0.0609$; $P = 0.0001$) and sulfur cycling genes ($z_c = 0.0367$, $z_f = 0.0724$; $P = 0.0001$).

For all phylogenetic groups obtained by mapping functional genes to their lineages, the mean *z* value was 0.0517 (±0.0121) in the fertilized plot and 0.0433 (±0.0130) in the control plot (see Table S1 in the supplemental material). Fertilization resulted in higher *z* values in alpha-, beta-, and gammaproteobacteria ($P < 0.05$), often considered copiotrophic bacteria, and a lower *z* value in deltaproteobacteria ($P < 0.05$), a class of oligotrophic anaerobic bacteria (29).

Factors affecting microbial spatial patterns. To determine whether fertilization, soil geochemical properties, plant diversity, and spatial distance affected microbial community composition, partial Mantel tests were performed (Table 2). The results showed significant correlations between all functional genes as a group and fertilization (Mantel correlation coefficient [r_M] = 0.138, $P = 0.010$), soil geochemical properties ($r_M = 0.198$, $P = 0.003$), plant species richness ($r_M = 0.181$, $P = 0.001$), and spatial distance ($r_M = 0.165$, $P = 0.001$). For C cycling genes, both C degradation and C fixation genes were significantly ($P < 0.05$) correlated with fertilization, geochemical properties, plant species, and distance. For N cycling genes, dissimilatory N reduction genes and N fixation were also significantly ($P < 0.05$) correlated with those four factors. Nitrification genes were significantly ($P < 0.05$) correlated with plant species and distance. Denitrification genes were significantly ($P < 0.05$) correlated with all except soil geochemical variables. In addition, both P and S cycling genes were also significantly ($P < 0.05$) correlated with all four factors.

Canonical correspondence analysis (CCA) was performed to link the most significant environmental variables to microbial spatial patterns (Fig. 2). The results for samples from the two plots were clearly separated, and plot 11/2C (fertilized) revealed a more divergent pattern, which could be confirmed by the permutational analysis of dispersion, showing that community dispersion increased significantly with fertilization ($P = 0.017$) (see Fig. S3 in the supplemental material). Of all the environmental variables selected, spatial distance (principal coordinates of neighbor matrices 1 [PCNM1]), fertilization treatment (total N [TN] and am-

TABLE 2 Correlation analysis between microbial functional gene and soil geochemical variables, plant, and distance by partial Mantel test[a]

	Fertilization		Soil		Plant		Distance	
	r_M	P	r_M	P	r_M	P	r_M	P
All functional genes	0.138	**0.010**	0.198	**0.003**	0.181	**0.001**	0.165	**0.001**
Functional groups								
C degradation	0.119	**0.011**	0.200	**0.002**	0.163	**0.001**	0.153	**0.002**
C fixation	0.158	**0.003**	0.250	**0.002**	0.201	**0.001**	0.208	**0.001**
Assimilatory N reduction	0.043	0.172	0.098	0.071	0.043	0.17	0.0502	0.066
Dissimilatory N reduction	0.090	**0.045**	0.145	**0.016**	0.165	**0.002**	0.108	**0.001**
Nitrification	0.015	0.362	0.045	0.275	0.100	**0.027**	0.067	**0.048**
Denitrification	0.088	**0.049**	0.111	0.051	0.169	**0.002**	0.124	**0.001**
N fixation	0.156	**0.004**	0.202	**0.006**	0.185	**0.001**	0.174	**0.001**
Phosphorus	0.115	**0.029**	0.119	**0.042**	0.141	**0.004**	0.145	**0.003**
Sulfur	0.166	**0.003**	0.232	**0.002**	0.189	**0.001**	0.199	**0.001**

[a] The significant values ($P \leq 0.05$) are indicated in boldface.

monium [NH_4^+]), and plant diversity were the most significant factors influencing microbial spatial scaling. The pH and moisture explained differences along the other axis, which were most likely due to lime application (see Text S1 in the supplemental material).

Variance partitioning analysis (VPA) was performed to further quantify the contributions of environmental variables to the microbial community variation (Fig. 3). Soil fertilization attributes, spatial distance, and plant diversity were found to contribute equally to the variations in microbial functional structure (4.7 to 4.8%), while soil geochemical properties contributed less (2.3%). The joint effect of fertilization, plant diversity, and spatial distance was 3.2%, and other joint effects were less than 1.0%. Meanwhile, a substantial amount of the variation in microbial community composition (78.4%) could not be explained by the environmental variables measured.

Comparative analysis of TARs across different groups of organisms. To obtain reliable insights on the spatial scaling of biodiversity across different organism types and the effect of long-term anthropogenic disturbance, the z values obtained in both control and fertilized plots in this study were compared with all available published data (806 datasets) (Fig. 4). Although detailed comparisons could not be made due to the wide ranges of organisms and habitats, the various spatial scales, and the differences in experimental design and analytical approaches used, we were able to show that microorganisms had lower z values than macroorganisms, 3 to 5 times lower than those in plants in this study. Furthermore, although long-term fertilization significantly increased the z values of bacteria, fungi, and archaea ($P < 0.05$), the influence did not change the general pattern that the rates of

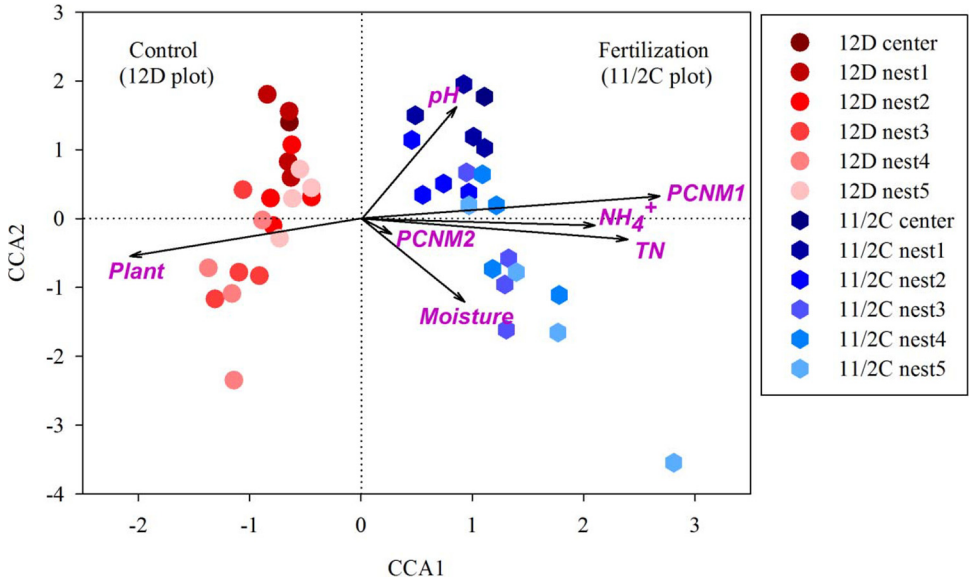

FIG 2 Canonical correspondence analysis (CCA) of GeoChip hybridization signal intensities and environmental variables that were significantly related to microbial variation. Subsets of soil variables, distance, and plant diversity were selected for analysis on the basis of variance in inflation factors. Distance was represented by eight primary variables from principal coordinates of neighbor matrices (PCNM), and the first two were used for CCA. Triangles represent 12D sample data (control), and circles represent 11/2C sample data (fertilization). Colors from dark to bright represent samples from the center to the periphery. The relationship was significant ($P = 0.005$).

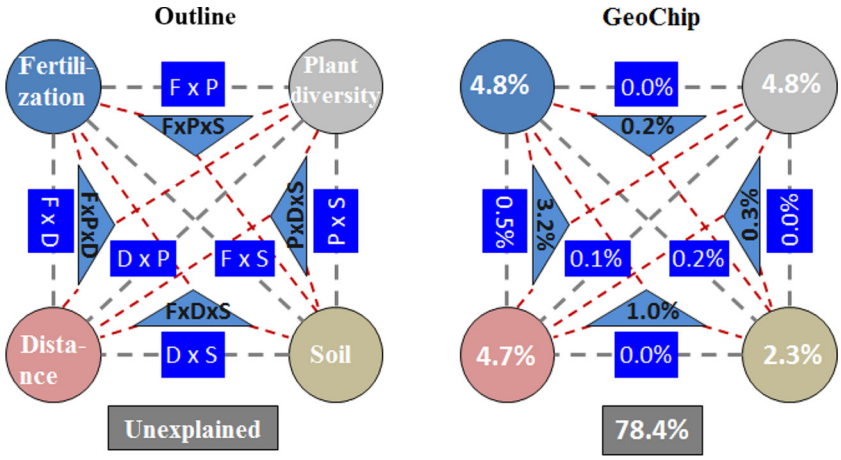

FIG 3 Variation partitioning analysis (VPA) of microbial distribution patterns explained by soil geochemical properties (S), fertilization (F), spatial distance (D), and plant diversity (P). Each diagram represents the biological variation partitioned into the relative effects of each factor or a combination of factors. Distance is represented by eight primary variables from principal coordinates of neighbor matrices (PCNM) analysis. Soil geochemical properties included soil pH and moisture. Fertilization was quantified by total nitrogen and ammonium.

change of microorganisms in space are much lower than those of other organisms (plants and animals).

DISCUSSION

Spatial scaling of biodiversity is a central issue in ecology. It has been shown that anthropogenic activities influence the SARs of animals and plants (27, 30–32). However, the impact of anthropogenic activities on microbial spatial scaling has rarely been explored. In this study, our results showed that more than 150 years of fertilization has had significant impacts on the spatial scaling of soil microbial communities and plant species simultaneously.

A number of short-term studies observed that fertilization altered soil microbial biomass, community structure, and certain functional groups (e.g., autotrophic ammonia-oxidizing bacteria) (20, 33). Such changes of microbes and fungi were more evident in studies of longer duration and with larger amounts of fertilizer used (20). For example, long-term application of inorganic N fertilizer (140 years) caused significant differences in the ability of soil to oxidize CH_4, while no significant short-term effects could be observed (34). Moreover, microbial community composition and abundance are influenced by changes in soil characteristics, which could be more significant in long-term fertilization regimes. For example, it has been proven that all forms of soil P are enriched when fertilized for over 100 years (26). In this study, the experimental plots have been fertilized for more than 150 years, which is the longest grassland experiment available in the world. Thus, we believe that the changes of microbial community structure observed in this site could represent permanent and stable, rather than transient, responses of microbial communities to fertilization. Consequently, the estimated *z* values are likely to be a reliable reflection of the effects of fertilization on the spatial scaling of soil microbial communities.

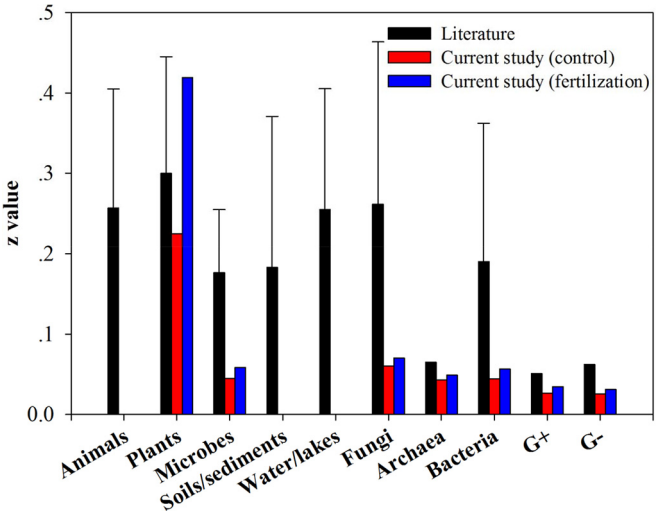

FIG 4 A comparison of *z* values of macroorganism and microbial taxonomic groups. Data were obtained from supplemental data of Drakare et al. (1) except for groups that are less defined taxonomically. Some recent data for microbial communities were also included. A total of 806 datasets were analyzed. Error bars show standard deviations.

Microbial TARs can be affected by long-term N fertilization in different ways. First, long-term fertilization may reduce the heterogeneity of nutrient availability, allowing a few species that are adapted for higher nutrient levels to spread into and dominate local communities. Second, long-term fertilization may unevenly magnify the initial differences, resulting in more divergent spatial patterns. Our study showed that long-term nutrient fertilization significantly increased the slopes of both microbial GARs ($P < 0.0001$) and plant TARs ($P < 0.0001$), supporting the second possibility. This is also consistent with a previous observation showing that N supply could cause communities to diverge to a more heterogeneous pattern (35). Slight variations in initial species composition could be unevenly magnified in the fertilized treatments, as observed from the data in Fig. S4 in the supplemental material. Agricultural practice could increase certain microbial species growth (36, 37) and community interactions (38–40), resulting in a decrease in species evenness and, eventually, species richness through their spatial aggregation around plants, the dominance of some species, and the loss of rare species (41). This could also happen with soil microbes. For example, N fertilization resulted in the dominance of *Nitrosospira* cluster 3 in soil (42) and

pronounced shifts in bacterial community composition (18). Further analysis with metagenomic sequencing and molecular ecological networks (43, 44) will be helpful to validate the microbial species spatial aggregation and competition processes.

We observed that long-term inorganic fertilization not only increased microbial and plant spatial turnover but also decreased the intercept of the GAR curves (*c* values), sometimes called the height of the curve, which were significantly different ($\ln c_{\text{control}} = 3.582$, $\ln c_{\text{fertilization}} = 3.509$; $P < 0.001$), as shown in Fig. 1. In species-area relationships, α diversity can be measured as the height of the curve at a given area (45), so the lower curves indicate that long-term fertilization decreased both plant and microbial α diversity. At the smallest spatial scales (0.1 m^2), the total gene numbers were 2,615 ± 74 in the control plot and 1,771 ± 346 in the fertilized plot. At the plot scale (5 m^2), the total gene numbers were 3,655 ± 96 in the control plot and 3,023 ± 165 in the fertilized plot. The α diversity was reduced more at the smallest spatial scales than at the largest plot scale, causing steeper spatial turnover (*z* values).

It has been recognized that plant and soil microbial communities are tightly linked together (1, 46, 47). Here, we also observed significant correlations between plant diversity and microbial functional genes ($P < 0.05$) (see Table S2 in the supplemental material). Plant diversity contributed ~5% to microbial community variation, equal to soil fertilization and spatial distance. A more divergent spatial pattern of plant species (Fig. S4) and lower plant species diversity (α diversity) (Fig. S5) under long-term N fertilization may result in a more heterogeneous habitat, such as the rhizospheric environment (48). Different microbial species may be favored in different patches, leading to greater spatial change in community composition. Furthermore, soils with different grass species differed in the composition and abundance of microbial communities (48, 49). In this study, more plant species were found in the control plot, including, among different quadrats, *Festuca rubra* (90.5%), *Briza media* (71.4%), *Agrostis capillaris* (52.4%), *Leontodon hispidus* (42.9%), and *Ranunculus* sp. (42.9%), while only *Poa pratensis* (81.0%) was dominant among the quadrats of the fertilized plot, with other plant species accounting for less than 40% (Table S3). Since plant species differ in both the quantity and quality of nutrients they return to soil, different dominant plant species could have important effects on components of the soil available to microbes and the processes that they regulate (47), which then affects the microbial spatial patterns of different functional groups. For example, soil C input varied among plant species (50) and could be altered by N addition (51). Thus, microbial communities functioning in C degradation may be more heterogeneous with different C input in the fertilized plot, resulting in a higher spatial turnover (higher *z* values). In all cases, the effect of agricultural practice on microbial spatial patterns and the linkage between these patterns and the plant is interesting yet little studied. More systematic studies, both empirical and theoretical, are needed to generalize these phenomena and to elucidate the underlying mechanisms.

Current knowledge about microbial TARs largely comes from studies of bacterial communities, and the extent to which this applies to fungal communities, which have very different life histories and dispersal mechanisms, remains to be elucidated. It was recently reported that spatial scaling of arbuscular mycorrhizal fungal diversity is affected by farming practice and that the *z* values were greater under organic than under conventional farm management, 0.61 and 0.45, respectively (52). Here, we also observed that long-term fertilization significantly increased the *z* values of fungi, which were 0.0704 in the fertilized plot and 0.0604 in the control plot. It has been proposed that N fertilization may affect fungal communities by altering plant C inputs (53). Thus, more internally heterogeneous patterns of plants under N addition may result in steeper spatial changes in fungal communities. In addition, the difference in estimated *z* values in these two studies might be due to the different approaches used with different ecosystems. Here, we estimated the TARs of all fungi in bulk soil of grassland and used a microarray hybridization-based approach, which contains tens of thousands of functional gene markers so that many microbial populations and functional groups can be simultaneously detected at the whole-community-wide scale (9). Thus, any direct comparison of *z* values with different methods would be difficult.

Generally, the mean *z* value for plants was 0.306 (±0.018) (Fig. 4), which was significantly affected by variables in terms of the sampling schemes, spatial scales, and habitats involved (31, 54, 55). In this study, the observed *z* values for plants were 0.225 and 0.419 in the control and fertilization plots, respectively. Our results are generally in accordance with a previous metastudy that showed that N enrichment reduced plant α diversity within plots by an average of 25% (ranging from a reduction of 61% to an increase of 5%) and frequently enhanced β diversity (31). The enhancement of β diversity was proposed to be related to increases in productivity following nutrient enrichment (56, 57).

The factors driving microbial spatial patterns could be very complex, depending on both environmental factors (e.g., temporal variation, spatial heterogeneity, and soil properties and disturbances) and biotic factors (e.g., reproductive behavior, dispersal ability, competition, niche differentiation and extinction, and interactions with the plant community) (55, 58, 59). Soil biogeochemical properties, fertilization, spatial distance, and plant species diversity have influences on the diversity patterns of microbial communities in the grassland soils, because significant correlations were detected for all functional genes and most of the functional gene groups examined using both partial Mantel test and variation partition analysis (see Table S2 in the supplemental material). However, all the environmental factors examined contributed only a small portion to the total variation in microbial functional structure (21.6%) (Fig. 2). Similar to other studies (6, 9), a large percentage of the total variation remained unexplained, which could be due to unmeasured biotic and abiotic factors, such as time span, the small spatial scale, and ecologically neutral processes of diversification (3, 6, 9, 59–61). The knowledge gained in this study may only be applicable at the meter scale used here and may not hold at smaller (e.g., micrometer or soil aggregate scales) or larger (e.g., tens of thousands of kilometers) scales of study. To avoid over- or underestimating the effects of agricultural practice like fertilization on microbial spatial scaling, further work is required to address the importance of multiple spatial scales for understanding the influence of human perturbations on microbial biogeography. In addition, it should be noted that if one would like to experimentally test the differences of *z* values between treatment and control, nested samples (21 in this case) from a minimum of three biological replicates under each condition are required. However, since the plots of the Park Grass Experiment were set up 150 years ago and have received different amounts and combinations of inorganic N, P, and K fertilizers and lime over the

years, no biologically replicated plots under each condition are available. As a result, we were unable to identify biologically replicated plots to perform such experimental tests. Due to high heterogeneity of the soil environment, the results from the current study based on a single plot might underestimate the microbial spatial turnover rates. Thus, more biologically replicated plots should be considered in examining microbial spatial patterns in future studies.

In conclusion, this study examined spatial patterns of microbial communities in response to over 150 years of fertilization at the Rothamsted Research site and demonstrated spatial scaling of microbial biodiversity and its relationship to plant diversity. The long-term experiment enabled us to avoid transient responses and, instead, provided a long-term response to assess the influence of farming practice on spatial patterns of plant and microbial communities. The results showed that long-term fertilization has dramatic impacts on the spatial scaling of microbial communities. These findings make a fundamental contribution to predictive understanding of microbial biogeography.

MATERIALS AND METHODS

Details for all methods are provided in Text S1 in the supplemental material. Briefly, soil samples were taken from the PGE using a spatially explicit nested sampling design (0.1 m^2, 0.25 m^2, 1 m^2, 2.5 m^2, and 5 m^2) in two treatment areas, of which one was fertilized with inorganic fertilizers for more than 150 years (11/2C) and the other was a control with no fertilizer or lime (12D), in September 2009. Plant species richness was also recorded in each of the nested squares (0.025 m^2, 0.1 m^2, 0.625 m^2, 1 m^2, 6.25 m^2, and 25 m^2) in plots 11/2C and 12D. Soil geochemical variables were measured, including soil pH, moisture, total N (TN), total C (TC), NO_3^--N, and NH_4^+-N, by Brookside Laboratories, Inc. (New Knoxville, OH).

GeoChip 3.0 was used for analyzing microbial community functional gene structure; it is a functional gene array containing ~28,000 probes covering approximately 57,000 gene variants from 292 functional gene families involved in C, N, P, and S cycling, energy metabolism, antibiotic resistance, metal resistance, and organic contaminant degradation. GeoChip hybridization, imaging, and data preprocessing were described previously (62–64).

The power law form of GAR ($S = cA^z$) was fitted by logarithmic transformation. The exponent z was estimated by linear regression as follows: $\log S = \log c + z \log A$, where S is observed gene richness (S_{obs}) and A is the area in the nested design (0.1 m^2, 0.25 m^2, 1 m^2, 2.5 m^2, or 5 m^2). The log-based linear equation was applied to all individual functional genes, as well as functional groups and phylogenetic groups. The estimated z values were compared with the observed z values by one-tailed t test after 10,000 bootstraps between area and richness (9). All the analyses were performed in R (version 2.11.1; http://www.r-project.org/), with packages vegan and ecodist.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00240-15/-/DCSupplemental.

Text S1, PDF file, 0.1 MB.
Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.3 MB.
Figure S3, PDF file, 0.04 MB.
Figure S4, PDF file, 0.1 MB.
Figure S5, PDF file, 0.05 MB.
Table S1, PDF file, 0.05 MB.
Table S2, PDF file, 0.1 MB.
Table S3, PDF file, 0.05 MB.

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