# Consistent responses of soil microbial communities to elevated nutrient inputs in grasslands across the globe

Jonathan W. Leff[a,b], Stuart E. Jones[c], Suzanne M. Prober[d], Albert Barberán[a], Elizabeth T. Borer[e], Jennifer L. Firn[f], W. Stanley Harpole[g,h,i], Sarah E. Hobbie[e], Kirsten S. Hofmockel[j], Johannes M. H. Knops[k], Rebecca L. McCulley[l], Kimberly La Pierre[m], Anita C. Risch[n], Eric W. Seabloom[o], Martin Schütz[n], Christopher Steenbock[b], Carly J. Stevens[p], and Noah Fierer[a,b,1]

[a]Cooperative Institute for Research in Environmental Sciences, University of Colorado, Boulder, CO 80309; [b]Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO 80309; [c]Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556; [d]Commonwealth Scientific and Industrial Research Organisation Land and Water Flagship, Wembley, WA 6913, Australia; [e]Department of Ecology, Evolution and Behavior, University of Minnesota, St. Paul, MN 55108; [f]School of Earth, Environmental and Biological Sciences, Queensland University of Technology, Brisbane, QLD 4001, Australia; [g]Department of Physiological Diversity, Helmholtz Center for Environmental Research UFZ, 04318 Leipzig, Germany; [h]German Centre for Integrative Biodiversity Research Halle-Jena-Leipzig, D-04103 Leipzig, Germany; [i]Institute of Biology, Martin Luther University Halle-Wittenberg, 06108 Halle (Saale), Germany; [j]Ecology, Evolution, and Organismal Biology Department, Iowa State University, Ames, IA 50011; [k]School of Biological Sciences, University of Nebraska, Lincoln, NE 68588; [l]Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY 40546; [m]Department of Integrative Biology, University of California, Berkeley, CA 94720; [n]Community Ecology, Swiss Federal Institute for Forest, Snow and Landscape Research, 8903 Birmensdorf, Switzerland; [o]Department of Ecology, Evolution, and Behavior, University of Minnesota, St. Paul, MN 55108; and [p]Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, United Kingdom

Edited by Peter M. Vitousek, Stanford University, Stanford, CA, and approved July 16, 2015 (received for review April 29, 2015)

**Soil microorganisms are critical to ecosystem functioning and the maintenance of soil fertility. However, despite global increases in the inputs of nitrogen (N) and phosphorus (P) to ecosystems due to human activities, we lack a predictive understanding of how microbial communities respond to elevated nutrient inputs across environmental gradients. Here we used high-throughput sequencing of marker genes to elucidate the responses of soil fungal, archaeal, and bacterial communities using an N and P addition experiment replicated at 25 globally distributed grassland sites. We also sequenced metagenomes from a subset of the sites to determine how the functional attributes of bacterial communities change in response to elevated nutrients. Despite strong compositional differences across sites, microbial communities shifted in a consistent manner with N or P additions, and the magnitude of these shifts was related to the magnitude of plant community responses to nutrient inputs. Mycorrhizal fungi and methanogenic archaea decreased in relative abundance with nutrient additions, as did the relative abundances of oligotrophic bacterial taxa. The metagenomic data provided additional evidence for this shift in bacterial life history strategies because nutrient additions decreased the average genome sizes of the bacterial community members and elicited changes in the relative abundances of representative functional genes. Our results suggest that elevated N and P inputs lead to predictable shifts in the taxonomic and functional traits of soil microbial communities, including increases in the relative abundances of faster-growing, copiotrophic bacterial taxa, with these shifts likely to impact belowground ecosystems worldwide.**

soil bacteria | soil fungi | shotgun metagenomics | soil ecology | fertilization

Human activities associated with fossil fuel combustion, agricultural fertilization, and dust or ash production have greatly increased nitrogen (N) and phosphorus (P) inputs to ecosystems around the globe relative to their preindustrial levels (1, 2). The impacts of elevated N and P inputs on grassland ecosystems, which cover 26% of the global land surface (3), are expected to occur on relatively short time scales, with potentially important effects on plant biodiversity and terrestrial carbon (C) dynamics (4–7). A large body of research focusing on plant community responses has demonstrated consistent loss of grassland plant diversity with nutrient additions (7, 8). In many cases, nutrient additions also shift the composition of plant communities with faster-growing plants that are good competitors for light being favored under conditions where nutrients are less limiting to growth (9, 10). The associated belowground microbial responses to nutrient additions, including general taxonomic and trait shifts, remain poorly understood, even though soil microbes represent a large fraction of the living biomass in grassland systems (11) and can have important effects on terrestrial C dynamics, soil fertility, and plant diversity (12). In particular, integrated, cross-site, experimental investigations of both plant and soil microbial responses to nutrient additions are needed to inform understanding of how the structure and functional attributes of soil microbial communities shift in response to anthropogenic inputs of N and P and whether these shifts are consistent across sites.

Soil microbial communities are often sensitive to nutrient inputs. For instance, N fertilization typically reduces microbial biomass and respiration rates (13–15), with specific functional groups of microbes, including ammonia oxidizers and mycorrhizal fungi, often being very sensitive to N additions (16–18). A

## Significance

Human activities have resulted in large increases in the availability of nutrients in terrestrial ecosystems worldwide. Although plant community responses to elevated nutrients have been well studied, soil microbial community responses remain poorly understood, despite their critical importance to ecosystem functioning. Using DNA-sequencing approaches, we assessed the response of soil microbial communities to experimentally added nitrogen and phosphorus at 25 grassland sites across the globe. Our results demonstrate that the composition of these communities shifts in consistent ways with elevated nutrient inputs and that there are corresponding shifts in the ecological attributes of the community members. This study represents an important step forward for understanding the connection between elevated nutrient inputs, shifts in soil microbial communities, and altered ecosystem functioning.

Author contributions: J.W.L., E.T.B., W.S.H., E.W.S., and N.F. designed research; J.W.L., S.M.P., E.T.B., J.L.F., W.S.H., S.E.H., K.S.H., J.M.H.K., R.L.M., K.L.P., A.C.R., E.W.S., M.S., and C.J.S. performed research; J.W.L., S.E.J., S.M.P., and C.S. analyzed data; and J.W.L., S.E.J., S.M.P., A.B., E.T.B., J.L.F., W.S.H., S.E.H., K.S.H., J.M.H.K., R.L.M., K.L.P., A.C.R., E.W.S., M.S., C.S., C.J.S., and N.F. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The raw sequence data have been deposited in the NCBI Sequence Read Archive (accession no. SRP052716 and BioProject accession no. PRJNA272747). The shotgun metagenomic sequences have been deposited in the Genomes Online Database (GOLD Study ID Gs0053063).

[1]To whom correspondence should be addressed. Email: noah.fierer@colorado.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1508382112/-/DCSupplemental.



ECOLOGY

few studies conducted at individual sites also have shown that elevated N inputs can alter the overall composition of bacterial or fungal communities (17, 19–22). Understanding of soil microbial community responses to elevated P inputs remains more limited, even though many regions experience elevated inputs of both N and P (2), and anthropogenic activities can alter N:P ratios in soil (1, 23). We are not aware of any studies that have used standardized nutrient treatments to evaluate the generality and local context dependence of soil bacterial, archaeal, and fungal communities to N and P amendments across a wide range of soil types. Individual studies conducted at specific sites are useful, but inconsistencies in methods and site characteristics limit the ability to make robust generalizations of how belowground microbial communities will respond to elevated nutrient inputs across sites.

Although previous studies have shown that soil microbial communities can shift in response to nutrient additions at individual grassland sites (18, 20, 22, 24), relating these taxonomic or phylogenetic shifts to changes in the functional attributes of these communities is not trivial. Simply documenting how communities shift in composition might not tell us how the aggregated traits of these communities change in response to nutrient additions because soil microorganisms are incredibly diverse, and most soil microbial taxa remain uncharacterized (25). Such trait-level information is arguably more important for linking changes in soil microbial communities to changes in belowground processes than simply documenting how nutrients increase or decrease the relative abundances of community members (26). Just as the aggregated traits of plant communities can shift in predictable directions with nutrient additions (9, 10), we expect that the aggregated traits of soil microbial communities will also shift in a predictable manner with fertilization. Here, we focus on the aggregated traits of bacterial communities, and specifically, we expect that increases in nutrient availability will tend to favor copiotrophic (i.e., fast-growing, low C use efficiency) bacterial taxa and reduce the abundances of more oligotrophic (i.e., slow-growing, high C use efficiency) taxa (20, 27). Although there is some evidence that we can use taxonomic information to place soil bacteria along this continuum in life history strategies (28), we can use shotgun metagenomic information to more accurately infer the aggregated traits of soil bacterial communities and determine whether copiotrophic traits are actually favored under conditions of elevated nutrient availability.

For this study, we sought to build a predictive understanding of the responses of diverse soil microbes to elevated nutrient inputs that is generalizable across grasslands. We collected soils from an N and P addition experiment replicated at 25 grassland sites spanning four continents and quantified shifts in bacterial, archaeal, and fungal community structure in response to experimentally increased soil nutrients using high-throughput sequencing of marker genes. In addition, we investigated potential shifts in bacterial community-level traits by analyzing functional gene metagenomic sequences from a subset of those sites. We hypothesized that N and P additions would induce shifts in fungal communities with mycorrhizal fungi decreasing in relative abundance; alter archaeal community composition by increasing the abundances of those taxa presumed to be capable of ammonia oxidation (29); and shift bacterial communities to favor copiotrophic over more oligotrophic taxa. Further, we hypothesized that the degree to which microbial communities shifted in response to nutrient additions would be positively correlated with the magnitude of the shifts in plant community composition. Those sites where nutrient additions have the largest effects on plant communities are also those sites where we would expect to see the largest responses in belowground microbial communities, due to the direct associations between plants and microbes or their shared responses to fertilization.

## Results and Discussion

**Effect of Nutrient Additions on Soil Fungal Communities.** Fungal diversity and community composition differed strongly across the 25 globally distributed grassland sites, regardless of nutrient treatment ($P < 0.001$ in all cases; Fig. S1). Mean fungal phylotype (i.e., species) richness ranged 1.7-fold across the sites, and there were large variations in the relative abundances of major taxonomic groups (Table S1). The strong site effects are not surprising, given the range in environmental conditions and soil characteristics found across sites spanning four continents and elevations from 50 to 2,320 m (Tables S2 and S3). In particular, the sites represented a broad range in soil acidity, climate, and plant community composition, factors that have previously been associated with differences in soil fungal community structure at these sites and others (30, 31).

We investigated the within-site effects of nutrient additions on fungal community structure by statistically controlling for the strong cross-site differences by including site as a random effect in our models. Fungal Shannon diversity responded weakly to nutrient additions, decreasing by only 2.7% on average when N and P were added together ($P = 0.05$), a response consistent with the weak response observed for plants (8).

In contrast to the weak effects of nutrients on fungal diversity, we observed significant effects of N ($R^2 = 0.003$; $P < 0.001$) and/or P ($R^2 = 0.002$; $P = 0.04$) additions on fungal community composition, with the same taxa generally responding to nutrient additions across sites, despite the large cross-site variation in fungal community types (Fig. 1). With combined addition of N and P, there were increases in *Ascomycota* and significant decreases in the relative abundances of *Glomeromycota* (Fig. 2*A*). The *Glomeromycota* phylum is composed almost entirely of arbuscular mycorrhizal fungi (32), and we expected these fungi to decrease in relative abundance with nutrient additions because they would be less valuable to their hosts and thus provided with less plant C under conditions of increased N and P availability (33–35). We further investigated nutrient effects on mycorrhizal fungi by assessing the collective responses of mycorrhizal fungi, including those taxa outside the *Glomeromycota* phylum that are reported in the literature as being mycorrhizal. These taxa also consistently decreased in plots receiving N and P relative to the control plots ($P = 0.016$), corroborating results from a meta-analysis demonstrating declines in mycorrhizal fungi with N additions (18). Interestingly, adding N and P together led to far larger decreases in the relative abundances of *Glomeromycota* than when these nutrients were added individually ($P > 0.1$; Table S4), suggesting a role for both of these nutrients in shaping arbuscular mycorrhizal communities.

The overall decrease in the proportion of mycorrhizal fungi with N and P additions—and shifts in fungal community composition more broadly—could be caused by plant community shifts, changes

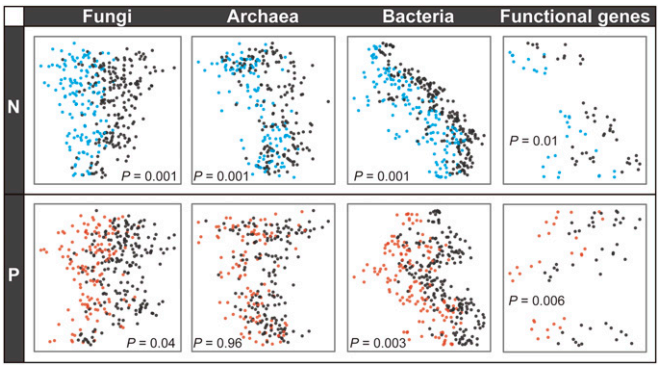


**Fig. 1.** Constrained ordinations showing differences between microbial communities from plots that did not receive the indicated nutrient (gray points) and from plots receiving N (blue) or P (red) additions (colored points). Colored points include samples receiving both nutrients. *P* values refer to permutational multivariate ANOVA results.

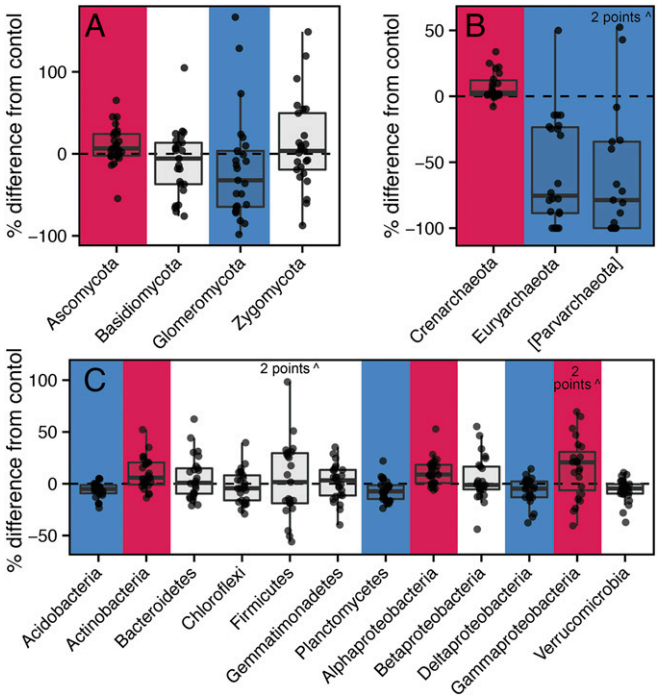


**Fig. 2.** Differences in the relative abundance of higher-level taxa between control and nutrient addition plots. Fungal (*A*) and bacterial (*C*) taxa differences are comparisons to +N,+P plots, and archaeal taxa differences (*B*) are comparisons to +N differences because P additions did not significantly affect the relative abundance of archaeal taxa, nor was there an interaction between N and P additions. Points represent site means, and boxplots show quartile values for each taxon. Red and blue backgrounds show significant increases and decreases in the relative abundances of specific taxa, respectively (false discovery rate-corrected $P < 0.05$). Only taxa with relative abundances >1% in any of the treatments are shown. Points with values greater than the plot axis maximum are indicated.

in plant biomass, and/or the direct effects of added nutrients. The magnitudes of the responses of major fungal taxonomic groups were not significantly correlated with changes in key soil characteristics (Table S5). However, the magnitude of fungal community composition response (i.e., the mean community dissimilarity between samples with added N and P and control samples) was significantly correlated with the magnitude of the response of plant community composition to added N and P ($r = 0.44$; $P = 0.03$; Fig. 3), helping to explain site-to-site variability in shifts in belowground communities. Those sites where nutrients had the largest impacts on plant communities were also the sites that had the strongest nutrient effects on fungal communities. This finding suggests either that shifts in plant community composition drive shifts in fungal community composition or that both plant and fungal communities respond similarly to changes in edaphic factors. Although overall fungal compositional shifts correlated with plant community composition shifts, changes in the relative abundance of *Glomeromycota* were not related to changes in live plant biomass with fertilization ($P > 0.1$), or to changes in surface soil N concentrations ($P > 0.1$; Table S5), suggesting that plant nutrient limitation was not a good predictor of the differential responses observed across the sites.

**Effect of Nutrient Additions on Soil Archaeal Communities.** Archaea were rare at most sites, and archaeal diversity (Fig. S1*A*) and community composition (Fig. S1*B*) were highly variable across sites, regardless of nutrient additions ($P < 0.001$). Archaeal phylotype richness ranged 3.7-fold across the sites, and the archaeal communities were dominated by *Crenarchaeota* (92% on average) and *Euryarchaeota* (4.3% on average; Table S1). The proportion of 16S rRNA reads that were of archaeal origin was also highly variable across the sites (Fig. S2*A*), ranging from 0 to 0.16. This variability in archaeal communities was likely due to the large cross-site differences in environmental conditions mentioned above. For instance, previous work has shown a correlation between archaeal relative abundances and soil nutrient content (36); we know that soil N concentrations varied 33-fold across the control plots, and archaea relative abundances were inversely related to soil C:N ratios ($r = -0.67$; $P < 0.001$).

We next assessed whether there were consistent shifts in archaeal relative abundance and community structure with nutrient additions by statistically controlling for the strong cross-site differences. Archaeal relative abundances generally increased with N additions ($P < 0.001$; Fig. S2*B*), and there was a mean 4.8% decrease in archaeal diversity with N additions compared with control plots ($P = 0.01$). This decrease in diversity was possibly related to an N-induced growth of specific archaeal taxa. Specifically, the phylum *Crenarchaeota*, which primarily comprised members of the family *Nitrososphaeraceae*, consistently increased in relative abundance with N additions across the majority of sites, whereas *Euryarchaeota* and the candidate division *Parvarchaeota* consistently decreased (Fig. 2*B*). These shifts are likely related to *Archaea* being active drivers of the soil N cycle. For example, *Nitrososphaeraceae* can oxidize ammonia (29, 37), a metabolism that is expected to be advantageous with elevated ammonium supply, which should have been elevated in the N addition plots, because urea is readily hydrolyzed to ammonium. Abundances of soil *Crenarchaeota* also are positively correlated with soil N content (36). Conversely, several reports have shown the potential for members of the *Euryarchaeota*, which are predominately methanogens, to fix atmospheric $N_2$ (38, 39). This characteristic could place them at a competitive disadvantage under conditions of elevated N availability and explain their strong proportional decrease with N fertilization. Although it has been shown that N can inhibit methanogenesis in vitro (40), this work is, to our knowledge, the first direct evidence that N additions may also decrease methanogen populations in non-wetland soils. Still, it is important to note that these shifts in the relative

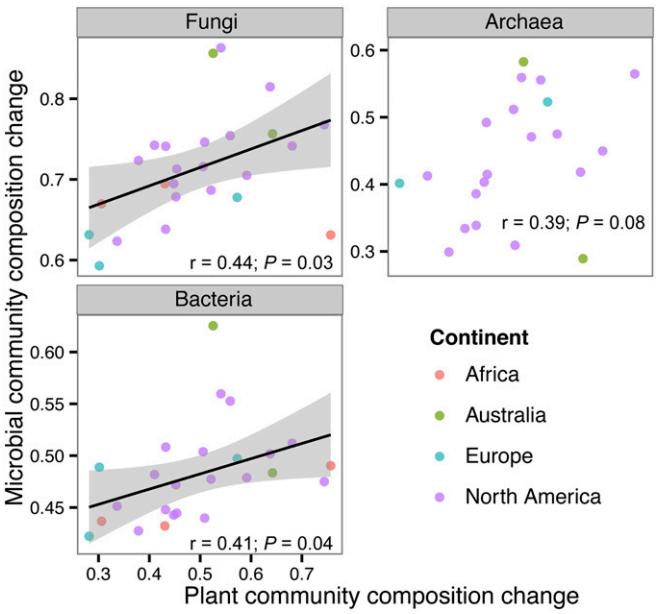


**Fig. 3.** Correlations between changes in microbial and plant community composition with N and P additions across the sites for fungal, archaeal, and bacterial communities. Change in community composition was calculated as the mean Bray–Curtis dissimilarity between control plots and those plots amended with nutrients. Relationships were assessed by using Pearson correlations.

ECOLOGY

abundances of archaeal phyla are not independent of one another, and decreased methanogen relative abundances could simply be the result of increased relative abundances of *Crenarchaeota*. Nonetheless, these results highlight that soil archaeal communities are sensitive to N additions, but additional research is required to determine whether these community responses are associated with changes in methane fluxes or soil N cycling rates.

**Effect of Nutrient Additions on Soil Bacterial Communities.** As with fungal and archaeal communities, bacterial diversity and community composition differed strongly across the 25 grassland sites (Fig. S1). These differences were likely due to factors such as acidity, climate, and plant community composition, as has been previously observed (30, 41, 42). Mean phylotype richness ranged 1.7-fold, and the abundant phyla, including *Proteobacteria*, *Acidobacteria*, *Verrucomicrobia*, *Actinobacteria*, and *Bacteroidetes*, all varied considerably in their relative abundances across the sites (Table S1).

Nutrient additions did not strongly alter bacterial diversity; P additions caused marginal (0.5%) increases in bacterial diversity ($P = 0.06$), and N had no significant effect. Our results stand in contrast to negative relationships between bacterial diversity and N additions reported from previous studies conducted at individual sites (19, 43). This finding points to the importance of local context and highlights the pitfalls associated with extrapolating results obtained from individual sites to other ecosystems or soil types.

Bacterial community composition was significantly affected by N ($R^2 = 0.002$; $P < 0.001$) and P additions ($R^2 = 0.002$; $P = 0.003$; Fig. 1). The community shifts corresponded to changes in the relative abundances of numerous major taxa. For example, the relative abundances of *Actinobacteria*, *Alphaproteobacteria*, and *Gammaproteobacteria* consistently increased with nutrient additions across sites, whereas those of *Acidobacteria*, *Planctomycetes*, and *Deltaproteobacteria* consistently decreased (Fig. 2*C*). However, these taxonomic shifts were not always in the same direction or magnitude when N or P was added alone (Table S4). Overall, the taxonomic patterns in our cross-site study were in agreement with previous work conducted at individual grassland sites (20), and they corroborate laboratory studies that have noted similar shifts in the relative abundances of these major bacterial groups with nutrient additions (13). Our findings are generally consistent with our hypothesized shifts in general life history strategies with bacterial taxa that are faster growing and more copiotrophic (28) being favored under conditions of elevated nutrient availability (27). In particular, soil bacterial groups that are generally considered to be more copiotrophic, including *Actinobacteria* and *Alphaproteobacteria,* increased in relative abundance with nutrient additions, and the largely oligotrophic *Acidobacteria* phylum decreased in relative abundance. Whereas original evidence for generalizations of these life history strategies across broad bacterial taxonomic groups was based on responses to labile C inputs (28, 44, 45), our results extend evidence for these ecological classifications to the direct or indirect bacterial responses to nutrient additions.

**Genomic and Metagenomic Evidence for Shifts in Bacterial Life History Strategy with Nutrient Additions.** We recognize that it is difficult to confidently assign bacterial clades into groups with copiotrophic and oligotrophic life history strategies, especially given the overwhelming amount of undescribed bacterial diversity found in soil (25). Thus, we used a combination of genomic and metagenomic approaches to provide independent assessments of how copiotroph:oligotroph ratios shifted in response to added nutrients. First, we estimated aggregate community growth rates because we expected increases in the relative abundance of copiotrophic taxa to be reflected by faster growth rates (28, 46). Thus, an increase in the estimated growth rate [i.e., a decrease in mean minimum generation time (MGT)] would suggest an increase in the relative abundance of copiotrophs. Mean MGTs were calculated for all samples from a combination of our bacterial marker gene data and published genomes; 757 of the 46,534 phylotypes could be matched to genomes. As with other attributes of community structure, estimates of MGT strongly varied across sites (Fig. S3*A*). Within-site differences between nutrient-amended and control samples showed that adding nutrients tended to decrease MGTs (Fig. S3*B*), but this trend was not significant for N additions ($P = 0.57$) or P additions ($P = 0.34$) individually. However, this analysis has important limitations in that only a small proportion (~10%) of the 16S rRNA gene sequences from our samples could be mapped to genomes for which we had MGT estimates, and this proportion differed across nutrient treatments (Fig. S3*C*). Thus, this analysis likely provides a conservative estimate of potential differences in MGTs associated with nutrient additions and is only weakly supportive of the hypothesis that soil bacterial MGT decreases with nutrient additions.

To further confirm the putative shifts in life history strategies in bacterial communities, we assessed functional attributes directly from functional gene (i.e., shotgun metagenomic) data collected from six of the sites used in the taxonomic analyses (Tables S2 and S3). These sites were selected because they spanned a wide geographic range and encapsulated a variety of environmental conditions, and the marker gene analyses suggested the N and P effects on microbial community composition were particularly strong. The shotgun metagenomic data (hereafter referred to as metagenomic data) were found to be almost entirely derived from bacterial genomes—94.8 ± 2.3% (mean ± SD) of the metagenomic small subunit rRNA gene reads were identified as bacterial. Just as the marker gene data revealed that bacterial diversity and community composition differed strongly across sites, the metagenomic data revealed that functional gene diversity and composition also varied strongly across sites (Fig. S1). In addition, the diversity of annotated genes identified from the metagenomic data were significantly correlated with the diversity of bacterial phylotypes across the samples ($r^2 = 0.27$, $P < 0.001$; Fig. S4*A*), and the dissimilarity in functional gene composition was strongly related to the dissimilarity in bacterial community composition across the six sites ($\rho = 0.87$, $P < 0.001$; Fig. S4*B*). These findings suggest that bacterial communities that are distinct in composition tend to have distinct functional attributes, and bacterial communities that are taxonomically more diverse also have more diverse metagenomes with a broader array of annotated genes. Correspondingly, the diversity of functional genes did not change with nutrient additions ($P > 0.1$), but there were significant shifts in overall functional gene composition with N additions ($P = 0.01$) and P additions ($P = 0.006$; Fig. 1), as was observed for bacterial taxa. These results are supported by previous work showing a relationship between the taxonomic structure of soil bacteria and functional genes across ecosystems (41) and significant N effects on functional gene composition at two North American sites (27).

The metagenomic data yielded additional lines of evidence to support our hypothesis that nutrient additions favor copiotrophic bacterial taxa. Previous work has suggested that soil microorganisms with larger genomes should be more successful in resource-poor environments (47), and, thus, we expect copiotrophic taxa to have smaller genomes. To assess this hypothesis, we calculated mean effective genome size—the estimated mean size of a genome in a given sample—and found that it significantly decreased with added N or P ($P < 0.03$ in both cases; Fig. 4*A*). More generally, this result highlights that genome size can be considered an important ecological trait, just as bacterial genome size is correlated with range size (48) and plant genome size is an important predictor of species' ability to invade (49).

We investigated the specific gene categories that changed in proportion with nutrient additions by analyzing the quality-filtered metagenomic sequences that could be annotated. First, it is important to note that only 28.7–32.7% of sequences could be annotated, and soils receiving N or P had a 0.3% higher annotation

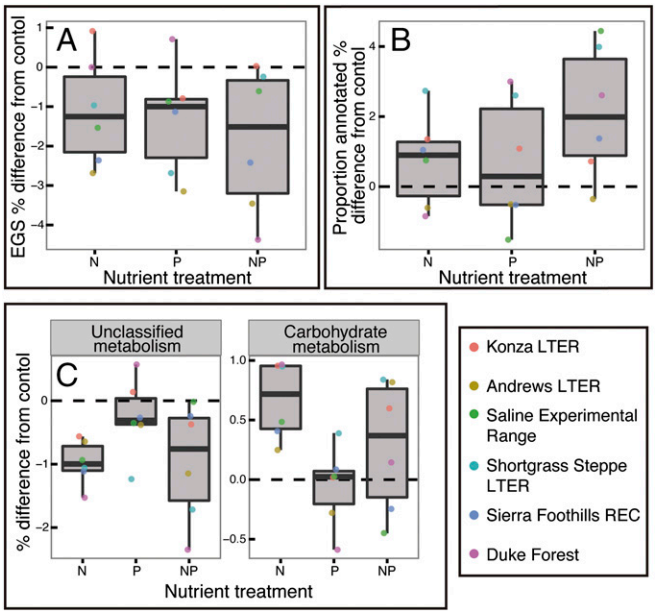


**Fig. 4.** Shifts in metagenomic characteristics with the addition of nutrients. Differences in the proportion of annotated genes (*A*), effective genome size (*B*), and the relative abundance of metabolic genes (*C*) are shown with boxplots and mean responses for each site (points). Gene categories in *C* were chosen by selecting those that most greatly differed between control and treatment plots ($P < 0.02$ for each; Table S6).

rate on average ($P \leq 0.01$ in both cases; Fig. 4*B*), a pattern likely driven by the overrepresentation of copiotrophic bacteria, which are easier to culture, and are thus more commonly found in genome databases. Similarly, soils receiving N amendments tended to have a lower relative abundance of annotated, but unclassified, metabolic genes compared with control samples, likely also reflecting the better representation of copiotrophs in genome databases (Fig. 4*C* and Table S6). We also observed a significant increase in the relative abundances of genes associated with carbohydrate metabolism (Fig. 4*C*) in fertilized plots. This finding is consistent with the added nutrients increasing copiotroph:oligotroph ratios and potentially increasing plant C inputs to soil. Although <33% of the sequence reads could be annotated—a percentage that is similar to that reported in other metagenomic analyses of diverse bacterial communities, e.g., ref. 27—our results highlight that the annotated reads can be used to infer shifts in the functional capabilities of communities, shifts that are consistent with nutrient additions increasing the proportional abundance of bacteria with copiotrophic life history strategies.

Nutrients can have both direct and indirect effects on belowground bacterial communities, making it difficult to unravel the mechanisms underlying the community responses described above. Potential mechanisms include direct effects of the nutrients themselves, nutrient effects on soil characteristics (e.g., pH), nutrient inputs increasing plant productivity and organic matter inputs to soils (20), and nutrient inputs mediating microbial shifts through changes in plant community composition. With N addition, soil pH decreased by an average of 0.16 units across the sites ($P < 0.001$), and pH has been shown to strongly drive shifts in soil bacterial communities (42, 50, 51). However, pH alone is not likely to have been a major driver of community shifts observed here, because the pH change was relatively small, it did not change with P additions ($P = 0.36$), and the magnitude of change in pH was unrelated to the change in the relative abundance of any of the major bacterial taxa with N and P additions across the sites (Table S7). Proportional changes in plant productivity were also unrelated to changes in the relative abundance of bacterial taxa, suggesting that elevated plant productivity in fertilized plots was not responsible for the bacterial community responses. Conversely, the magnitude of shifts in plant community composition was directly related to the magnitude of shifts in bacterial community composition ($r = 0.41$, $P = 0.04$; Fig. 3), a pattern that mirrored that observed for fungi (Fig. 3). These findings suggest that changes in plant community composition may be more important for mediating bacterial community responses to elevated nutrient inputs than changes in edaphic characteristics or plant growth.

## Conclusions

Together, our results demonstrate that, although microbial community composition varied considerably across the diverse grassland sites examined, nutrient availability elicits changes to the composition of microbial communities in consistent ways across sites by selecting for microbial groups that have certain functional traits. Understanding the responses of soil microbial communities to changes in nutrient availability is critical, given that ecosystems across the globe are receiving increasing inputs of N and P. Our analyses represent one of the first attempts to empirically assess whether there are generalizable patterns in these responses across a wide range of climatic and edaphic environments and confirm their existence, despite large cross-site differences in microbial community structure. The observed patterns correspond to broader ecological theory and set the stage for more targeted hypothesis testing. For example, nutrient-induced shifts in copiotrophic vs. oligotrophic traits could have important implications for soil C cycling (52) if their traits elicit effects rather than solely reflect responses (53). Likewise, decreases in mycorrhizae and methanogens could have important impacts on ecosystem-level processes (39, 54). This work moves us toward a more mechanistic understanding of how shifts in microbial community composition mediate and reflect the effects of anthropogenically elevated nutrient inputs on terrestrial ecosystems.

## Materials and Methods

Complete documentation of the experimental design, sample collection, and analytical methods are provided in *SI Materials and Methods*.

Identical full factorial N and P addition experiments were established at each of the 25 sites used in this study, which included temperate-zone grasslands in Africa, Australia, Europe, and North America (Tables S2 and S3). Nutrients were added annually in 10 g of N or P per $m^2 \cdot y^{-1}$. Plant communities and soil characteristics were assessed as in ref. 30. Fungal, archaeal, and bacterial community structure were characterized by using barcoded Illumina sequencing of the internal transcribed spacer region of the ribosomal operon and the 16S rRNA gene for fungi and bacteria, respectively, using a described approach (30). These raw sequence data are available in the Sequence Read Archive at the National Center for Biotechnology Information (accession no. SRP052716). The shotgun metagenomic sequences were collected and processed by using an approach similar to ref. 55, with annotation performed using the Kyoto Encyclopedia of Genes and Genomes hierarchy (56). These data are available at the Integrated Microbial Genomes and Metagenomes website (img.jgi.doe.gov) and referenced in the Genomes Online Database (GOLD Study ID Gs0053063). We estimated MGTs for bacterial communities by calculating MGTs in available whole-bacterial genomes by using the method described in ref. 57 and mapping the 16S rRNA sequences we collected to these genomes.

**ACKNOWLEDGMENTS.** We thank Monte Lunacek (University of Colorado Research Computing) for valuable computational support; Elizabeth DeLorenze and Ryan Williams for feedback on earlier drafts of this manuscript; and Jessica Henley and Xavier Rojas for help with the sample processing. The shotgun metagenomic analyses were supported by the Department of Energy Joint Genome Institute and their Community Sequencing Program (CSP-672). This work was supported by National Science Foundation (NSF) Grant DEB0953331 (to N.F.). The Nutrient Network (nutnet.org) experiment is funded at the site scale by individual researchers. Coordination and data management are supported by funding to E.T.B. and E.W.S. from NSF Research Coordination Network Grant NSF-DEB-1042132 and Long Term Ecological Research (LTER) Grant NSF-DEB-1234162 (to Cedar Creek LTER) programs and the University of Minnesota Institute on the Environment (DG-0001-13). This work used the Janus supercomputer, which is supported by NSF Award CNS-0821794 and the University of Colorado Boulder.

1. Galloway JN, et al. (2004) Nitrogen cycles: Past, present, and future. *Biogeochemistry* 70:153–226.
2. Wang R, et al. (2015) Significant contribution of combustion-related emissions to the atmospheric phosphorus budget. *Nat Geosci* 8:48–54.
3. Foley JA, et al. (2011) Solutions for a cultivated planet. *Nature* 478(7369):337–342.
4. Clark CM, Tilman D (2008) Loss of plant species after chronic low-level nitrogen deposition to prairie grasslands. *Nature* 451(7179):712–715.
5. Craine JM, Morrow C, Stock WD (2008) Nutrient concentration ratios and co-limitation in South African grasslands. *New Phytol* 179(3):829–836.
6. LeBauer DS, Treseder KK (2008) Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology* 89(2):371–379.
7. Suding KN, et al. (2005) Functional- and abundance-based mechanisms explain diversity loss due to N fertilization. *Proc Natl Acad Sci USA* 102(12):4387–4392.
8. Borer ET, et al. (2014) Herbivores and nutrients control grassland plant diversity via light limitation. *Nature* 508(7497):517–520.
9. Tilman D, Wedin D (1991) Plant traits and resource reduction for five grasses growing on a nitrogen gradient. *Ecology* 72:685–700.
10. Grime JP (1977) Evidence for the existence of three primary strategies in plants and its relevance to ecological and evolutionary theory. *Am Nat* 111(982):1169–1194.
11. Fierer N, Strickland MS, Liptzin D, Bradford MA, Cleveland CC (2009) Global patterns in belowground communities. *Ecol Lett* 12(11):1238–1249.
12. van der Heijden MGA, Bardgett RD, van Straalen NM (2008) The unseen majority: Soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecol Lett* 11(3):296–310.
13. Ramirez KS, Craine JM, Fierer N (2012) Consistent effects of nitrogen amendments on soil microbial communities and processes across biomes. *Glob Change Biol* 18: 1918–1927.
14. Janssens IA, et al. (2010) Reduction of forest soil respiration in response to nitrogen deposition. *Nat Geosci* 3:315–322.
15. Treseder KK (2008) Nitrogen additions and microbial biomass: A meta-analysis of ecosystem studies. *Ecol Lett* 11(10):1111–1120.
16. Wessén E, Nyberg K, Jansson JK, Hallin S (2010) Responses of bacterial and archaeal ammonia oxidizers to soil organic and fertilizer amendments under long-term management. *Appl Soil Ecol* 45:193–200.
17. Egerton-Warburton L, Johnson N, Allen E (2007) Mycorrhizal community dynamics following nitrogen fertilization: A cross-site test in five grasslands. *Ecol Monogr* 77: 527–544.
18. Treseder K (2004) A meta-analysis of mycorrhizal responses to nitrogen, phosphorus, and atmospheric CO2 in field studies. *New Phytol* 164:347–355.
19. Campbell BJ, Polson SW, Hanson TE, Mack MC, Schuur EAG (2010) The effect of nutrient deposition on bacterial communities in Arctic tundra soil. *Environ Microbiol* 12(7):1842–1854.
20. Ramirez KS, Lauber CL, Knight R, Bradford MA, Fierer N (2010) Consistent effects of nitrogen fertilization on soil bacterial communities in contrasting systems. *Ecology* 91(12):3463–3470, discussion 3503–3514.
21. Allison SD, Hanson CA, Treseder KK (2007) Nitrogen fertilization reduces diversity and alters community structure of active fungi in boreal ecosystems. *Soil Biol Biochem* 39: 1878–1887.
22. Coolon JD, Jones KL, Todd TC, Blair JM, Herman MA (2013) Long-term nitrogen amendment alters the diversity and assemblage of soil bacterial communities in tallgrass prairie. *PLoS One* 8(6):e67884.
23. Peñuelas J, et al. (2013) Human-induced nitrogen-phosphorus imbalances alter natural and managed ecosystems across the globe. *Nat Commun* 4:2934.
24. Pan Y, et al. (2014) Impact of long-term N, P, K, and NPK fertilization on the composition and potential functions of the bacterial community in grassland soil. *FEMS Microbiol Ecol* 90(1):195–205.
25. Ramirez KS, et al. (2014) Biogeographic patterns in below-ground diversity in New York City's Central Park are similar to those observed globally. *Proc Biol Sci* 281(1795): 20141988.
26. Fierer N, Barberán A, Laughlin DC (2014) Seeing the forest for the genes: Using metagenomics to infer the aggregated traits of microbial communities. *Front Microbiol* 5:614.
27. Fierer N, et al. (2012) Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. *ISME J* 6(5): 1007–1017.
28. Fierer N, Bradford MA, Jackson RB (2007) Toward an ecological classification of soil bacteria. *Ecology* 88(6):1354–1364.
29. Leininger S, et al. (2006) Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature* 442(7104):806–809.
30. Prober SM, et al. (2015) Plant diversity predicts beta but not alpha diversity of soil microbes across grasslands worldwide. *Ecol Lett* 18(1):85–95.
31. Tedersoo L, et al. (2014) Fungal biogeography. Global diversity and geography of soil fungi. *Science* 346(6213):1256688.
32. Redecker D, Raab P (2006) Phylogeny of the glomeromycota (arbuscular mycorrhizal fungi): Recent developments and new gene markers. *Mycologia* 98(6):885–895.
33. van Diepen LT, Lilleskov EA, Pregitzer KS, Miller RM (2007) Decline of arbuscular mycorrhizal fungi in northern hardwood forests exposed to chronic nitrogen additions. *New Phytol* 176(1):175–183.
34. Wei C, et al. (2013) Nitrogen deposition weakens plant-microbe interactions in grassland ecosystems. *Glob Change Biol* 19(12):3688–3697.
35. Johnson NC, Wilson GWT, Bowker MA, Wilson JA, Miller RM (2010) Resource limitation is a driver of local adaptation in mycorrhizal symbioses. *Proc Natl Acad Sci USA* 107(5):2093–2098.
36. Bates ST, et al. (2011) Examining the global distribution of dominant archaeal populations in soil. *ISME J* 5(5):908–917.
37. Gubry-Rangin C, et al. (2011) Niche specialization of terrestrial archaeal ammonia oxidizers. *Proc Natl Acad Sci USA* 108(52):21206–21211.
38. Leigh JA (2000) Nitrogen fixation in methanogens: The archaeal perspective. *Curr Issues Mol Biol* 2(4):125–131.
39. Offre P, Spang A, Schleper C (2013) Archaea in biogeochemical cycles. *Annu Rev Microbiol* 67:437–457.
40. Klüber HD, Conrad R (1998) Inhibitory effects of nitrate, nitrite, NO and N2O on methanogenesis by Methanosarcina barkeri and Methanobacterium bryantii. *FEMS Microbiol Ecol* 25:331–339.
41. Fierer N, et al. (2012) Cross-biome metagenomic analyses of soil microbial communities and their functional attributes. *Proc Natl Acad Sci USA* 109(52):21390–21395.
42. Lauber CL, Hamady M, Knight R, Fierer N (2009) Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Appl Environ Microbiol* 75(15):5111–5120.
43. Koyama A, Wallenstein MD, Simpson RT, Moore JC (2014) Soil bacterial community composition altered by increased nutrient availability in Arctic tundra soils. *Front Microbiol* 5:516.
44. Bastian F, Bouziri L, Nicolardot B, Ranjard L (2009) Impact of wheat straw decomposition on successional patterns of soil microbial community structure. *Soil Biol Biochem* 41:262–275.
45. Eilers KG, Lauber CL, Knight R, Fierer N (2010) Shifts in bacterial community structure associated with inputs of low molecular weight carbon compounds to soil. *Soil Biol Biochem* 42:896–903.
46. Pianka E (1970) On r-and K-selection. *Am Nat* 104:592–597.
47. Konstantinidis KT, Tiedje JM (2004) Trends between gene content and genome size in prokaryotic species with larger genomes. *Proc Natl Acad Sci USA* 101(9):3160–3165.
48. Barberán A, et al. (2014) Why are some microbes more ubiquitous than others? Predicting the habitat breadth of soil bacteria. *Ecol Lett* 17(7):794–802.
49. Suda J, Meyerson LA, Leitch IJ, Py P (2015) The hidden side of plant invasions: The role of genome size. *New Phytol* 205(3):994–1007.
50. Fierer N, Jackson RB (2006) The diversity and biogeography of soil bacterial communities. *Proc Natl Acad Sci USA* 103(3):626–631.
51. Rousk J, Brookes PC, Glanville HC, Jones DL (2011) Lack of correlation between turnover of low-molecular-weight dissolved organic carbon and differences in microbial community composition or growth across a soil pH gradient. *Appl Environ Microbiol* 77(8):2791–2795.
52. Wieder WR, Bonan GB, Allison SD (2013) Global soil carbon projections are improved by modelling microbial processes. *Nat Clim Chang* 3:909–912.
53. Lavorel S, Garnier E (2002) Predicting changes in community composition and ecosystem functioning from plant traits: Revisiting the Holy Grail. *Funct Ecol* 16:545–556.
54. Van der Heijden MGA, et al. (1998) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature* 396:69–72.
55. Fierer N, et al. (2013) Reconstructing the microbial diversity and function of pre-agricultural tallgrass prairie soils in the United States. *Science* 342(6158):621–624.
56. Kanehisa M, Goto S, Sato Y, Furumichi M, Tanabe M (2012) KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res* 40(Database issue): D109–D114.
57. Vieira-Silva S, Rocha EPC (2010) The systemic imprint of growth and its uses in ecological (meta)genomics. *PLoS Genet* 6(1):e1000808.
58. Edgar RC (2013) UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* 10(10):996–998.
59. Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26(19):2460–2461.
60. McDonald D, et al. (2012) An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J* 6(3):610–618.
61. Abarenkov K, et al. (2010) The UNITE database for molecular identification of fungi–recent updates and future perspectives. *New Phytol* 186(2):281–285.
62. Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 73(16):5261–5267.
63. Magoč T, Salzberg SL (2011) FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27(21):2957–2963.
64. Schmieder R, Edwards R (2011) Quality control and preprocessing of metagenomic datasets. *Bioinformatics* 27(6):863–864.
65. Markowitz VM, et al. (2012) IMG: The Integrated Microbial Genomes database and comparative analysis system. *Nucleic Acids Res* 40(Database issue):D115–D122.
66. Kent WJ (2002) BLAT–the BLAST-like alignment tool. *Genome Res* 12(4):656–664.
67. Quast C, et al. (2013) The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res* 41(Database issue):D590–D596.
68. Haegeman B, et al. (2013) Robust estimation of microbial diversity in theory and in practice. *ISME J* 7(6):1092–1101.
69. R Core Team (2013) R: A language and environment for statistical computing (R Foundation for Statistical Computing, Vienna).

# Supporting Information

## Leff et al. 10.1073/pnas.1508382112

### SI Materials and Methods

**Site Characteristics and Experimental Design.** All 25 sites used in this study (Tables S2 and S3) were from temperate-zone grasslands in Africa, Australia, Europe, and North America, and all were part of the Nutrient Network experiment (8) and are described in ref. 30. In brief, the sites ranged in many environmental characteristics, including mean annual precipitation (262–1,898 $mm \cdot y^{-1}$), mean annual temperature (0–18 °C), elevation (50–2,320 m), soil pH (4.5–8.4), total soil P (1–253 ppm), soil %N (0.03–1.5%), and aboveground plant productivity (15–1482 $g^{-2} \cdot y^{-1}$).

Identical full factorial N and P addition experiments were established at each of the 25 sites (8). We used samples from three +N, three +P, and three +N/+P 1-$m^2$ plots, and six 1-$m^2$ plots with no added nutrients. Nutrients were added annually as 10 g of N per $m^2 \cdot yr^{-1}$ timed-release urea [$(NH_2)_2CO$] and 10 g of P per $m^2 \cdot yr^{-1}$ triple-super phosphate [$Ca(H_2PO_4)_2$]. Samples were treated between 2 and 4 y before collection (Table S2), but this timing did not significantly relate to the proportional changes in the major microbial taxon relative abundances ($P > 0.5$ in all cases). Fences to exclude herbivores surrounded three of the plots at each site (8), but because fencing had no effect on belowground microbial communities ($P > 0.1$), we included these plots as controls.

Plant community composition and biomass were assessed and soil samples were collected during the growing season of 2011 or 2012. Plant species were identified within the plots, and plant biomass and soil cores were sampled directly adjacent to the plots to determine soil characteristics and assess microbial community structure (8). Soil cores were shipped on ice to a central processing facility (Corvallis, OR) immediately after collection, and samples for microbial community analysis were preserved at −20 °C. Analyses to determine soil pH and C, N, and P content are described in ref. 30.

**Assessment of Microbial Community Composition and Diversity.** Microbial community diversity and composition were assessed by using targeted marker gene surveys focusing on the 16S rRNA gene for the Bacteria and Archaea domains and the internal transcribed spacer (ITS) region for Fungi as in Prober et al. (30). DNA was extracted by inserting a sterile swab into each soil sample and cutting the swab tip off into a well in a bead plate of the PowerSoil-htp 96-well DNA extraction kit (Mo Bio Laboratories, Inc.). The downstream extraction procedure was followed according to the manufacturer's instructions, and two wells on each extraction plate were left empty to serve as blanks. Marker genes in isolated DNA were PCR-amplified and barcoded in triplicate reactions for both the 16S rRNA gene (using the 515f/806r primer pair) and the ITS1 region (using the ITS1-F/ITS2 primer pair). PCR products from the triplicate reactions were combined and visualized on an agarose gel to ensure successful amplification and to verify no amplification from blanks. PCR product from samples that were successfully amplified was combined in equimolar ratios for each of the marker genes. This procedure was conducted separately for two sets of samples, one including samples from 19 of the sites and the other including the samples from the remaining 6 sites. For the first set, 16S rRNA and ITS amplicon pools were each sequenced on separate Illumina HiSeq 2000 lanes by using 100-bp paired-end sequencing, and for the second set, each amplicon type was sequenced on a separate Illumina MiSeq run by using 151-bp paired-end sequencing. All sequencing was conducted at the University of Colorado at Boulder.

Raw sequences from 16S rRNA and ITS amplicons were processed by using the UPARSE pipeline (58). Sequences were demultiplexed according to the raw sequenced barcodes, and because there were generally many more sequences for samples sequenced on the HiSeq run, a random subset of the sequences from each of these samples was included in further processing such that the number of sequences per sample was generally within two orders of magnitude across samples. Sequences from both sequencing runs were combined after trimming to a uniform length of 100 bp. A de novo database of ≥97% similar sequence clusters was created in USEARCH (Version 7; ref. 59) by (*i*) quality filtering sequences using a "maxee" value of 0.5 (i.e., sequences with a predicted error rate of 0.5 bases per sequence were discarded), (*ii*) dereplicating identical sequences, (*iii*) removing singleton sequences, (*iv*) clustering those sequences, and (*v*) filtering poor-quality sequences by removing sequences that were not ≥75% similar to any sequence in Greengenes (Version 13_5; ref. 60) or UNITE (Version 12_11; ref. 61) databases for 16S rRNA and ITS1 sequences, respectively. Raw demultiplexed sequences were then mapped against these de novo databases to generate counts of sequences matching clusters (i.e., phylotypes) for each sample. Taxonomy was assigned to each phylotype by using the RDP classifier with a confidence threshold of 0.5 (62) and trained on the databases indicated above. Among 16S rRNA sequences, phylotypes classified as bacteria were separated from those classified as archaea before further processing. Mycorrhizal fungi phylotypes were identified by comparing to known mycorrhizal ITS sequences, as detailed in ref. 30. To normalize the sequencing depth across samples, samples were rarefied to 18,000 bacterial, 100 archaeal, and 485 fungal sequences per sample. Because of insufficient sequence coverage, bacterial data from 4 samples, archaeal data from 90 samples, and fungal data from 29 samples were discarded. One sample ("NN10") was removed from downstream analyses due to a large disparity in diversity and community composition from all other sequences. The raw sequence data are available in the Sequence Read Archive at the National Center for Biotechnology Information (BioProject accession no. PRJNA272747).

**Shotgun Metagenomic Analyses.** All replicate samples from a subset of six of the sites (90 samples; Fig. S1) used for the compositional analyses were prepared and submitted for shotgun metagenomic sequencing at the Joint Genome Institute. The sites were selected because they represented a broad range in environmental characteristics and exhibited the largest bacterial community composition responses to N and P. For this analysis, DNA was extracted by using the same method as above, except that ∼0.25 g of each sample was loaded into the first extraction plate. Library preparation and sequencing were conducted at the Department of Energy Joint Genome Institute (Walnut Creek, CA) using their standard protocols. Briefly, 270-bp DNA fragments were prepared and sequenced on a HiSeq instrument using 150-bp paired-end sequencing. Sequencing efforts yielded a total of 850 million read pairs across all samples (range = 428,000 to 65 million). Raw sequence data can be accessed at IMG (img.jgi.doe.gov) and are referenced in the Genomes Online Database (GOLD Study ID Gs0053063).

Sequence processing was conducted in a method similar to Fierer et al. (55). Paired-end sequences were first merged by using FLASH (Version 1.2.6; ref. 63) with the parameters, "-r 150 –f 210 –s 21." Merged sequences were quality-filtered by using PRINSEQ-lite (Version 0.20.3; ref. 64) to remove sequences containing more than five undefined bases and exact duplicates. On average, 49% (range = 23–62%) of sequences were successfully merged and passed quality filtering from those samples that were successfully sequenced. Sequences from samples with high sequence coverage

were then randomly subsampled to reduce computational processing time. The merged and quality-filtered sequences were annotated by mapping them against the IMG (Version 350) amino acid sequences (65) using BLAT (66). Sequences that could be mapped within a minimum percentage identity of 0.55 and an e-value cutoff of 1e-03 were provided Kyoto Encyclopedia of Genes and Genomes Orthology (KO) identifiers. Of the quality-filtered sequences, 28.7–32.7% could be annotated with specific gene category information, a similar percentage to that noted in previous studies exploring soil metagenomes (27), but larger than studies using comparatively short sequences (41). Only sequences that were annotated were considered for further analysis, and samples were rarefied to an equal sequence depth of 500,000 annotated sequences per sample. This process resulted in a total of 75 shotgun metagenomic samples spread across treatments and sites. When KO identifiers were categorized into coarser functional categories (56), those that belonged to multiple categories were counted in each of those categories. Functional diversity and composition calculations were performed identically to those used for taxonomic analyses, except that KO identifiers were used instead of phylotypes.

To determine what proportion of the shotgun data represented bacterial genes, we compared merged and quality-filtered reads to the SILVA 111 database (67), which contains representative small subunit rRNA sequences from all three domains of life. Shotgun reads were mapped to this database at the 94% similarity level by using UCLUST (59). We used a low similarity threshold to account for the diverse soil taxa that are not represented in the database.

**MGT Analysis.** To estimate mean MGTs (i.e., community-aggregated growth rates) from 16S rRNA sequence data, MGTs were estimated from published genomes and linked to bacterial community composition by matching 16S rRNA gene sequences. Fasta files of all ORFs from 1,136 genomes were obtained from the Joint Genome Institute's IMG database. These genomes were selected to only include non-host-associated organisms based upon information available in the GOLD. Both IMG and GOLD were accessed in September 2012. MGT was estimated for each genome based upon the methods described by ref. 57 and python code provided by the authors. Briefly, two measures of codon use bias (ENC′ and S) were combined via regression to generate a predictive model of MGT. MGTs were linked to phylotypes observed through 16S rRNA data by matching the 16S rRNA sequences in our dataset to the 16S rRNA sequences from the genomes at a 97% similarity threshold using the "usearch_global" function in USEARCH (59), with "maxaccepts" and "maxrejects" set to 0 to disable approximate clustering. Mean MGT was calculated on a per-sample basis by calculating proportional abundances of the phylotypes that matched represented genomes, excluding all other phylotypes, and multiplying its estimated MGT by its proportional abundance. The mean MGT was the sum of these values.

**Statistical Analysis.** Microbial diversity was calculated by using Shannon diversity because this method has been found to be a more reliable metric than species richness with microbial sequence data from complex communities (68). However, richness values are provided for interpretation and comparative purposes. Kruskal–Wallis tests were used to test whether there were significant differences in diversity among sites. Linear mixed effect models were used to test for differences in diversity with nutrient additions (+N, +P, +NP) by using Genstat (Version 14.0). We excluded the +NP term if not significant and treated site as a random variable.

We tested for significant shifts in overall community composition or functional gene composition across sites or with nutrient additions using permutational multivariate ANOVA (PERMANOVA) implemented in the "Adonis" function in the vegan package in R (Version 3.0.2; ref. 69). Community or genetic composition was represented by Bray–Curtis dissimilarity matrices computed from square-root-transformed abundance tables. N and P addition were used as predictor variables, and the geographic site was included as "strata," which restricts permutations to within sites. N × P interactions were found to be nonsignificant in all cases and therefore removed from final PERMANOVA models. When visualizing potential treatment differences, we used constrained ordination as implemented in the "capscale" function in the vegan package in R. To test for significant differences in the relative abundance of specific taxa, gene categories, and MGTs between control and nutrient treatment plots, we used linear mixed-effects models in which each nutrient treatment (N and P additions) was a fixed factor and site was a random effect. Relative abundances were rank-transformed to meet the assumptions of the model. Tests were run for each taxon represented by a median of at least 1% of the sequences in any of the nutrient treatments, and false discovery rate (FDR) corrections were used to account for the multiple comparisons.

Relationships between pretreatment environmental variables and changes in the relative abundance of taxa were assessed by using Pearson correlations with mean difference from control plots (on a per-site basis) as the response variable. We chose environmental variables to test based on their relationships with one another and avoided including multiple variables that were highly correlated with one another ($r^2 > 0.8$). Differences in posttreatment soil pH were investigated by using a linear mixed-effects model with N and P additions as fixed effects and site as a random effect. This analysis was conducted by using the "nlme" package in R.

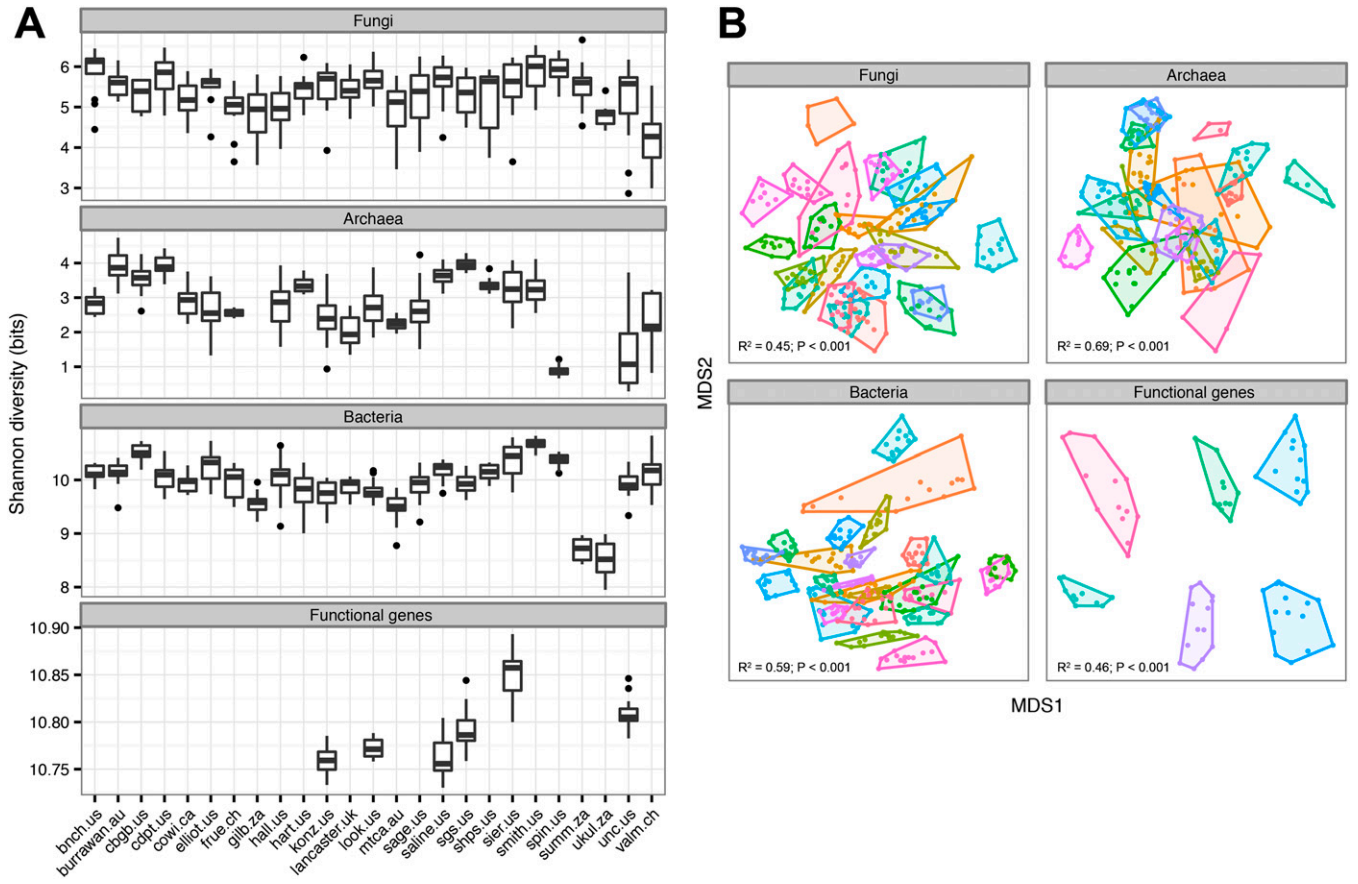


**Fig. S1.** Microbial community structure and functional gene structure across sites. Boxplots show Shannon diversity (*A*) and nonmetric multidimensional scaling ordinations showing compositional differences (*B*) across sites for the three microbial taxonomic groups and the functional genes. Points in *B* represent individual samples and are colored by site. PERMANOVA statistics refer to site effects.

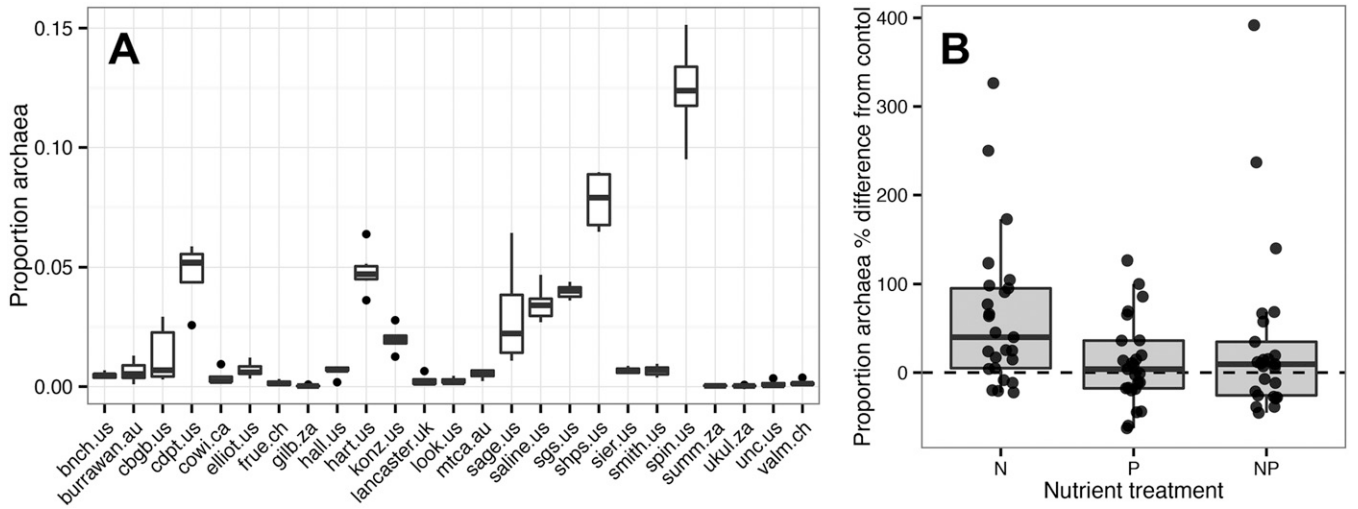


**Fig. S2.** The proportion of 16S rRNA gene sequences identified as archaeal (vs. bacterial) across sites (*A*) and changes in this proportion with nutrient additions within sites (*B*). Proportions were calculated after rarefying the full 16S rRNA dataset (including bacterial and archaeal sequences) to 18,000 sequences per sample.

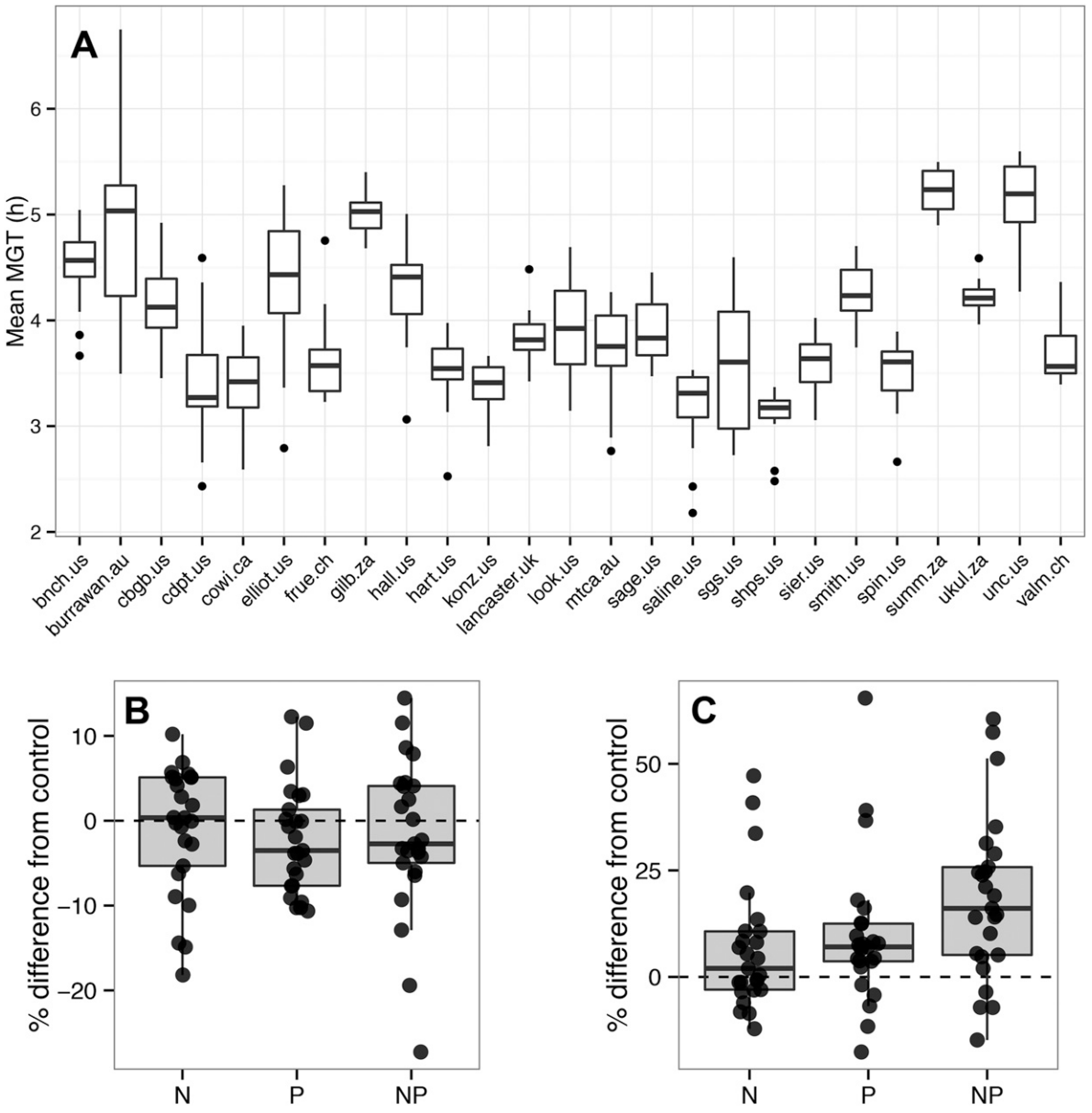


**Fig. S3.** MGTs estimated by matching 16S rRNA gene sequences to whole genomes. (*A* and *B*) Variability across sites (*A*) and differences from control plots vs. plots receiving nutrient additions within sites (*B*). (*C*) Differences in the proportion of sequences matching whole genomes with nutrient additions within sites.

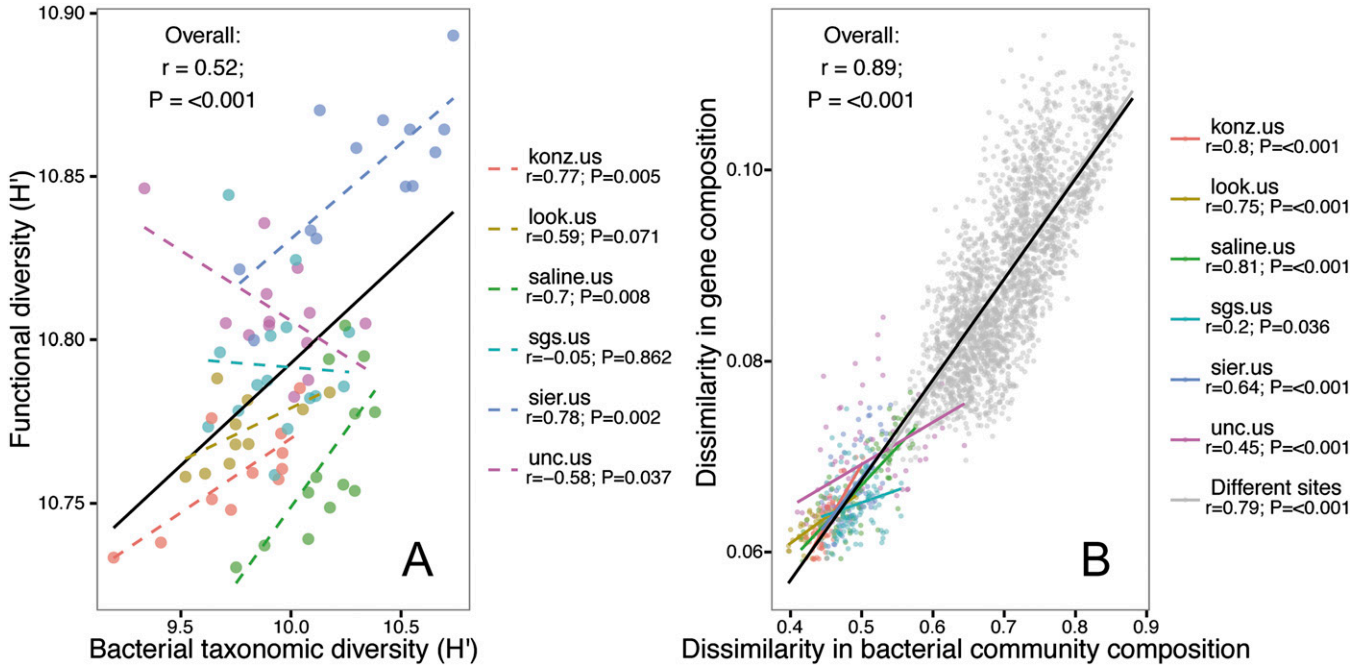


**Fig. S4.** Relationships between functional gene diversity and bacterial taxonomic diversity (*A*) and between pairwise differences in functional gene composition and pairwise differences in bacterial community composition across all samples (*B*). Colored lines show within-site relationships, and black lines show cross-site relationships.

**Table S1. Summary statistics for phylotype richness and the relative abundances of major phyla (mean ≥ 2%) from the three microbial groups**

| Statistic | Mean | SD | Min | Max |
|---|---|---|---|---|
| Fungal richness | 105 | 14 | 78 | 134 |
| Archaeal richness | 17 | 5 | 7 | 26 |
| Bacterial richness | 3,533 | 442 | 2,560 | 4,410 |
| Relative abundances | | | | |
| Fungal phyla | | | | |
| Ascomycota | 0.558 | 0.122 | 0.306 | 0.809 |
| Basidiomycota | 0.134 | 0.054 | 0.037 | 0.262 |
| Glomeromycota | 0.034 | 0.024 | 0.002 | 0.072 |
| Zygomycota | 0.169 | 0.124 | 0.010 | 0.465 |
| Unclassified fungi | 0.097 | 0.044 | 0.039 | 0.245 |
| Archaeal phyla | | | | |
| Crenarchaeota | 0.922 | 0.066 | 0.769 | 0.994 |
| Euryarchaeota | 0.043 | 0.047 | 0.002 | 0.181 |
| [Parvarchaeota] | 0.033 | 0.035 | 0.000 | 0.121 |
| Bacterial phyla | | | | |
| Acidobacteria | 0.203 | 0.059 | 0.130 | 0.379 |
| Actinobacteria | 0.112 | 0.044 | 0.058 | 0.227 |
| Bacteroidetes | 0.105 | 0.041 | 0.031 | 0.202 |
| Chloroflexi | 0.036 | 0.021 | 0.016 | 0.106 |
| Firmicutes | 0.033 | 0.019 | 0.003 | 0.070 |
| Planctomycetes | 0.043 | 0.012 | 0.023 | 0.074 |
| Proteobacteria | 0.205 | 0.041 | 0.114 | 0.274 |
| Verrucomicrobia | 0.182 | 0.092 | 0.036 | 0.433 |

Values were calculated from all samples, including those from nutrient-treated plots. Bacterial, archaeal, and fungal samples were rarefied to 18,000; 100; and 485 sequences, respectively.

**Table S2. Location of study sites and selected environmental data**

| Site code | Site name | Continent | Country | Ecosystem | Latitude | Longitude | Elev, m | Years treated | Used in metagenomic analysis |
|---|---|---|---|---|---|---|---|---|---|
| bnch.us | Bunchgrass (Andrews LTER) | N. America | USA | Montane grassland | 44.28 | −121.97 | 1,318 | 4 | |
| burrawan.au | Burrawan | Australasia | Australia | Semiarid grassland | −27.73 | 151.14 | 425 | 4 | |
| cbgb.us | Chichaqua Bottoms | N. America | USA | Tallgrass prairie | 41.79 | −93.39 | 275 | 2 | |
| cdpt.us | Cedar Point Biological Station | N. America | USA | Shortgrass prairie | 41.2 | −101.63 | 965 | 4 | |
| cowi.ca | Cowichan | N. America | Canada | Old field | 48.46 | −123.38 | 50 | 4 | |
| elliot.us | Elliott Chaparral | N. America | USA | Annual grassland | 32.88 | −117.05 | 200 | 3 | |
| frue.ch | Fruebuel | Europe | Switzerland | Pasture | 47.11 | 8.54 | 995 | 3 | |
| gilb.za | Mt Gilboa | Africa | South Africa | Montane grassland | −29.28 | 30.29 | 1,748 | 2 | |
| hall.us | Hall's Prairie | N. America | USA | Tallgrass prairie | 36.87 | −86.7 | 194 | 4 | |
| hart.us | Hart Mountain | N. America | USA | Shrub steppe | 42.72 | −119.5 | 1,508 | 4 | |
| konz.us | Konza LTER | N. America | USA | Tallgrass prairie | 39.07 | −96.58 | 440 | 4 | Y |
| lancaster.uk | Lancaster | Europe | United Kingdom | Mesic grassland | 53.99 | −2.63 | 180 | 3 | |
| look.us | Lookout (Andrews LTER) | N. America | USA | Montane grassland | 44.21 | −122.13 | 1,500 | 4 | Y |
| mtca.au | Mt. Caroline | Australasia | Australia | Savanna | −31.78 | 117.61 | 285 | 3 | |
| sage.us | Sagehen Creek UCNRS | N. America | USA | Montane grassland | 39.43 | −120.24 | 1,920 | 4 | |
| saline.us | Saline Experimental Range | N. America | USA | Mixedgrass prairie | 39.05 | −99.1 | 440 | 4 | Y |
| sgs.us | Shortgrass Steppe LTER | N. America | USA | Shortgrass prairie | 40.82 | −104.77 | 1,650 | 4 | Y |
| shps.us | Sheep Experimental Station | N. America | USA | Shrub steppe | 44.24 | −112.2 | 910 | 4 | |
| sier.us | Sierra Foothills REC | N. America | USA | Annual grassland | 39.24 | −121.28 | 197 | 4 | Y |
| smith.us | Smith Prairie | N. America | USA | Mesic grassland | 48.21 | −122.62 | 62 | 4 | |
| spin.us | Spindletop | N. America | USA | Pasture | 38.14 | −84.5 | 271 | 4 | |
| summ.za | Summerveld | Africa | South Africa | Mesic grassland | −29.81 | 30.72 | 679 | 2 | |
| ukul.za | Ukulinga | Africa | South Africa | Mesic grassland | −29.67 | 30.4 | 842 | 3 | |
| unc.us | Duke Forest | N. America | USA | Old field | 36.01 | −79.02 | 141 | 4 | Y |
| valm.ch | Val Mustair | Europe | Switzerland | Alpine grassland | 46.63 | 10.37 | 2,320 | 3 | |

**Table S3. Selected environmental data for study sites**

| Site code | Mean annual temp., °C | Mean annual precip., mm | Soil C, % | Soil N, % | Soil P, ppm | Soil pH | Sand, % | Silt, % | Clay, % |
|---|---|---|---|---|---|---|---|---|---|
| bnch.us | 5.5 | 1,647 | 8.3 | 0.6 | 13.1 | 5.6 | 70.4 | 26.5 | 2.9 |
| burrawan.au | 18.4 | 683 | 1.2 | 0.1 | 18.0 | 5.6 | 82.2 | 9.1 | 8.6 |
| cbgb.us | 9 | 855 | 0.6 | 0.1 | 63.0 | 6.1 | 88.7 | 6.4 | 4.8 |
| cdpt.us | 9.5 | 445 | 1.5 | 0.1 | 31.9 | 6.7 | 68.0 | 21.7 | 10.2 |
| cowi.ca | 9.8 | 764 | 5.3 | 0.4 | 41.1 | 5.6 | 31.5 | 40.2 | 28.3 |
| elliot.us | 17.2 | 331 | 2.0 | 0.1 | 16.3 | 5.7 | 54.2 | 25.6 | 20.1 |
| frue.ch | 6.5 | 1,355 | 3.8 | 0.4 | 69.3 | 5.5 | 38.2 | 41.0 | 20.6 |
| gilb.za | 13.1 | 926 | 20.4 | 1.2 | 17.9 | 5.1 | NA | NA | NA |
| hall.us | 13.6 | 1,282 | 1.4 | 0.1 | 33.2 | 5.2 | 25.1 | 59.3 | 15.5 |
| hart.us | 7.4 | 272 | 1.1 | 0.1 | 64.6 | 7.2 | 47.7 | 22.7 | 29.5 |
| konz.us | 11.9 | 877 | NA | NA | NA | NA | NA | NA | NA |
| lancaster.uk | 8 | 1,322 | 20.9 | 1.1 | 33.2 | 4.7 | 50.2 | 31.1 | 18.6 |
| look.us | 4.8 | 1,898 | 16.7 | 1.2 | 54.9 | 5.1 | 70.0 | 29.1 | 0.8 |
| mtca.au | 17.3 | 330 | 1.3 | 0.1 | 8.7 | 5.2 | 82.0 | 11.3 | 6.6 |
| sage.us | 5.7 | 882 | 8.9 | 0.7 | 35.5 | 6.1 | 44.8 | 30.9 | 24.1 |
| saline.us | 11.8 | 607 | NA | NA | NA | NA | NA | NA | NA |
| sgs.us | 8.4 | 365 | 0.8 | 0.1 | 65.9 | 6.1 | 73.0 | 15.1 | 11.8 |
| shps.us | 5.5 | 262 | 2.2 | 0.2 | 35.0 | 8.0 | 50.7 | 37.8 | 11.5 |
| sier.us | 15.6 | 935 | 2.1 | 0.2 | 14.7 | 6.0 | 38.7 | 42.4 | 18.8 |
| smith.us | 9.8 | 597 | 7.5 | 0.6 | 76.3 | 6.1 | 78.0 | 15.1 | 6.8 |
| spin.us | 12.5 | 1,140 | 2.7 | 0.3 | 233.4 | 6.4 | 29.3 | 49.8 | 20.8 |
| summ.za | 18.2 | 939 | 6.8 | 0.3 | 12.5 | 5.1 | NA | NA | NA |
| ukul.za | 18.1 | 880 | 5.0 | 0.3 | 9.3 | 5.8 | 18.3 | 37.0 | 44.6 |
| unc.us | 14.6 | 1,163 | 2.2 | 0.2 | 21.3 | 5.3 | 56.0 | 22.6 | 21.3 |
| valm.ch | 0.3 | 1,098 | 7.4 | 0.6 | 46.0 | 5.5 | 57.6 | 29.1 | 13.3 |

NA, not available.

**Table S4. Median relative abundances of higher-level taxa among the control and nutrient treatment samples**

| | *P* (FDR corrected) | Control, % | N, % | P, % | NP, % |
|---|---|---|---|---|---|
| Fungi | | | | | |
| Glomeromycota | 0.000 | 2.5 | 2.3 | 2.9 | 1.2 |
| Glomeraceae | 0.000 | 1.9 | 1.3 | 1.4 | 0.8 |
| Ascomycota | 0.027 | 54.2 | 58.1 | 56.7 | 61.2 |
| Nectriaceae | 0.001 | 4.3 | 6.4 | 5.2 | 6.8 |
| Mycosphaerellaceae | 0.011 | 0.4 | 0.4 | 0.4 | 0.6 |
| Plectosphaerellaceae | 0.084 | 0.6 | 1.0 | 1.2 | 0.7 |
| Pleosporaceae | 0.229 | 1.6 | 1.4 | 1.6 | 1.8 |
| Basidiomycota | 0.058 | 12.0 | 10.6 | 11.1 | 10.4 |
| Filobasidiaceae | 0.005 | 0.4 | 0.8 | 0.6 | 0.9 |
| Zygomycota | 0.910 | 12.0 | 10.9 | 13.6 | 11.4 |
| Mortierellaceae | 0.867 | 11.3 | 10.1 | 11.5 | 11.2 |
| Bacteria | | | | | |
| Alphaproteobacteria | 0.002 | 7.8 | 8.0 | 8.0 | 8.5 |
| Deltaproteobacteria | 0.002 | 4.3 | 3.8 | 4.3 | 3.8 |
| Gammaproteobacteria | 0.002 | 2.9 | 3.2 | 3.2 | 3.7 |
| Acidobacteria | 0.007 | 19.8 | 18.4 | 20.0 | 18.0 |
| Planctomycetes | 0.006 | 4.1 | 4.0 | 4.3 | 3.7 |
| Actinobacteria | 0.014 | 10.2 | 10.8 | 10.1 | 10.3 |
| Bacteroidetes | 0.064 | 9.7 | 9.3 | 10.8 | 9.8 |
| Verrucomicrobia | 0.455 | 16.7 | 16.7 | 15.7 | 16.6 |
| Chloroflexi | 0.501 | 2.9 | 2.9 | 2.7 | 2.9 |
| Firmicutes | 0.516 | 2.2 | 2.7 | 2.7 | 2.2 |
| Gemmatimonadetes | 0.576 | 1.6 | 1.6 | 1.6 | 1.5 |
| Betaproteobacteria | 0.570 | 3.6 | 3.6 | 3.7 | 3.7 |
| Archaea | | | | | |
| Crenarchaeota | 0.000 | 94.0 | 98.0 | 94.0 | 98.0 |
| Euryarchaeota | 0.000 | 2.0 | 1.0 | 2.5 | 1.0 |
| [Parvarchaeota] | 0.001 | 2.0 | 0.5 | 1.0 | 1.0 |

Only taxa ≥0.5% of sequences within at least one treatment are shown. *P* value corrections were made within taxonomic level for fungi.

**Table S5. Correlations between change in environmental variables and changes in the relative abundances of Fungi with N and P additions**

| | Ascomycota | | Glomeromycota | |
|---|---|---|---|---|
| Variable | *r* | *P* | *r* | *P* |
| (soil pH change)$^2$ | −0.38 | 0.065 | 0.01 | 0.95 |
| ln(soil P change) | −0.33 | 0.119 | 0.17 | 0.428 |
| sqrt(soil N change) | 0.37 | 0.075 | −0.16 | 0.462 |
| sqrt(live plant biomass change) | −0.15 | 0.491 | −0.17 | 0.431 |

Correlations were assessed using mean values for each site. Variables refer to percent differences from mean values in control plots. Numbers in bold type identify correlations that $P \leq 0.05$.

**Table S6. Correlations between change in environmental variables and changes in the relative abundances of Bacteria with N and P additions**

| | Acidobacteria | | Actinobacteria | | Alphaproteobacteria | | Deltaproteobacteria | | Gammaproteobacteria | | Planctomycetes | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Variable | *r* | *P* | *r* | *P* | *r* | *P* | *r* | *P* | *r* | *P* | *r* | *P* |
| (soil pH change)$^2$ | 0.03 | 0.889 | 0.06 | 0.794 | −0.21 | 0.32 | −0.1 | 0.644 | −0.23 | 0.283 | 0.17 | 0.422 |
| ln(soil P change) | −0.04 | 0.847 | 0.38 | 0.065 | **0.45** | **0.027** | −0.18 | 0.406 | 0.11 | 0.605 | −0.12 | 0.592 |
| sqrt(soil N change) | −0.34 | 0.101 | 0.07 | 0.729 | −0.01 | 0.954 | −0.14 | 0.513 | 0.35 | 0.098 | 0.02 | 0.929 |
| sqrt(live plant biomass change) | 0.11 | 0.602 | 0.32 | 0.131 | 0.25 | 0.233 | −0.07 | 0.741 | −0.11 | 0.602 | 0.1 | 0.635 |

Correlations were assessed using mean values for each site. Variables refer to percent differences from mean values in control plots. Numbers in bold type identify correlations that $P \leq 0.05$.

**Table S7. Median percent differences in relative abundances of gene categories of nutrient-treated samples from within-site control plots**

| | | | Difference from control, % | | |
|---|---|---|---|---|---|
| Gene category | *P* | *P* (FDR-corrected) | +N | +P | +N,+P |
| Metabolism (unclassified) | 0.002 | 0.048 | −1.00 | −0.31 | −0.76 |
| Translation | 0.016 | 0.180 | 0.04 | 0.70 | 0.80 |
| Carbohydrate metabolism | 0.017 | 0.129 | 0.72 | 0.02 | 0.37 |
| Genetic information processing | 0.031 | 0.178 | 0.83 | −1.82 | −1.39 |
| Enzyme families | 0.032 | 0.145 | −0.89 | −1.64 | −1.16 |
| Replication and repair | 0.069 | 0.264 | −0.07 | 0.54 | 0.96 |
| Nucleotide metabolism | 0.111 | 0.364 | 0.22 | 0.44 | 0.63 |
| Folding, sorting, and degradation | 0.120 | 0.344 | −0.17 | 0.25 | −0.75 |
| Metabolism of other amino acids | 0.262 | 0.669 | −0.12 | −0.50 | 0.05 |
| Cell motility | 0.331 | 0.762 | −0.82 | 0.74 | 1.46 |
| Energy metabolism | 0.334 | 0.699 | 0.44 | 0.62 | 0.53 |
| Metabolism of terpenoids and polyketides | 0.345 | 0.660 | 0.80 | −0.38 | 0.46 |
| Cellular processes and signaling | 0.373 | 0.660 | −1.19 | 0.46 | 0.05 |
| Xenobiotics biodegradation and metabolism | 0.463 | 0.760 | 0.18 | −0.11 | 0.12 |
| Amino acid metabolism | 0.480 | 0.737 | 0.01 | 0.39 | 0.67 |
| Lipid metabolism | 0.536 | 0.770 | 0.20 | −0.72 | −0.21 |
| Transcription | 0.542 | 0.733 | 0.73 | −0.75 | −0.64 |
| Glycan biosynthesis and metabolism | 0.711 | 0.908 | −0.22 | 0.44 | 0.29 |
| Metabolism of cofactors and vitamins | 0.742 | 0.898 | −0.13 | 0.10 | 0.01 |
| Biosynthesis of other secondary metabolites | 0.806 | 0.927 | 0.42 | −0.86 | 0.32 |
| Membrane transport | 0.808 | 0.885 | −0.62 | 0.16 | 0.25 |
| Signal transduction | 0.863 | 0.902 | −0.58 | −0.16 | −0.26 |
| Poorly characterized | 0.918 | 0.918 | −0.15 | −0.17 | −0.15 |