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Spatial patterning of soil microbial communities created by fungus-farming termites

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Abstract

Spatially overdispersed mounds of fungus-farming termites (Macrotermitinae) are hotspots of nutrient availability and primary productivity in tropical savannas, creating spatial heterogeneity in communities and ecosystem functions. These termites influence the local availability of nutrients in part by redistributing nutrients across the landscape, but the links between termite ecosystem engineering and the soil microbes that are the metabolic agents of nutrient cycling are little understood. We used DNA metabarcoding of soils from *Odontotermes montanus* mounds to examine the influence of termites on soil microbial communities in a semi-arid Kenyan savanna. We found that bacterial and fungal communities were compositionally distinct in termite-mound topsoils relative to the surrounding savanna, and that bacterial communities were more diverse on mounds. The higher microbial alpha and beta diversity associated with mounds created striking spatial patterning in microbial community composition, and boosted landscape-scale microbial richness and diversity. Selected enzyme assays revealed consistent differences in potential enzymatic activity, suggesting links between termite-induced heterogeneity in microbial community composition and the spatial distribution of ecosystem functions. We conducted a large-scale field experiment in which we attempted to simulate termites' effects on microbes by fertilizing mound-sized patches; this altered both bacterial and fungal communities, but in a different way than natural mounds. Elevated levels of inorganic nitrogen, phosphorus and potassium may help to explain the distinctive fungal communities in termite-mound soils, but cannot account for the distinctive bacterial communities associated with mounds.

KEYWORDS

African savannas, DNA metabarcoding, nitrogen cycling, soil microbial communities, spatial structure, termites

1 | INTRODUCTION

Spatial heterogeneity is an important contributor to the productivity, diversity and robustness of many ecological communities (Bonachela et al., 2015; Loreau, Mouquet, & Gonzalez, 2003; Tilman, 1994). In tropical savannas, fungus-farming termites (Macrotermitinae) act as ecosystem engineers and are major sources of spatial heterogeneity (Bignell & Eggleton, 2000; Jouquet, Dauber, Lagerlöf, Lavelle, & Lepage, 2006; Jouquet, Traoré, Choosai, Hartmann, & Bignell, 2011; Muvengwi, Witkowski, Davies, & Parrini, 2017).

Within their subterranean nests (hereafter mounds), termites locally increase macro- and micronutrient concentrations (Holdo & McDowell, 2004; Seymour et al., 2014) and modify soil texture and moisture (Holt & Lepage, 2000; Wood, 1988) in ways that alter plant assemblages and enhance primary productivity (Sileshi, Arshad, Konaté, & Nkunika, 2010) compared to the surrounding savanna (hereafter matrix). These edaphic changes are likely both to involve and to produce changes in soil microbial communities; yet to date, there is limited information about how fungus-farming termites influence soil microbes.

In East African vertisol savannas, spatially overdispersed mounds of the fungus-farming termite *Odontotermes montanus* Harris, 1960 form a regularly patterned template that influences numerous aspects of the ecosystem (Table S1; Brody, Palmer, Fox-Dobbs, & Doak, 2010; Palmer, 2003; Pringle, Doak, Brody, Jocque, & Palmer, 2010). Mature mounds are up to 10–20 m in diameter and 1–2 m deep (Figure S1), containing hundreds of chambers in which the termites cultivate a symbiotic *Termitomyces* fungus (Darlington, 2005). Vegetation on mounds has elevated foliar nutrients (Fox-Dobbs, Doak, Brody, & Palmer, 2010) and is visibly greener than vegetation in the matrix during rainy seasons (Brody et al., 2010). Plant community composition also differs on mounds compared to the matrix; for example, *Odontotermes* mounds in central Kenya are dominated by the grass *Pennisetum stramineum* and rarely support woody vegetation, whereas matrix plant assemblages are more diverse in plant species and life-forms (Brody et al., 2010; Fox-Dobbs et al., 2010; Odadi, Young, & Okeyo-Owuor, 2007; Palmer, 2003; Riginos & Grace, 2008).

These spatial patterns in the vegetation reflect the influence of termites on soil physical and chemical properties around mounds. Like those of other African fungus-farming termites (Sileshi et al., 2010), *O. montanus* mounds are enriched in total nitrogen and phosphorus, nitrate, and total and organic carbon (Brody et al., 2010; Fox-Dobbs et al., 2010). *Odontotermes* mounds are also lower in clay and higher in sand content than the surrounding clay-heavy vertisols, owing to translocation of particles from lower soil horizons (Brody et al., 2010). This tends to improve water infiltration and aeration on termite mounds, aided by the presence of termite galleries (Bignell, 2006) and a network of shallow cracks promoted by bioturbation (DeCarlo & Caylor, 2019).

However, it remains unclear how such local environmental modifications affect free-living microbial communities, their biogeochemical functions and their distribution across the landscape. We hypothesized that the enrichment of soil macronutrients close to mounds causes localized changes in soil microbial communities, creating landscape-scale spatial patterns in community composition and microbe-driven ecosystem functions. Nutrient limitations play a key role in the ecology of tropical savannas (Pellegrini, 2016). Furthermore, studies conducted over large spatial scales across a wide range of ecosystems and climates have documented changes in microbial community composition in response to anthropogenic nutrient addition (Fierer et al., 2011; Ramirez, Lauber, Knight, Bradford, & Fierer, 2010). Investigating the patterns and drivers of spatial heterogeneity in soil microbes is an important step towards understanding heterogeneity throughout the savanna biome, because soil microbes play important roles in nutrient cycling, including the conversion of nutrients between forms that differ in availability to plants.

Here, we used DNA metabarcoding to examine spatial patterning in free-living soil microbial communities created by *O. montanus* termite mounds in central Kenya. We anticipated two broad trends. First, we predicted that mound communities would have lower richness and evenness (alpha diversity; Whittaker, 1972) than matrix communities. We reasoned that the lower diversity of plants and the greater abundance of simple, accessible nutrients (e.g., nitrates, Fox-Dobbs et al., 2010) on mounds would favour a limited number of microbial taxa with high growth rates, whereas the higher diversity of plants and of complex, recalcitrant molecules (e.g., celluloses, lignins) in the matrix would promote a greater diversity of microbes that specialize in breaking down these different substrates. Second, we predicted that mound communities would differ in composition from matrix communities (beta diversity), generating large-scale spatial structure patterned on the overdispersed template of termite mounds. Specifically, we expected mounds to have higher relative abundances of copiotrophic microbes that thrive in nutrient-rich environments (e.g., the bacterial groups Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Bacteroidetes) and lower relative abundances of oligotrophic groups that perform better in nutrient-poor conditions (e.g., Acidobacteria and Deltaproteobacteria) compared to the matrix. The placement of these microbial groups along a copiotrophic–oligotrophic spectrum has found empirical support in a range of environments, albeit with substantial heterogeneity among taxa within the groups (Campbell, Polson, Hanson, Mack, & Schuur, 2010; Fierer, Bradford, & Jackson, 2007; Leff et al., 2015; Ramirez et al., 2010).

As an initial attempt to probe the mechanisms responsible for the predicted differences in microbial community composition between mounds and matrix habitats, we conducted a large-scale field experiment involving the repeated addition of inorganic nitrogen–potassium–phosphorus (NPK) fertilizer to replicated mound-sized patches. Although the addition of these three major macronutrients is a crude way to imitate the effects of termites—mound soils are characteristically elevated in a wide range of macro- and micronutrients, in addition to differing in pH and physical structure (Seymour et al., 2014; Sileshi et al., 2010)—N, P and K frequently have strong influences on both plant and microbial communities (Güsewell, 2004; Pan et al., 2014) and could thus account directly and/or indirectly for a major part of the effects of mounds on microbes. Accordingly, we expected microbial communities in fertilized patches to be more similar to those on mounds, with lower alpha diversity and greater relative abundances of copiotrophic bacterial groups compared to control patches.

Finally, to complement our metabarcoding data, we explored microbial community function by measuring extracellular enzyme activities linked to carbon, nitrogen and phosphorus cycles in a subset of our mounds. Extracellular enzyme activity is commonly viewed as

a proxy for microbial nutrient demand, because these enzymes catalyse nutrient acquisition from complex organic matter (Sinsabaugh et al., 2008). We expected nutrient demand to be lower on mounds than in the matrix because microbes should take advantage of more readily accessible simple nutrients close to mounds (Allison & Vitousek, 2005).

2 | MATERIALS AND METHODS

Here we provide an overview of our materials and methods, including all essential information necessary to understand the results. Additional technical details are provided in Document S1.

2.1 | Field site

We conducted fieldwork in July 2016 and May–June 2017 at the Mpala Research Centre and Conservancy (MRC) in the Laikipia Highlands of Kenya (0.2924°N, 36.8980°E, ~1,800 m elevation). *Odontotermes montanus* mounds occur in poorly drained, clay-rich vertisols, known locally as "black cotton," which are found across large areas of MRC (Pringle, Prior, Palmer, Young, & Goheen, 2016) and elsewhere across Laikipia (Ahn & Geiger, 1987) and East Africa more broadly. Mature mounds at MRC are characteristically overdispersed at spatial scales of <100 m (Bonachela et al., 2015; Pringle et al., 2010; Tarnita et al., 2017).

2.2 | Field sampling: Transects between termite mounds

To examine the effects of termite mounds on soil microbes, we sampled soil in 2016 from 21 transects grouped in three clusters (north, central and south; Figure S2a–d). Each transect began at a focal termite mound and ran into the matrix towards a neighbouring mound; six transects spanned the entire distance between two neighbouring mounds, and the remainder spanned half the distance. At 2.5-m intervals within the first 10 m along each transect and at 5-m intervals thereafter, we took soil cores from the top ~10 cm, recorded the dominant grass species (that with the highest areal cover within ~0.5 m of the sampling point) and classified each point as being located on a mound or in the matrix. We measured moisture content and pH for each soil sample and extracted extracellular DNA for metabarcoding. Subsamples from seven mound samples and seven matrix samples were used for measurements of enzymatic activity (see "Extracellular enzyme activity assays" below).

We resampled parts of these transects in 2017 to assess consistency over time and for comparison with experimental samples from 2017 (see "Field sampling: Fertilization experiment" below). Instead of resampling entire transects, we only sampled from the centre of the focal mound and the midpoint between the focal mound and its neighbour.

2.3 | Field sampling: Fertilization experiment

In 2015, we established an experiment in which we semi-annually fertilized a set of termite-mound-sized patches with inorganic NPK fertilizer in 96 patches of 10-m diameter (see Document S1 "Field sampling: fertilization experiment" for further details). These fertilized patches were arranged in six plots of 16 patches each; the plots, in turn, were arranged in three blocks of two plots each (north, central and south; see Figure S2a,g–i). All three blocks were located between 0.5 and 2.3 km from the north cluster of transects (for comparison, the north and south transect clusters were ~ 3.4 km apart). In May–June 2017, after the experiment had been running for 2 years (and ~5 months after the most recent fertilization), we sampled topsoil from the centres of 84 fertilized patches across all six plots (14 patches per plot). For each of these fertilized samples, we also collected a nearby control sample from the unfertilized area within the experimental plot but between treatment patches. These samples were processed in the same way as the transect samples.

2.4 | DNA metabarcoding

We used DNA metabarcoding to characterize microbial community composition in each soil sample. Extracellular DNA was extracted from each 15-g soil subsample using the methods in Taberlet et al. (2012). We PCR-amplified a ~258-bp fragment of the bacterial 16S rRNA gene (Fliegerova et al., 2014) and a ~185-bp fragment covering the fungal internal transcribed spacer I region (Epp et al., 2012) using tagged primers. We then constructed multiplexed amplicon libraries and sequenced them using paired-end reads of 400 (bacteria) or 350 (fungi) cycles on an Illumina MiSeq. We processed our sequence data using the OBITOOLS pipeline (Boyer et al., 2016) and then used SUMATRA for clustering using a 97% similarity threshold (Mercier, 2015).

We rarefied our operational taxonomic unit (OTU) tables to 1,500 reads per sample to minimize statistical artefacts arising from sequencing depth variation (Goodrich et al., 2014; Hughes & Hellmann, 2005; Weiss et al., 2017). We chose our rarefaction depth to strike a balance between excluding samples with low read counts and retaining adequate read depth. From our initial 452 samples, this led us to drop 36 bacterial samples and 54 fungal samples, leaving us with 416 samples and 398 samples in our bacterial and fungal data sets, respectively.

2.5 | Analysis of site characteristics

We compared pH and soil moisture between mounds and matrix, and between fertilized and control sampling points, using linear mixed-effects models and likelihood-ratio (LR) tests. We compared grass species richness between mounds and matrix, and between fertilized and control sites, using Wilcoxon rank-sum tests. We used

TABLE 1 Extracellular enzymes assessed for potential activity (see Figure 5)

Enzyme	Enzyme Commission number	Substrate	Function
β-Glucosidase	EC 3.2.1.21	4-Methylumbelliferyl-β-D-glucopyranoside	Release of glucose from cellulose
Phosphatase	EC 3.1.3.1	4-Methylumbelliferyl-phosphate	Phosphorus mineralization
Chitinase	EC 3.2.1.52	4-Methylumbelliferyl-N-acetyl-β-D-glucosaminide	Degradation of chitin compounds
Leucine aminopeptidase	EC 3.4.11.1	L-Leucine–7-amino–4-methylcoumarin	Degradation of protein into amino acid

permutational χ^2 tests to compare the frequency distributions of dominant grass species between mounds and matrix, and between fertilized and control sites.

2.6 | Analysis of microbial alpha and beta diversity

We compared OTU richness and Shannon diversity between mounds and matrix, and between fertilized and control samples, using linear mixed-effects models and LR tests. We visualized microbial beta diversity using nonmetric multidimensional scaling (NMDS) ordinations of Bray–Curtis dissimilarities (Anderson, Ellingsen, & McArdle, 2006). We tested for differences between mound and matrix samples, and between fertilized and control samples, using permutational multivariate analysis of variance (MANOVA) (Anderson, 2001; McArdle & Anderson, 2001). We used Kruskal–Wallis tests to compare pairwise dissimilarities between mound and fertilized samples to pairwise dissimilarities between matrix and control samples. We likewise compared pairwise dissimilarities between mound and fertilized samples to pairwise dissimilarities between mound and control samples. We used Mantel tests to evaluate the Pearson correlation between Bray–Curtis dissimilarities and pairwise differences in pH, and we visualized the relationship between pH and community composition by plotting the position of each sample along the first NMDS axis against sample pH.

To explore the effects of termite mounds on particular bacterial and fungal groups, we calculated median read counts for phyla, classes and fungal orders that accounted for >1% of reads from the 2016 transect samples. We used Wilcoxon rank-sum tests to compare read counts for each of these taxonomic groups between mound and matrix samples, and between fertilized and control patches.

To examine the effects of termites and fertilization on individual bacterial and fungal taxa, we used an analysis of microbial communities (ANCOM) (Mandal et al., 2015) to identify OTUs showing a strong association with mounds or fertilized patches. We employed the ANCOM method on a set of "core OTUs" that we defined by taking OTUs that were (a) present in ≥50% of bacterial samples or ≥20% of fungal samples, and (b) in the top decile of bacterial OTUs by total reads or the top two deciles of fungal OTUs. Document S1 provides further details of the definition and analysis of these core OTUs.

2.7 | Analysis of the spatial extent of mound influence

To visualize the spatial extent of the termite mounds' influence on soil bacterial and fungal communities, we first plotted the Bray–Curtis compositional dissimilarities (based on all OTUs) between each of the 2016 transect samples and the centroid of the 2016 mound samples as a function of distance from the mound edge. Next, we described the relationship between microbial community dissimilarity and distance from the mound edge by using the R function NLS to fit the model

$$\text{dissimilarity} = \alpha + \beta e^{\gamma \cdot \text{distance}}.$$

We chose this functional form, with free parameters α, β and γ, for its ability to generate a curve that approximated the shape of our observed data. We visualized these results across a representative portion of the landscape as described in Document S1.

2.8 | Analysis of microbial gamma diversity

Microbial diversity at the landscape scale (gamma diversity) is a function of diversity at smaller spatial scales. Having already compared the diversity of individual mound and matrix samples (alpha diversity), we next compared beta diversity between pairs of mound and matrix samples, both within and among transects. First, we compared mound–sample pairs and matrix–sample pairs from the same transect. For this analysis, we calculated for each transect the Bray–Curtis dissimilarities between 0 and 5 m (i.e., from the centre and toward the edge of the same mound) and between 25 and 30 m (i.e., matrix samples separated by the same distance as those on the mound); then, taking all transects together, we used Kruskal–Wallis tests to compare the dissimilarities between the mound pairs and the matrix pairs. Second, we examined among-transect beta diversity by using permutational MANOVA to compare the pairwise Bray–Curtis dissimilarities between all mound samples at 0 m (i.e.,

mound centres) to the pairwise Bray–Curtis dissimilarities between all matrix samples at 25 m from the mound centre. Third, we used sample-wise rarefaction curves to evaluate the overall effect of termite mounds on soil microbial gamma diversity (as described in detail in Document S1).

2.9 | Assays of extracellular enzyme activity

We measured hydrolytic extracellular enzyme activity associated with carbon, nitrogen and phosphorus cycles (Table 1) in seven mound samples and seven matrix samples collected in 2016. We also measured fluorescein diacetate hydrolysis to estimate overall soil microbial activity (Green, Stott, & Diack, 2006). Enzyme assays were performed according to Marx, Wood, and Jarvis (2001) with the modifications of Puissant et al. (2015) as detailed in Document S1. Enzyme activities were calculated in nanokatal (nmol of product per second) normalized by dry soil mass. We also calculated ratios between the measured activities of different enzymes to assess relative resource allocation to the acquisition of carbon (β-glucosidase), nitrogen (leucineaminopeptidase) and phosphorus (phosphatase) (Sinsabaugh et al., 2008; Stone, Plante, & Casper, 2013). To compare enzyme activity and activity ratios between mound and matrix samples, we fitted mixed-effects models with location (mound vs. matrix) as a fixed effect and transect as a random effect. We used LR tests to compare these models to random effects models with transect as a random effect.

3 | RESULTS

3.1 | Site characteristics

Mound samples had higher pH than matrix samples (Figure 1a; Figure S3a). In contrast, fertilized samples had lower pH than control samples (Figure 1b). Both termite mounds and experimental fertilization tended to reduce soil moisture, but there was also substantial intersample variation (Figure S3b–d).

The median species richness of grasses at sampling points on mounds was 1 in both 2016 and 2017, compared to 3 in 2016 and 2 in 2017 in the matrix (2016: $W = 671$, $n_{mounds} = 49$, $n_{matrix} = 141$, $p < .001$; 2017: $W = 93.5$, $n_{mounds} = 21$, $n_{matrix} = 22$, $p = .001$). The

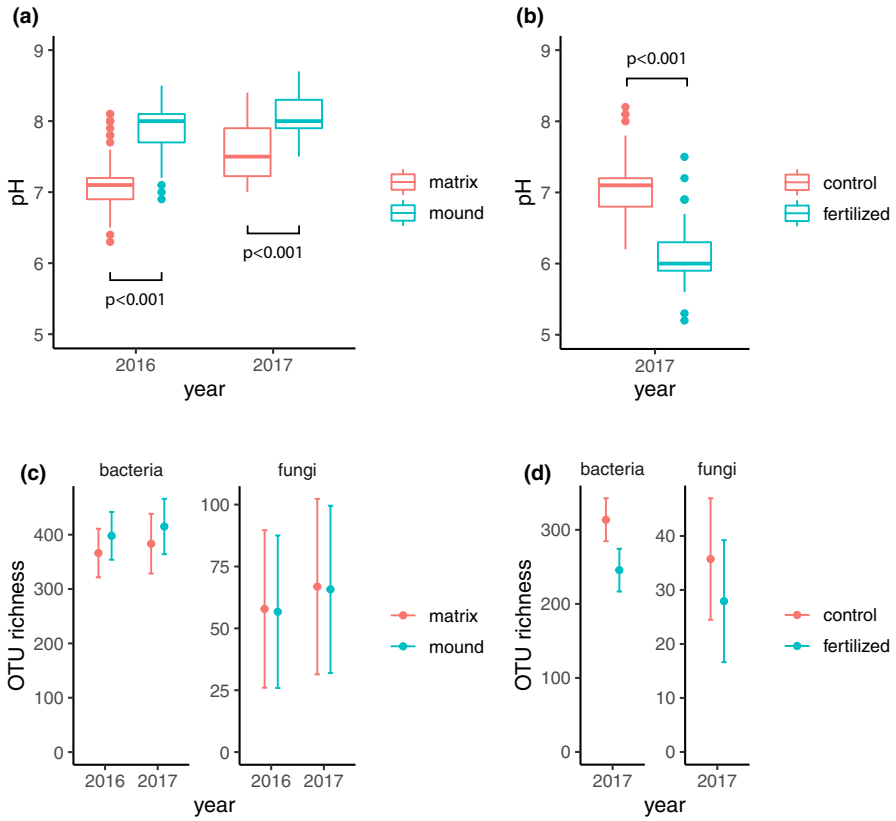

FIGURE 1 Effects of termite mounds and experimental fertilization on soil pH and microbial community richness. (a) In the transect samples, termite mounds had higher soil pH than the surrounding matrix (2016: $\chi^2 = 202.7$, $df = 1$, $n = 258$, $p < .001$; 2017: $\chi^2 = 19.4$, $df = 1$, $n = 43$, $p < .001$). (b) In the experimental samples, fertilized patches had lower pH than control samples ($\chi^2 = 202.7$, $df = 1$, $n = 168$, $p < .001$). (c) Estimated OTU richness for transect samples at 1:10 PCR template dilution. Mounds had higher bacterial OTU richness than matrix soils, but similar fungal OTU richness (bacteria: $\chi^2 = 11.1$, $df = 1$, $n = 250$, $p < .001$; fungi: $\chi^2 = .10$, $df = 1$, $n = 241$, $p = .76$). (d) Estimated OTU richness for experimental samples. Experimental fertilization decreased bacterial and fungal OTU richness relative to control samples (bacteria: $\chi^2 = 46.3$, $df = 1$, $n = 166$, $p < .001$; fungi: $\chi^2 = 8.71$, $df = 1$, $n = 156$, $p = .003$). Error bars in (c) and (d) show 95% confidence intervals [Colour figure can be viewed at wileyonlinelibrary.com]

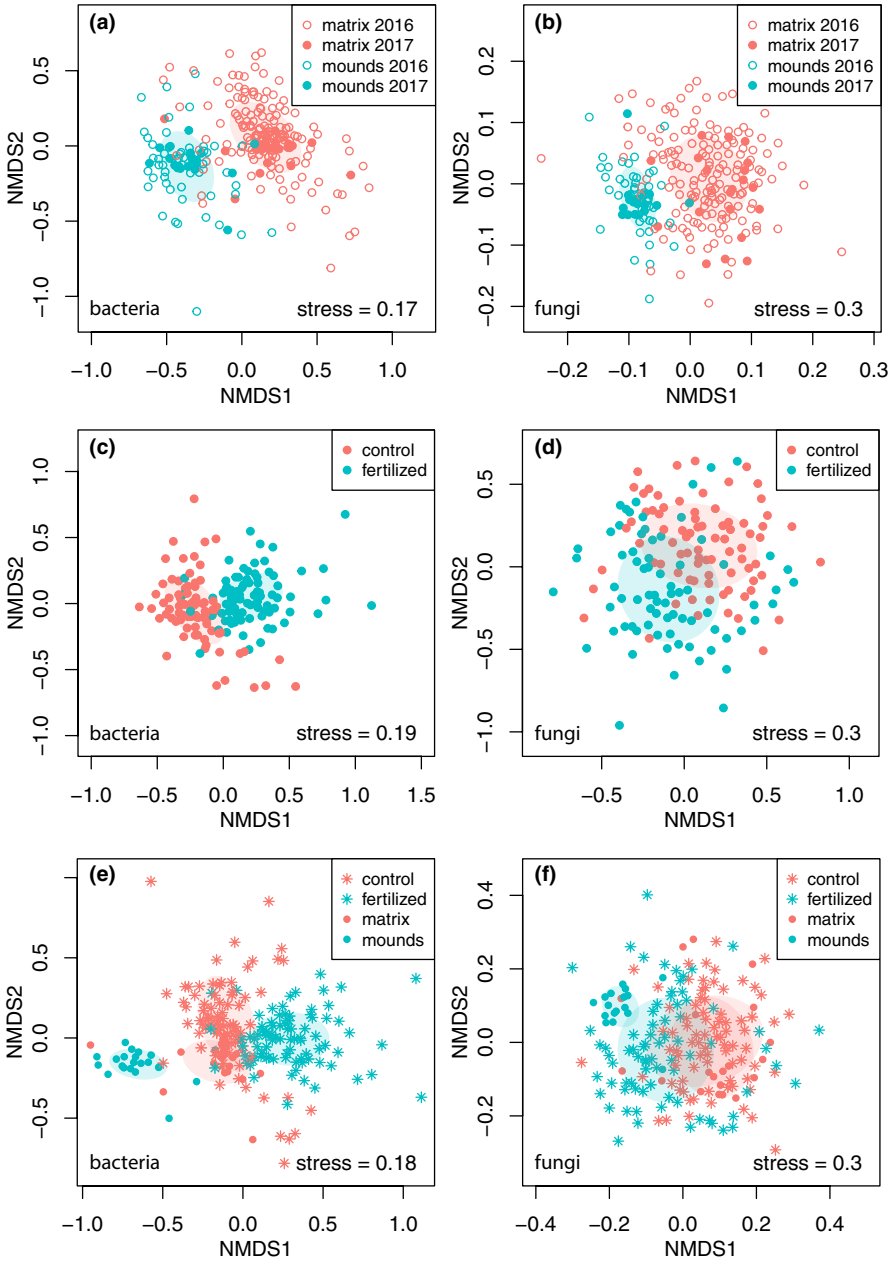

FIGURE 2 Compositional dissimilarity of microbial communities in mound, matrix, fertilized and control soils. All plots show nonmetric multidimensional scaling ordinations based on Bray–Curtis dissimilarities. (a) Bacterial and (b) fungal communities from transects in 2016 and 2017. Bacterial and fungal communities were compositionally distinct between mounds and matrix (bacteria: pseudo-$F = 37.2$, $df = (1, 221)$, $p = .001$; fungi: pseudo-$F = 11.2$, $df = (1, 215)$, $p = .001$). (c) Bacterial and (d) fungal communities were likewise compositionally distinct between fertilized and control patches in 2017 (bacteria: pseudo-$F = 20.2$, $df = (1, 159)$, $p = .001$; fungi: pseudo-$F = 3.64$, $df = (1, 149)$, $p = .001$). (e) Bacterial and (f) fungal communities from transects and fertilization experiment together in 2017, showing that mounds and fertilized patches were compositionally distinct (bacteria: pseudo-$F = 20.0$, $df = (1, 103)$, $p = .001$; fungi: pseudo-$F = 4.11$, $df = (1, 90)$, $p = .001$), as were matrix samples and control patches (bacteria: pseudo-$F = 6.30$, $df = (1, 102)$, $p = .001$; fungi: pseudo-$F = 1.58$, $df = (1, 99)$, $p = .01$). These plots additionally illustrate that fertilization made bacterial communities more dissimilar and fungal communities less dissimilar to those on termite mounds (see Figure S9). In all plots, each point represents the microbial community from a single sample, and the distances between points reflects the degree of dissimilarity. Shaded ellipses show standard deviations of mound, matrix, fertilized patch or control samples around their centroids. While some ordinations, especially for fungi, had high stress values, there was no clear breakpoint in screeplots for the transect, experimental or combined data sets, and higher-dimension ordinations did not alter our interpretation of the data (see Figures S6–S8 for screeplots and ordinations in three dimensions) [Colour figure can be viewed at wileyonlinelibrary.com]

relative frequencies of dominant grass species also differed between mound and matrix locations. *Pennisetum stramineum* was the dominant grass species for almost all of the mound sampling points, whereas we recorded *Brachiaria lachnantha* and *Pennisetum mezianum* was the most common dominant species in the matrix in 2016 and 2017 respectively (Figure S3e,f). Like real mounds, sampling

points in experimentally fertilized patches had lower grass species richness than control samples in 2017 (median 1 species in fertilized patches; median 2 species in control patches; $W = 2{,}617$, $n_{\text{fertilized}} = n_{\text{control}} = 84$, $p = .001$). *P. mezianum* was the most common dominant species for both fertilized and control sampling points in 2017, although *P. stramineum* was more frequently dominant in fertilized than in control patches, possibly reflecting a trend towards increased vegetation similarity between fertilized patches and mounds (Figure S3f).

3.2 | Microbial alpha diversity

Contrary to our prediction, mound soils had higher bacterial OTU richness (Figure 1c) and Shannon diversity (Figure S4a) than matrix soils; the richness and diversity of fungal OTUs did not differ significantly between mounds and matrix (Figure 1c; Figure S4). In contrast, experimental fertilization decreased both bacterial and fungal richness by ~20% relative to control samples (Figure 1d) and likewise suppressed bacterial and fungal diversity (Figure S4b). Thus, although this effect of fertilization was consistent with our prediction, NPK addition and termite mounds had opposing effects on microbial diversity.

3.3 | Microbial beta diversity

The most common bacterial phyla across both transect and experimental samples were Actinobacteria and Proteobacteria (Figure S5a; median 64% and 18% of reads per sample respectively). In the fungal data set, the phylum Ascomycota accounted for the majority of reads (Figure S5b; median 81% of reads per sample), of which Sordariomycetes and Dothideomycetes were the most abundant classes.

Mound samples had distinctive bacterial and fungal communities compared to matrix samples (Figure 2a,b; see also Figure S6). Likewise, experimental fertilization produced distinctive bacterial and fungal communities relative to controls (Figure 2c,d; see also Figure S7).

Termites and fertilization had contrasting effects on bacterial, but not fungal, community composition (Figure 2e,f; see also Figure S8). Although both bacterial and fungal communities differed subtly between matrix and control samples (Figure 2e,f), possibly reflecting minor differences in background soil conditions between the transect and experimental plot locations, the pairwise dissimilarities in bacterial communities were significantly greater between mound and fertilized-patch samples than they were between matrix and control samples (Figure S9a). Moreover, experimental fertilization caused soil bacterial communities to become more dissimilar to those on termite mounds than either matrix or control samples (Figure 2e; Figure S9b). In contrast to the bacterial patterns, the pairwise dissimilarities in fungal communities were not significantly greater between mound and fertilized samples than they were between matrix and control samples (Figure S9a), and fertilization tended to make fungal communities more similar to those on termite mounds, although mound samples still formed a largely discrete cluster in the NMDS ordination (Figure 2f; Figure S9b).

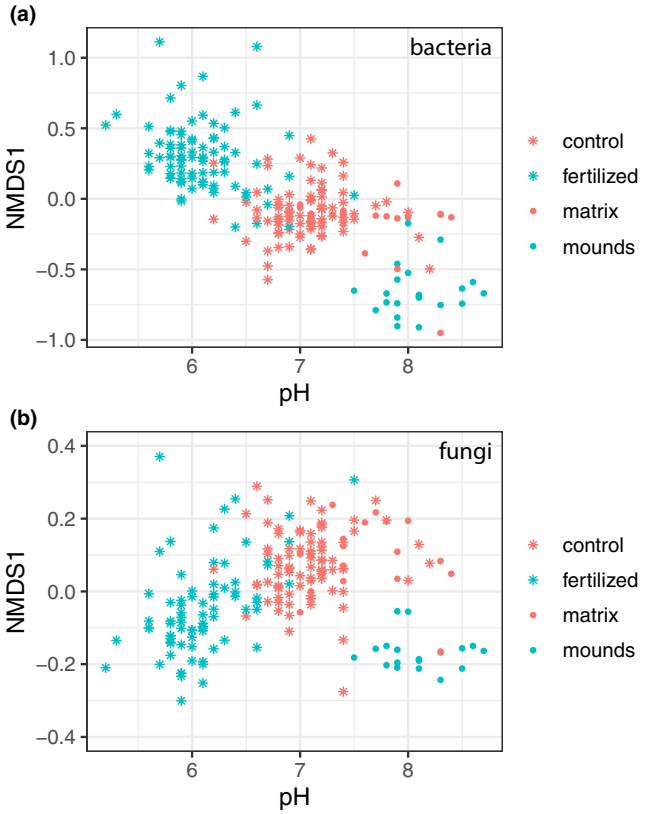

FIGURE 3 Relationship between microbial community composition and pH. Plots of scores on the first nonmetric multidimensional scaling axis (NMDS1) for (a) bacterial communities and (b) fungal communities against pH for all samples collected in 2017. Each point represents a single sample, and NMDS1 functions here as one measure of variation in microbial community composition. NMDS was performed in two dimensions using Bray–Curtis dissimilarities (see Figure 2e,f) [Colour figure can be viewed at wileyonlinelibrary.com]

Pairwise dissimilarities in both bacterial and fungal communities were positively correlated with pairwise differences in pH between samples (bacteria: $r = .36$, $p = .001$; fungi: $r = .094$, $p = .001$), suggesting that soil pH (Figure 1a,b) might explain some of the differences in microbial communities observed among mound, matrix, fertilized and control samples. Variation in pH corresponded to variation in bacterial community composition as indexed by the first NMDS axis from the combined transects and experimental fertilization data sets, with samples to the left of the ordination (lower values on the NMDS axis) having higher pH than those on the right (Figure 3a). For fungal communities, the samples on the left of the NMDS ordination comprised both mound samples (high pH) and fertilized samples (low pH), such that pH did not correspond well to the variation in community composition among sample types (Figure 3b).

Several taxonomic groups occurred at higher or lower relative abundances in mound samples compared to the matrix (Table S2).

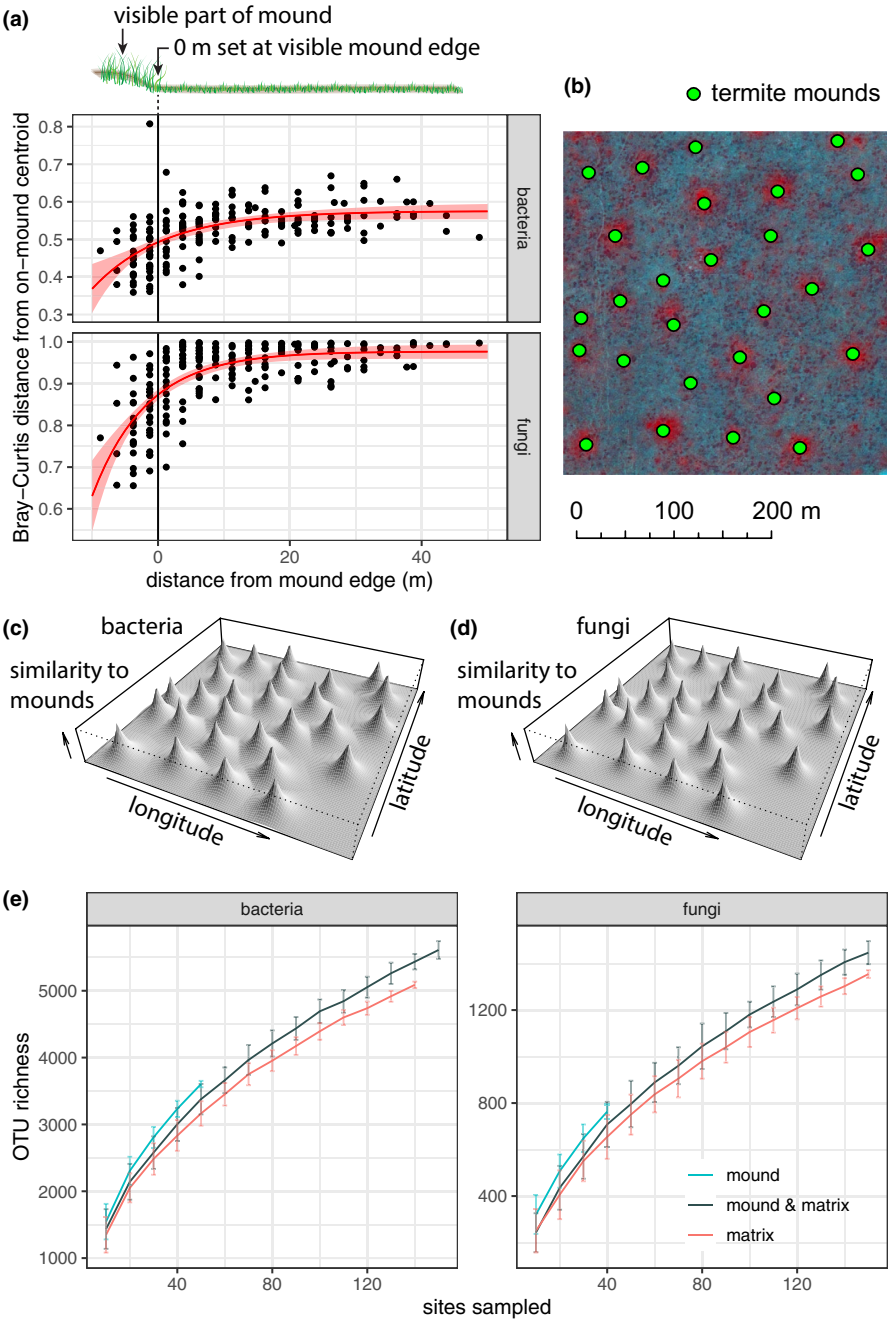

FIGURE 4 Spatial influences of termite mounds on microbial community composition and richness. Bray–Curtis dissimilarities from the centroid of the 2016 transect mound samples continue to increase for several metres beyond the visible mound edge. (a) Bacterial community dissimilarities with fitted curve $\text{dissimilarity} = 0.58 - 0.083e^{-0.091\,\text{distance}}$ (top panel), and fungal community dissimilarities with fitted curve $\text{dissimilarity} = 0.98 - 0.10e^{-0.12\,\text{distance}}$ (bottom panel). Shaded areas show 95% confidence intervals for fitted curves. Note that to facilitate comparisons between mounds of different sizes, the visible mound edge is set at 0 m for all transects, with mound centres located at negative distances; this labelling differs from other analyses in this study, in which 0 m refers to the mound centre. (b) Locations of termite mounds (green dots) as inferred from a multispectral Quickbird satellite image (2.4-m resolution, here in false-colour infrared); red patches in the image are areas of high primary productivity corresponding to termite mounds (Pringle et al., 2010). Given the inferred termite mound locations in (b), extrapolating regression results from (a) across the landscape reveals spatial patterning in (c) bacterial and (d) fungal microbial communities, as indicated by Bray–Curtis dissimilarities (inverted here to reflect similarity for visual clarity) from the 2016 on-mound centroid. (e) Sample-wise rarefaction curves of OTU richness for bacterial communities (left panel) and fungal communities (right panel). Blue curves at top show the accumulation of richness for mound samples only; red curves at bottom show the richness of samples collected in the matrix only; and grey curves in the middle show a mix of mound and matrix samples in proportion to the areal coverage of mounds and matrix across the landscape. The higher richness of mixed mound/matrix samples relative to matrix-only samples shows that termite mounds increase microbial alpha diversity relative to landscapes without mounds. Error bars show standard deviations from 100 random rarefactions [Colour figure can be viewed at wileyonlinelibrary.com]

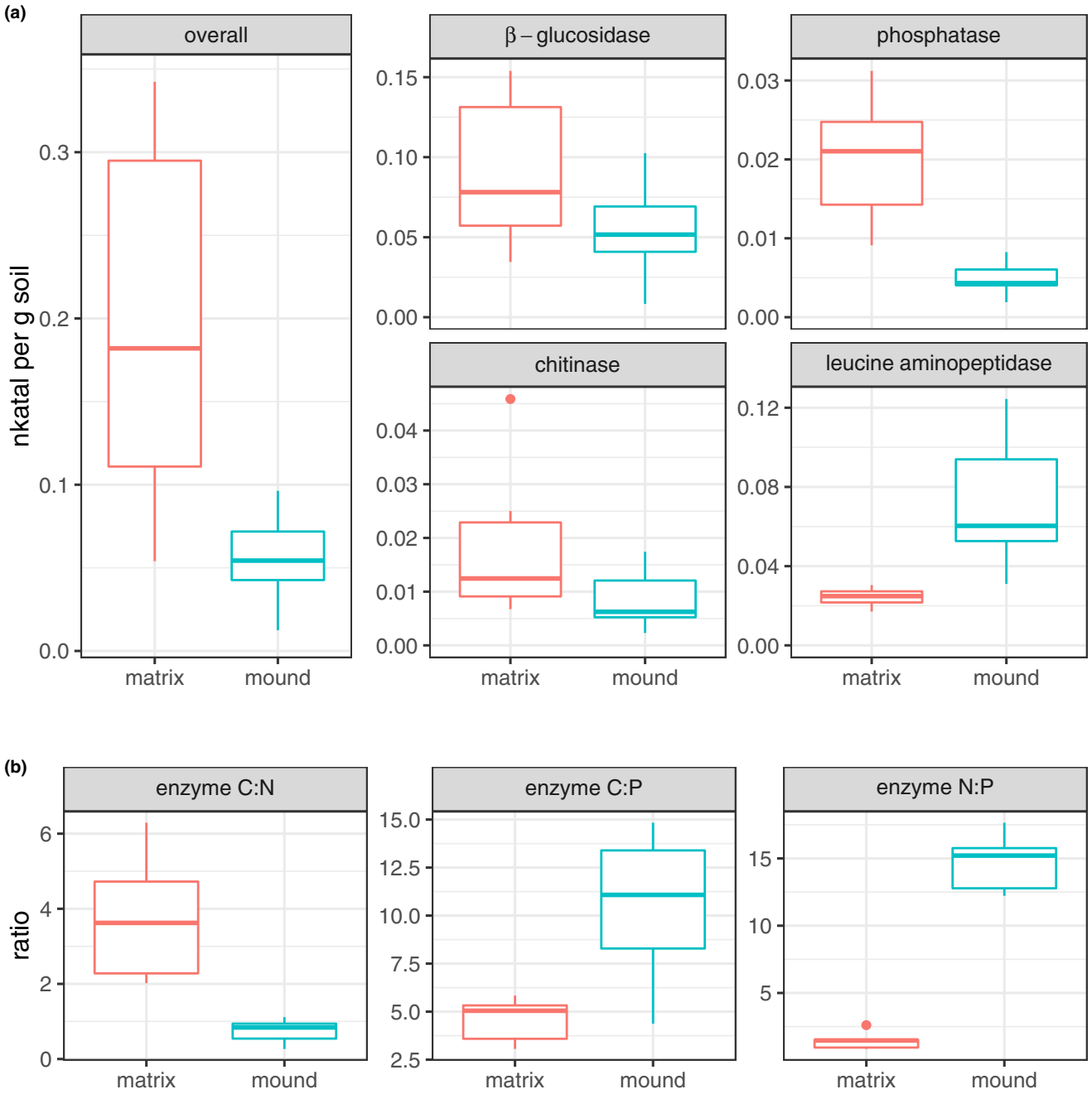

FIGURE 5 Effects of termite mounds on soil enzymatic activity. (a) Measured soil extracellular enzyme activities normalized by dry soil mass. Mound samples show lower overall hydrolytic enzyme activity compared to matrix samples ($\chi^2 = 43.2$, $df = 1$, $p < .001$; $n_{matrix} = n_{mound}$ = for all panels in this figure), as well as lower β-glucosidase, phosphatase and chitinase activity (respectively: $\chi^2 = 36.0$, $df = 1$, $p < .001$; $\chi^2 = 60.1$, $df = 1$, $p < .001$; $\chi^2 = 19.3$, $df = 1$, $p < .001$). Leucine aminopeptidase activity, however, was higher on mounds than in the matrix ($\chi^2 = 43.2$, $df = 1$, $p = .001$). (b) Measured enzymatic activity ratios. Enzyme C/N (β-glucosidase/leucine aminopeptidase) was lower on mounds than in matrix samples ($\chi^2 = 51.3$, $df = 1$, $p < .001$; $n = 7$ per sampling location), whereas both enzyme C/P (β-glucosidase/phosphatase) and enzyme N/P (leucineaminopeptidase/phosphatase) were higher on mounds than in the matrix (C/P $\chi^2 = 46.3$, $df = 1$, $p < .001$; N/P $\chi^2 = 134.4$, $df = 1$, $p < .001$) [Colour figure can be viewed at wileyonlinelibrary.com]

Among bacterial phyla, Actinobacteria and Verrucomicrobia were less abundant on mounds than in the matrix, whereas Proteobacteria, Bacteroidetes and Chloroflexi were more abundant on mounds than in the matrix. Among fungi, Ascomycota were less abundant on mounds than in the matrix, whereas Basidiomycota were more abundant on mounds than in the matrix. At the level of individual OTUs, ANCOM analysis identified 139 bacterial core OTUs and 30 fungal core OTUs with significantly different relative abundances on mounds compared to the matrix, or in fertilized relative to control samples (Table S3).

Although previous studies (e.g., Jouquet, Ranjard, Lepage, & Lata, 2005) have speculated that *Termitomyces* fungi might contribute to soil communities around fungus-farming termite nests, we found little evidence for this. *Termitomyces* OTUs were present in only four of 76 mound samples, comprising just 0.01%, 0.5%, 0.8% and 3.0% of reads in those samples.

3.4 | Spatial extent of mound influence

The effects of mounds on soil bacterial and fungal communities extended several metres beyond the mound edge. Communities >10 m from mound edges were markedly dissimilar to those at mound centres, but the dissimilarities saturated as distance increased beyond 10 m (Figure 4a). Termites' relatively localized influence on soil microbial communities thus scales up to generate regular spatial patterning in community composition across the landscape, corresponding to the spatially overdispersed mounds (Figure 4b–d).

3.5 | Microbial gamma diversity

Sample-to-sample turnover of bacterial OTUs within transects was higher on mounds than in the matrix, but this was not true of fungal OTUs (Figure S10a). The mean Bray–Curtis dissimilarity in bacterial communities between 0- and 5-m mound samples on the same transect was significantly higher than between 25- and 30-m matrix samples. For fungal communities, however, Bray–Curtis dissimilarities did not differ significantly between mound and matrix sample pairs.

Bacterial OTU turnover between transects was also higher among mound samples than among matrix samples, but this was not true of fungal OTUs (Figure S10b). Pairwise Bray–Curtis dissimilarities in bacterial communities among 0-m samples (i.e., mound centres) were higher than dissimilarities among 25-m samples (i.e., matrix) on different transects. For fungal communities, however, OTU turnover across transects did not differ significantly between mound and matrix.

Sample-based rarefaction curves illustrate the extent to which termite mounds increase soil bacterial and fungal diversity at the landscape scale. Bacterial OTU richness was estimated to be 6.3% higher in 100 mixed mound/matrix samples than in 100 matrix-only samples, and fungal OTU richness was estimated to be 8.0% higher (Figure 4e,f). Bacterial and fungal Shannon diversity were estimated to be 2.2% and 2.1% higher, respectively, in mixed samples compared to matrix-only samples (Figure S11).

3.6 | Measured extracellular enzyme activity

Overall microbial activity potential was lower on mounds than in the matrix (Figure 5a). β-Glucosidase, chitinase and phosphatase activity were all lower on mounds; leucine aminopeptidase activity, however, was higher on mounds than in the matrix (Figure 5a). Enzyme C/N (β-glucosidase/leucine aminopeptidase) was lower on mounds than in matrix samples, whereas both enzyme C/P (β-glucosidase/phosphatase) and enzyme N/P (leucine aminopeptidase/phosphatase) were higher on mounds than in the matrix (Figure 5b).

4 | DISCUSSION

In this study, we used DNA metabarcoding in conjunction with observational and experimental sampling to explore the effects of *Odontotermes montanus* termites on free-living soil microbial communities. Using samples from transects, we showed that bacterial and fungal communities differed in composition between mounds and matrix, and that bacterial (but not fungal) communities were more diverse on mounds. Our analyses indicate that the relatively localized effects of termites on microbes scale up to create regular spatial patterning in community composition, and that this patterning increases the total microbial diversity of the savanna landscape. A field manipulation designed to simulate the effects of termites on the concentrations of three major nutrients altered the diversity and composition of bacterial and fungal communities, but in different ways. Experimental fertilization caused fungal communities to become more similar to those on real termite mounds, but caused bacterial communities to become markedly more dissimilar to those on real mounds.

Although fungus-farming termites have dramatic effects on the structure and functioning of tropical savannas (Davies, Baldeck, & Asner, 2016; Pringle et al., 2010; Sileshi et al., 2010), and these effects are undoubtedly mediated to some degree by microbial activity, few studies have characterized the influence of termites on soil microbes. Our study extends previous work that has reported distinctive mound-associated soil microbial communities on the basis of community fingerprinting methods (automated ribosomal intergenic spacer analysis, Jouquet et al., 2005) or metabarcoding of a limited number of soil samples (Makonde, Mwirichia, Osiemo, Boga, & Klenk, 2015). Our study also begins to explore the potential mechanisms underlying termite-driven heterogeneity in soil microbial communities by coupling observational sampling with manipulative experimentation. Our results show that elevated levels of inorganic N, P and K cannot account for the elevated microbial diversity and distinctive bacterial communities associated with *O. montanus* mounds, although these nutrients may contribute to the patterns observed in fungal community composition.

There are many potential reasons why the application of inorganic NPK fertilizer did not replicate the effects of mounds on bacterial communities. We briefly review the available evidence below. We conclude with a consideration of how future work might further clarify the ecological roles of fungus-farming termites in African savannas.

4.1 | Potential mechanisms of mound-induced shifts in microbial community composition

Soil pH is likely to act in conjunction with soil nutrients to determine microbial responses to termite mounds and experimental

fertilization. While fertilization lowered soil pH relative to controls, pH was higher on mounds relative to the matrix, consistent with a previous study of *O. montanus* mounds in our study area (Petipas & Brody, 2014). pH is frequently cited as a major driver of bacterial communities at spatial scales ranging from tens of metres (Baker et al., 2009), to tens of kilometres (Bru et al., 2011) and beyond (Chu et al., 2010; Fierer & Jackson, 2006; Fierer, Strickland, Liptzin, Bradford, & Cleveland, 2009; Lauber, Hamady, Knight, & Fierer, 2009). The mechanisms underlying the influence of pH on bacterial communities are not well understood, but may include differences in salinity or nutrient availability, altered microbial enzyme activity, interference with microbial metabolisms, and changes to the thermodynamics and kinetics of redox reactions (Jin & Kirk, 2018a, 2018b). Studies have also suggested that soil fungi may be less responsive than bacteria to pH (Lauber, Strickland, Bradford, & Fierer, 2008; Rousk et al., 2010). Indeed, we found that differences in pH were strongly correlated with dissimilarities in bacterial community composition, but less so for fungal communities (Figure 3). Thus, we propose that the contrasting effects of termites and experimental fertilization on soil pH may help to explain the strongly divergent responses of bacteria, but not fungi, to these influences.

Termite-driven changes to soil nutrients might select directly for microbes that are able to exploit those resources. We attempted to replicate this using inorganic NPK fertilizer in our experiment because these nutrients are generally elevated on termite mounds (Sileshi et al., 2010) and are probably limiting nutrients for savanna grasses (Ries & Shugart, 2008), at least in the matrix. However, our metabarcoding data provided very little evidence that inorganic NPK availability is a major driver of the distinctive mound-associated communities. Of the 96 bacterial and 27 fungal OTUs that were significantly elevated or depressed on mounds relative to matrix in our ANCOM results, only four bacterial and five fungal OTUs showed a significant response in the same direction to experimental fertilization.

Semi-annual fertilization over the 2 years prior to sampling was adequate to induce consistent changes in soil microbial communities. We think it likely that any strong transient short-term effects of fertilization would have passed within a couple of months after fertilizer application and thus would not have been evident when we sampled 5 months later (e.g., see review by Geisseler & Scow, 2014). On the other hand, the 2-year duration of our treatment is short relative to the age of termite mounds, which may persist for centuries (Darlington, 1985). It is possible that repeated fertilizer applications over much longer periods might produce stronger (or even qualitatively different) effects on soil microbial communities. Studies have suggested that soil nutrient levels may take decades to come to equilibrium following changes in land use or nutrient inputs (e.g., Oberholzer, Leifeld, & Mayer, 2014; Sebilo, Mayer, Nicolardot, Pinay, & Mariotti, 2013). Furthermore, chronic nutrient inputs may lead to "permanent" state shifts in microbial community composition by stimulating evolutionary change (e.g., in N-fixing rhizobial bacteria, Weese, Heath, Dentinger, & Lau, 2015). It would be useful to assess such long-term trajectories by tracking soil microbial community composition through time under repeated fertilization.

Another limitation to our experimental inferences is that we employed only a single fertilization regime and did not measure soil-nutrient content in fertilized patches to evaluate the extent to which our treatment resulted in levels of N, P and K similar to those on real mounds. We selected the rate of fertilization with the goal of replicating the direction and approximate magnitude of the nutrient gradient on termite mounds. We benchmarked our treatment regime against local agricultural practices and previous nutrient manipulation studies, and used a small pilot to verify that our treatment elevated the foliar nitrogen content of trees in fertilized patches by an amount roughly comparable to the difference between mound and matrix trees (see Document S1 "Field sampling: fertilization experiment" for further details). However, a detailed accounting of soil nutrients on both mounds and fertilized patches would have been helpful in clarifying nutrient dynamics and identifying specific similarities and differences between mounds and our experiment.

Although our results did not provide much evidence that mound-associated microbial communities are driven by inorganic NPK availability, they do not rule out the possibility that microbes respond to the elevated availability of other nutrients on *O. montanus* mounds. Organic carbon, for example, is elevated on *O. montanus* mounds (Brody et al., 2010; Palmer, 2003), and this is also likely to be the case for other complex organic substrates. Organic nutrient additions can alter soil microbial community composition (Cline, Huggins, Hobbie, & Kennedy, 2018; Pérez-Piqueres, Edel-Hermann, Alabouvette, & Steinberg, 2006; Yao, Merwin, Abawi, & Thies, 2006) and may produce different outcomes than inorganic nutrient additions, as the microbes best able to exploit these resources may differ. Although many microbes probably use the inorganic nutrients available on mounds in preference to complex organic substrates (Allison & Vitousek, 2005), certain microbial taxa may thrive in mound soils by degrading complex substrates to obtain nutrients that remain limiting for microbial growth. The elevated leucine aminopeptidase activity that we measured on mounds may reflect such use of complex substrates to obtain nitrogen, as this enzyme is involved in breaking down proteins and peptides. In contrast, lower β-glucosidase and phosphatase activity on mounds suggests less use of complex substrates to obtain carbon or phosphorus. The lower ratio of β-glucosidase to leucine aminopeptidase activity on mounds compared to the matrix, and the higher ratio of leucine aminopeptidase to phosphatase activity, in turn suggest that nitrogen (but not carbon or phosphorus) remains limiting on mounds, despite elevated soil nitrogen levels, as costly enzyme production should only be favoured where nutrients cannot be adequately obtained in more accessible inorganic forms.

Although the effects of termites and fertilization on soil microbes could in principle be mediated by plant community composition (de Vries et al., 2012; O'Donnell, Seasman, Macrae, Waite, & Davies, 2001), our data suggest that this was not a prevailing mechanism. We defined mound edges based on the extent of the distinctive mound-associated grass community dominated by *P. stramineum*,

and this boundary was usually sharply visible. Yet our data showed that mound-associated soil microbial communities persist 5–10 m beyond that edge, suggesting that the effects of termites on soil microbes may be best understood in terms of resources that can either diffuse (perhaps aided by the greater porosity and extensive shallow cracking of mound soils; DeCarlo & Caylor, 2019) or be transported (e.g., by termites moving soil particles) beyond mound edges.

Other edaphic properties might also contribute independently and interactively to shaping microbial communities. Soil moisture, for example, can affect microbes (Lauber, Ramirez, Aanderud, Lennon, & Fierer, 2013; Lipson, 2007) and was lower both on mounds compared to the matrix and in fertilized patches compared to controls. The lowering of moisture by both termites and fertilization implies that moisture cannot entirely explain the contrasting responses of bacterial communities to these two influences. However, we cannot rule out a role for soil moisture in explaining the fungal community results or in contributing to the bacterial responses. The contrasting effects of termites and fertilization on bacterial communities could also be explained if the effect of nutrient-enrichment on microbes interacts with physical characteristics that differ between mound and matrix soils, such as altered porosity (Brody et al., 2010; Neumann, Heuer, Hemkemeyer, Martens, & Tebbe, 2013), compaction and cracking (DeCarlo & Caylor, 2019).

4.2 | Conclusions

We have shown that the topsoil of *O. montanus* mounds has distinctive bacterial and fungal communities compared to the surrounding matrix, and we report evidence from an initial experimental inquiry into the potential mechanisms behind these patterns. The assembly of these different microbial communities may be influenced heavily by the redistribution of organic nutrients by termites, and/or by subsequent changes in pH, but for the most part appear not to be driven directly by inorganic macronutrient availability. Microbial communities remain similar to those on mounds for several metres beyond the mound edge, but past that distance the effect of mound proximity is minimal. Termites thus generate spatial heterogeneity in the composition and function of free-living soil microbes across the landscape, which mirrors the regular patterning of the mounds themselves.

Such regular patterning of spatially overdispersed social-insect nests is observed in ecosystems worldwide (Pringle & Tarnita, 2017) and can be explained theoretically by territorial competition between neighbouring colonies (Tarnita et al., 2017; see also Korb & Linsenmair, 2001). A key outstanding question is how these patterns influence other ecosystem properties and processes (Pringle & Tarnita, 2017). A previous study from our system found that the regular patterning of *O. montanus* mounds boosted net productivity across the landscape (Pringle et al., 2010). Here, we found that termite mounds influenced the alpha diversity of soil microbiota, and that the spatially patterned template of termite mounds created pronounced beta diversity, such that the overall richness and diversity of soil bacteria and fungi was greater in the real landscape than in simulated landscapes lacking mounds. As soil microbes are agents of nutrient cycling, decomposition and other ecosystem functions, these results deepen our understanding of the mechanisms that generate spatial heterogeneity in tropical savanna ecosystems, paralleling previous work on the influence of *O. montanus* termites on symbiotic nitrogen fixation (Fox-Dobbs et al., 2010).

We suggest that future studies proceed by investigating the roles of nutrient availability and pH in shaping the distinctive soil microbial communities associated with fungus-farming termite mounds. It would be helpful to begin with a more detailed accounting of specific organic and inorganic nutrients. Stable-isotope tracers, manipulative experiments, metatranscriptomic sequencing, and measurements of microbial biomass and activity could all help to understand the effects of different nutrients in different forms, as well as the relationships between soil metabolic activity and pH. Developing a more detailed understanding of the soil microbial ecology will contribute to a clearer picture of how termite-induced spatial heterogeneity in microbial communities influences other aspects of the ecosystem, and the ways in which such connections might apply in other ecosystems.

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AUTHOR CONTRIBUTIONS

C.C.M.B. and C.E.T. conceived the study, and all authors contributed to research design through discussions. C.C.M.B. conducted sampling, generated molecular data and performed analyses. J. Puissant conducted enzyme assays. C.C.M.B. wrote the manuscript with the contribution of all co-authors.

DATA AVAILABILITY STATEMENT

Data from sequencing and enzyme assays are available through Dryad, along with code for bioinformatic processing with OBITOOLS

and statistical analysis in R (Baker et al., 2020), https://datadryad.org/stash/share/IICLI2teEvVh-98uoHmjUcXQ2e7I7ETRK5P0YqTVHew (https://doi.org/10.5061/dryad.mw6m905th).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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