

Global long-term agricultural experiments reveal consequences of mineral fertilization for soil microbiomes

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Supplementary Method 1. Determination of soil physicochemical properties

Soil pH was determined from soil-water suspensions (soil: water=1: 2.5 (w/v)). **Soil organic carbon (SOC)**, **total nitrogen (TN)**, and **C/N ratio** were quantified using an elemental analyzer (Elementar, Vario MAX CN, Co., Ltd, Germany) using soil pretreated with hydrogen chloride. **Dissolved organic carbon (DOC)** and **microbial biomass carbon (MBC)** were determined using the K₂SO₄ extraction and chloroform fumigation-extraction method¹. To determine DOC, 5 g soil was combined with 25 mL of 0.5M K₂SO₄ and placed on a shaker for 1 hour, followed by filtration. A second set of soils were placed in a desiccator with 20 mL chloroform under a vacuum and fumigated for 48 hours before extraction. Filtrate C contents were determined on a Total Organic Carbon analyzer (Multi-N/C 2100S; Analytik Jena, Jena, Germany). The MBC was calculated as the difference between fumigated and unfumigated samples divided by the extraction efficiency ($k_{EC} = 0.45$). **Ammonium (NH₄⁺-N)** and **nitrate (NO₃⁻-N)** were extracted from soil with 1 M KCl (1:7.5 (w/v)) and measured using a continuous flow analyzer (Skalar SAN++ System, Skalar Analytical B.V., Breda, Netherlands). **Available phosphorus (AP)** was measured as Olsen P from air-dried soil samples. **Available potassium (AK)** measurements were performed by inductively coupled plasma-optical emission spectrometry (Optima 8300 ICP-OES, Perkin Elmer, USA). All physicochemical measurements were performed in three replicates per treatment.

Supplementary Method 2. Analysis of soil microbial community

Phospholipid fatty acids (PLFAs) were extracted from 2 g of freeze-dried soil using a modified Bligh and Dyer method, in triplicate for each treatment². Soil samples were extracted twice with 22.8 mL one-phase chloroform-methanol-citrate buffer (0.15 M, pH 4.0)³. Lipid fractions were separated into neutral lipids, glycolipids, and phospholipids on a silica gel column (Supelco, USA), with the phospholipid fraction eluted using methanol. Phospholipids were transesterified to fatty acid methyl esters (FAMES) by mild alkaline methanolysis and subsequently analyzed using gas chromatography (7890B, Agilent, USA) equipped with a flame ionization detector and a capillary column. Individual FAMES were identified by comparing retention times with known standards (MIDI Inc., USA) and quantified based on internal standards. PLFA biomarkers were assigned to major microbial groups, including Gram-positive and Gram-negative bacteria, fungi, according to established nomenclature⁴. Specifically, the PLFA classification encompasses bacterial PLFA, including Gram-positive (G⁺) and Gram-negative (G⁻) types, where G⁺ comprises *Firmicutes* (i14:0, i15:0, i16:0, i17:0, i18, a15:0, a16:0, a17:0, a18:0, a19:0) and *Actinobacteria* (10Me16:0, 10Me17:0, 10Me18:0), while G⁻ includes cy17:0, cy19:0, 16:1 ω 7, 16:1 ω 9, 17:1 ω 8, and 18:1 ω 7; fungal PLFA covers AMF (16:1 ω 5c), *Zygomycota* (18:1 ω 9c), *Ascomycota* and *Basidiomycota* (18:2 ω 6c), and unspecific fungal PLFA (18:3 ω 6,9,12); and unspecific microbial PLFA includes 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 20:4 ω 6,9,12,15.

Soil DNA was extracted from 0.5 g of freeze-dried soil using the DNeasy PowerSoil Kit (QIAGEN, Germany) following the manufacturer's instructions. The DNA quality was assessed with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The **16S rRNA gene** (V4-V5 region) was amplified using primers 515F (Illumina adapter sequence 1 + GTGCCAGCMGCCGCGG) and 907R (Illumina adapter

sequence 2 + CCGTCAATTCMTTTRAGTTT), while fungal **ITS1** regions were amplified using primers ITS1F (Illumina adapter sequence 1+ CTTGGTCATTAGAGGAAGTAA) and ITS2 (Illumina adapter sequence 2+ GCTGCGTTCTTCATCGATGC). Sample-specific barcodes were introduced, and amplicons were purified with AMPure XP beads. Libraries were quantified, pooled at equimolar concentrations, and quality-checked using an Agilent 2100 Bioanalyzer (Agilent, USA). Sequencing was performed on the Illumina NovaSeq 6000 platform (PE250) at G&C Biotechnology (Shanghai, China). Paired-end reads were processed using the QIIME2 (version 2024.10) pipeline⁵. The adaptor and primer sequences were trimmed using the *cutadapt* plugin. Sequencing data were quality-filtered, trimmed, and denoised using DADA2 plugin⁶. Taxonomic assignment was performed on filtered amplicon sequence variants (ASVs) against pre-trained (the Naïve Bayes classifier) QIIME2-compatible databases (16S: Greengenes version 13.8, ITS: UNITE version 10.0). Feature tables were rarefied to normalize sequencing depth across samples prior to further analysis.

Metagenomic libraries were constructed from high-quality genomic DNA using a ligation-based protocol. DNA was randomly sheared to ~200–600 bp fragments using a Covaris system (Covaris, USA), followed by end repair, adapter ligation, and PCR amplification. Size-selected libraries were purified using magnetic beads (Transgen, China), quantified with Library Quantification Kit (KAPA Biosystems), and quality-checked on an Agilent 2100 Bioanalyzer (Agilent, USA). Sequencing was performed on the Illumina NovaSeq X Plus platform (PE150), generating approximately 15 Gb of paired-end reads per sample. Metagenomic reads were assembled *de novo* using MEGAHIT (version 1.2.9) with a multi *k-mer* strategy (--k-list 27,47,67,87,107,127,141). Contigs $\geq 3,000$ bp were retained for **viral identification**. Putative viral sequences were detected using VirSorter2 (version 2.2.4), geNomad (version 1.11.1), and viralVerify (version 1.1) in non-concentrated mode (--non-con). CheckV (version 1.5) was used to assess genome completeness and remove host-derived regions. High-quality viral contigs ($\geq 50\%$ completeness) were binned into viral metagenome-assembled genomes (vMAGs) using vRhyme (version 1.1.0), and de-replicated with dRep (version 3.4.2; --min-seq-id 0.95 --cov-mode 1 -c 0.9) to generate a non-redundant genome set^{7,8}. High-confidence viral genomes were clustered into viral operational taxonomic units (vOTUs; $\geq 95\%$ ANI and $\geq 85\%$ alignment fraction). Viral abundance was estimated by mapping quality-filtered reads to vOTU representative sequences using Bowtie2 (version 2.5.4) with --end-to-end and --very-sensitive parameters⁹. Read counts were normalized to transcripts per million (TPM) based on contig length and library size.

Within our targeted sampling dataset, microbial community **α -diversity** was quantified using the observed richness (number of ASVs/vOTUs) and Shannon index following the *mothur* implementation (<https://mothur.org/wiki/shannon/>), and **β -diversity** was assessed as Bray–Curtis dissimilarity among samples.

Supplementary Method 3. Determination of soil microbial activities

Basal respiration was determined by incubating 10 g (dry weight equivalent) of pre-incubated soil at 25 °C for 24 h in sealed 120 mL serum bottles. Headspace gas samples were collected and analyzed for CO₂ concentration using a gas chromatograph (7890B, Agilent, USA) equipped with a thermal conductivity

detector. The **metabolic quotient ($q\text{CO}_2$)** was calculated as the ratio of basal respiration to MBC ($\text{mg CO}_2\text{-C mg}^{-1}\text{ MBC d}^{-1}$).

All enzyme activities were analyzed using assay kits (Boxbio Science & Technology Co., Ltd, China) following the manufacturer's protocols, and measurements were performed using a microplate method (SpectraMax iD3, Molecular Devices, USA). Activities of soil **β -D-glucosidase (βG)**, **β -xylosidase (βX)**, **cellobiohydrolase (CBH)**, **N-acetylglucosaminidase (NAG)**, were measured using their respective p-nitrophenyl-linked substrates, and the amount of p-nitrophenol (p-NP) released was determined colorimetrically at 400 nm¹⁰. **Phosphatase (PHO)** activities were determined depending on soil pH. Both acid and alkaline phosphatases were assayed based on the amount of phenol released from the hydrolysis of disodium phenyl phosphate, measured colorimetrically at 660 nm¹¹. **Leucine aminopeptidase (LAP)** activity was determined using L-leucine-p-nitroanilide as substrate, with p-nitroaniline quantified at 405 nm¹². **Urease activity (URE)** was measured based on the formation of a blue complex from the reaction between released NH_4^+ and a phenol chromogenic agent in alkaline conditions, with absorbance read at 578 nm¹³. **Phenoloxidase (PO)** activity was determined using l-3,4-dihydroxyphenylalanine (L-DOPA) as substrate, with dopachrome formation measured at 475 nm¹⁴. **Invertase (INV)** activity was quantified based on reducing sugar production from sucrose, using the 3,5-dinitrosalicylic acid (DNS) method at 540 nm¹⁵. **Dehydrogenase (DHA)** activity was measured via the reduction of triphenyltetrazolium chloride (TTC) to triphenyl-formazan (TPF), detected at 460 nm¹⁶.

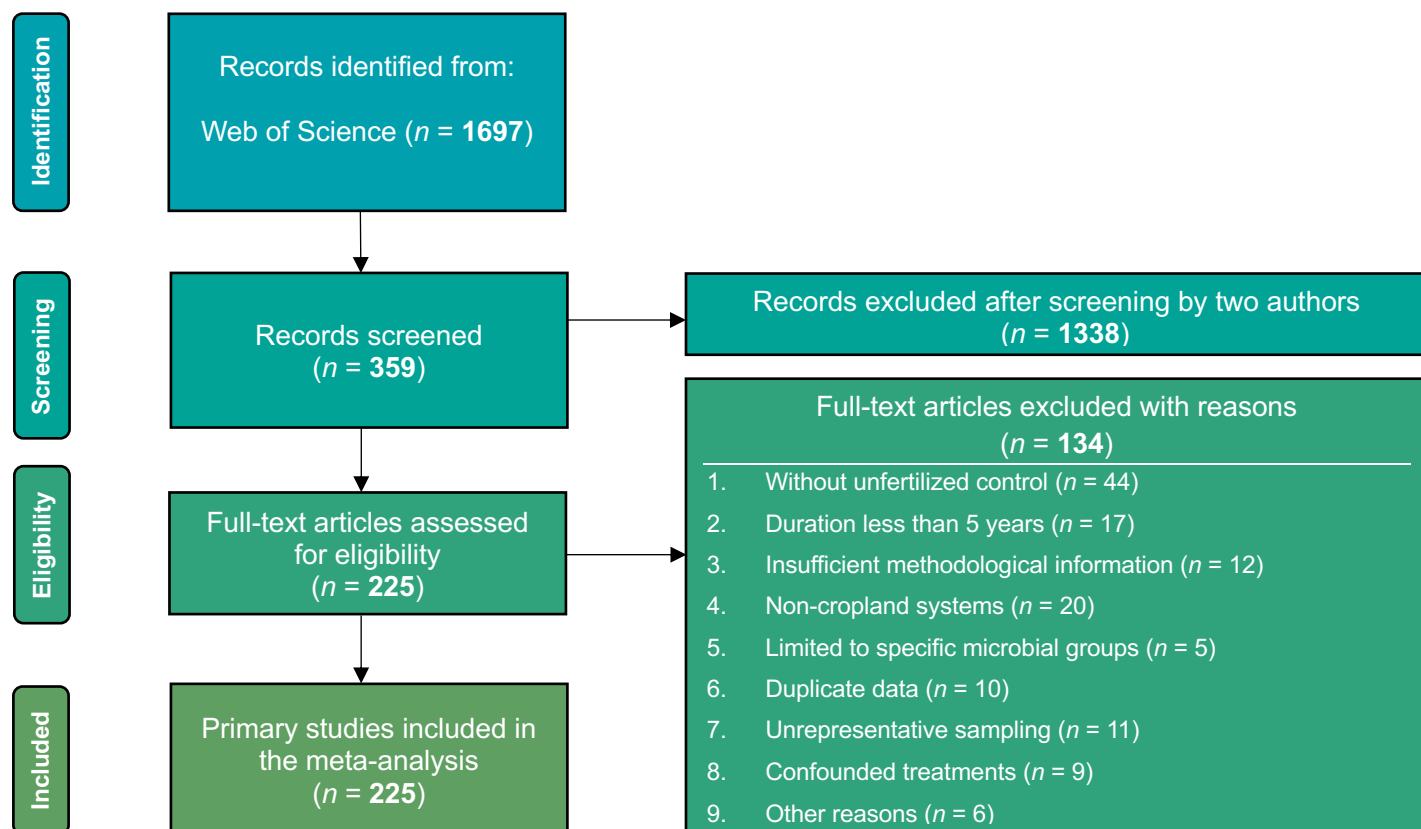
Supplementary Note 1. Interpretation of β -dispersion and centroid shift

Both β -dispersion and centroid shift were calculated from unconstrained ordination (*e.g.*, principal coordinates analysis, PCoA) based on dissimilarity matrices derived from microbial abundance data. **β -dispersion** quantifies within-group variability by computing the average pairwise Euclidean distance among replicate samples within a group in the ordination space, serving as a measure of community heterogeneity¹⁷. Here we used pairwise distances rather than distances to the group centroid to improve robustness under small sample sizes and reduce sensitivity to outliers. In contrast, **centroid shift** measures the Euclidean distance between the centroids of different groups (*e.g.*, treatment vs. control), reflecting a directional shift in average community composition¹⁸. Together, these two metrics differentiate between compositional divergence (centroid shift) and variability in community responses (β -dispersion) under long-term mineral N fertilization. To aid interpretation, conceptual illustrations and corresponding formulas are provided in Supplementary Figs. 9 and 10. The conceptual basis for distinguishing these two components of β -diversity is also detailed by Houseman et al¹⁷.

As centroid shift is inherently non-negative, we employed a nonparametric bootstrap approach to derive unbiased estimates of both effect sizes and their sampling variances. For each study observation, group coordinates (control vs. treatment) on the first two PCoA axes were resampled 1,000 times with replacement, and the mean Euclidean distance across bootstrap replicates was taken as the effect size (y_i), with its standard

error (SE) providing the sampling variance ($V_i = SE^2$). These bootstrapped estimates were then synthesized in a multilevel random-effects meta-analysis (Supplementary Fig. 5)

Supplementary Table 1. PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) flow diagram for the current meta-analysis.



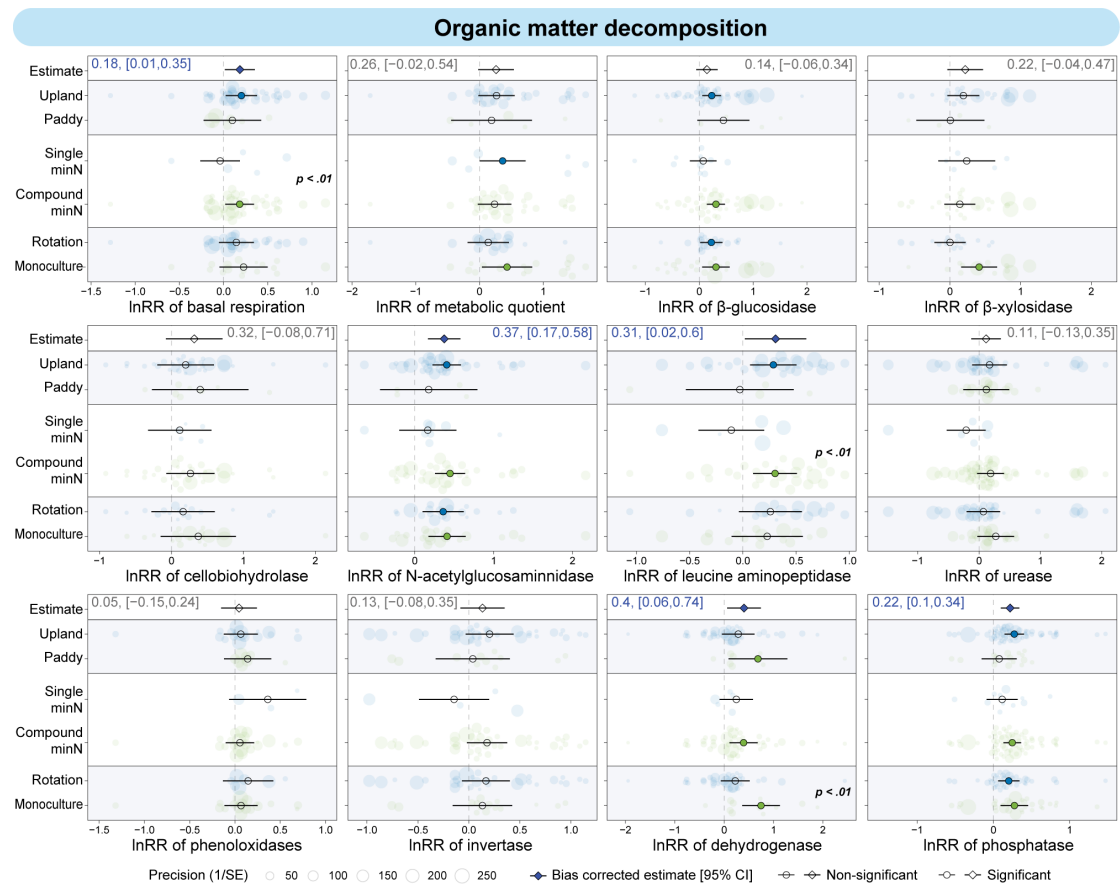
Supplementary Table 2. Sensitivity analysis of generalized additive models (GAMs) using a drop-one approach to assess the influence of individual predictors on Akaike information criterion (AIC) and explained variance (R^2).

Model	AIC	R^2	Deviance explained
Microbial biomass C response			
^a Full model	79.2	0.33	0.42
-s(pH)	87.3	0.25	0.33
-s(Soil organic C)	87.0	0.23	0.31
-s(Mean annual temperature)	94.7	0.16	0.25
-s(Mean annual precipitation)	79.5	0.31	0.39
<i>Proteobacteria</i> response			
^a Full model	-57.4	0.56	0.66
-s(pH)	-42.3	0.47	0.59
-s(Soil organic C)	-48.5	0.50	0.60
-s(Mean annual temperature)	-58.1	0.56	0.65
-s(Mean annual precipitation)	-58.2	0.56	0.65
<i>Firmicutes</i> response			
^a Full model	96.3	0.54	0.66
-s(pH)	96.5	0.51	0.61
-s(Soil organic C)	97.7	0.49	0.59
-s(Mean annual temperature)	93.8	0.56	0.66
-s(Mean annual precipitation)	96.3	0.55	0.68

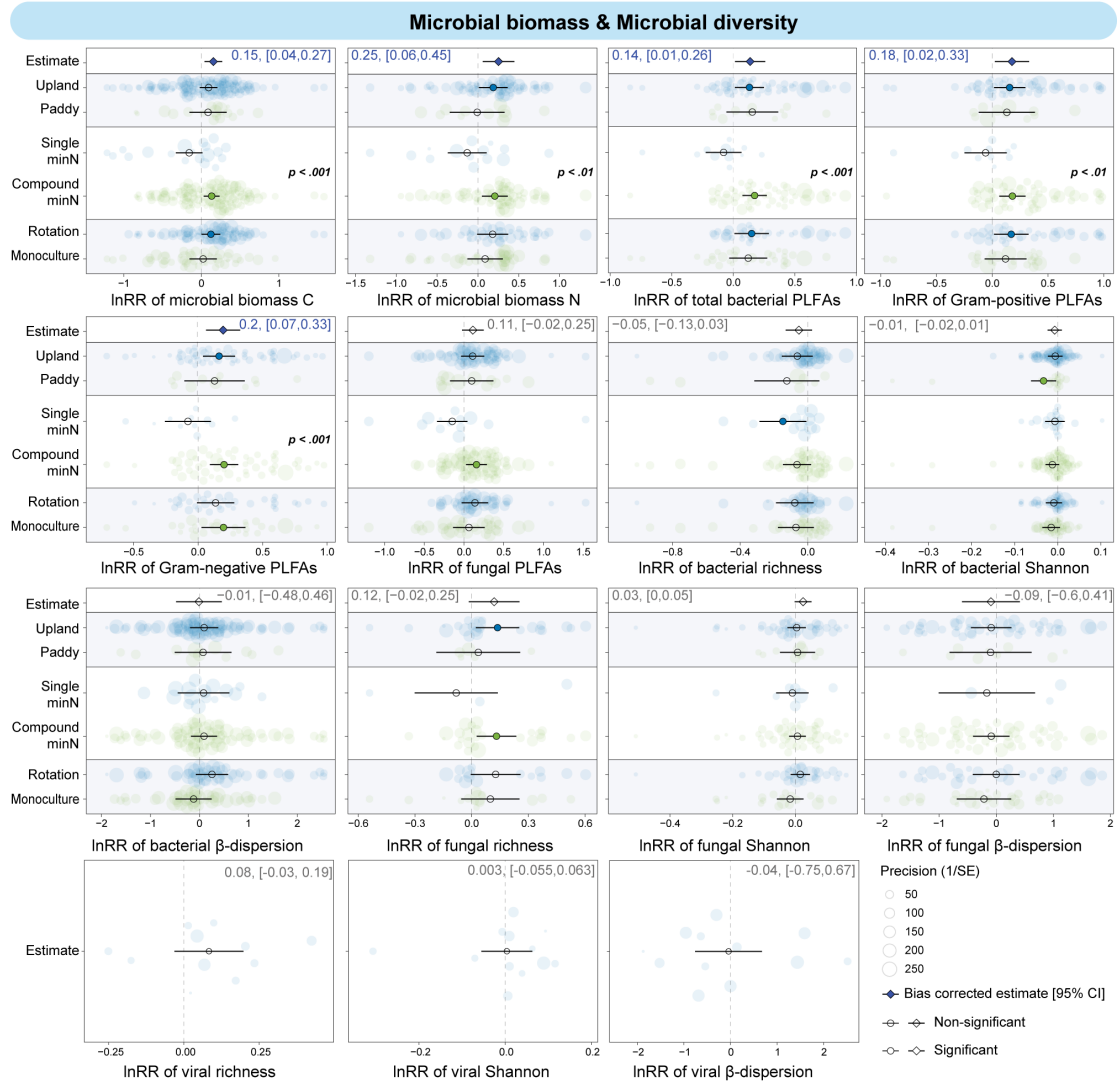
^alnRR = s(pH) + s(Soil organic C) + s(Mean annual temperature) + s(Mean annual precipitation) + s(Annual rate of mineral N addition) + s(Longitude, Latitude).



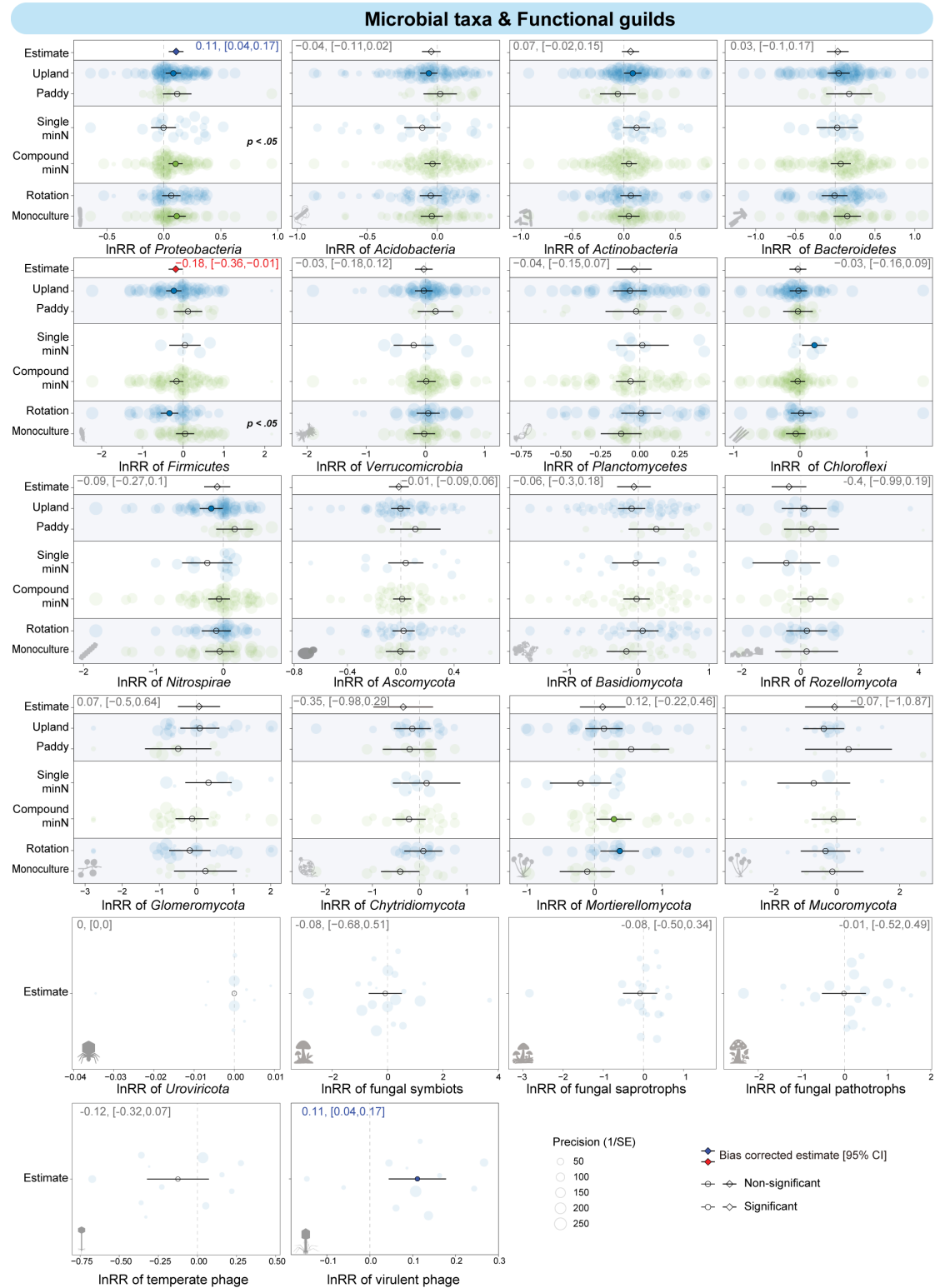
Supplementary Fig. 1. Effect of long-term mineral fertilization on soil properties and crop productivity. Bias-corrected estimates ($\pm 95\%$ confidence intervals) are shown for overall effects (diamonds) and subgroup-level estimates (circles) grouped by agroecosystem type (upland vs. paddy), fertilization regime (single vs. compound mineral N), and cropping regime (rotation vs. monoculture). Changes in soil pH are expressed as mean differences between fertilized and control; all other variables are expressed as log response ratios (lnRR). Solid blue and red diamonds indicate significant overall positive and negative effects, respectively; open diamonds indicate non-significant overall effects. Circles represent subgroup estimates, with point size scaled by precision (1/standard error); open circles denote non-significant effects. p values indicate the significance of subgroup contrasts, reported only when the overall effect is significant. Source data are provided as a Source Data file.



Supplementary Fig. 2. Effects of long-term mineral fertilization on microbial activities relevant to organic matter decomposition. Overall effects are shown as diamonds, subgroup-level estimates as circles, with point size proportional to precision (1/standard error). Other legend details are as described in Supplementary Fig. 1. Source data are provided as a Source Data file.

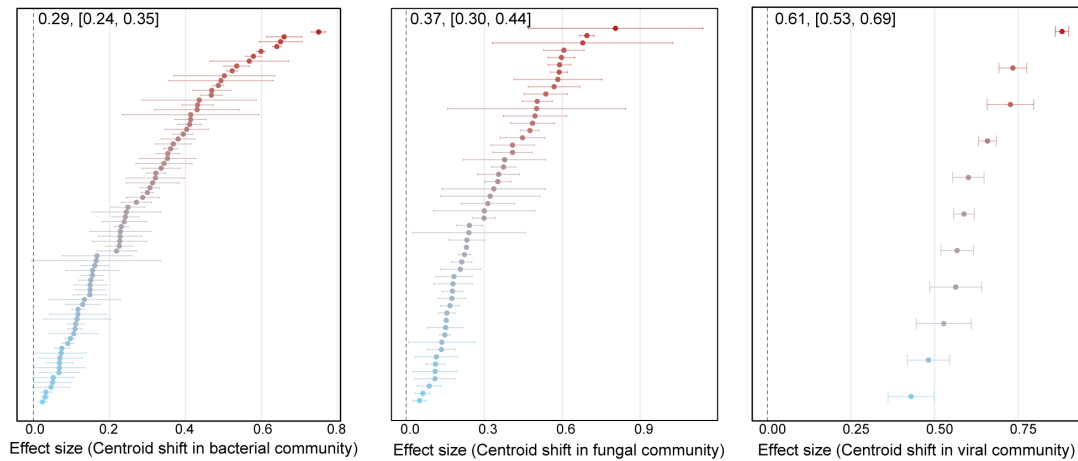


Supplementary Fig. 3. Effects of long-term mineral fertilization on microbial biomass and diversity. Overall effects are shown as diamonds, subgroup-level estimates as circles, with point size proportional to precision (1/standard error). Other legend details are as described in Supplementary Fig. 1. Viral diversity metrics were excluded from subgroup analysis and publication bias correction because they were exclusively derived from our targeted field sampling. Source data are provided as a Source Data file.

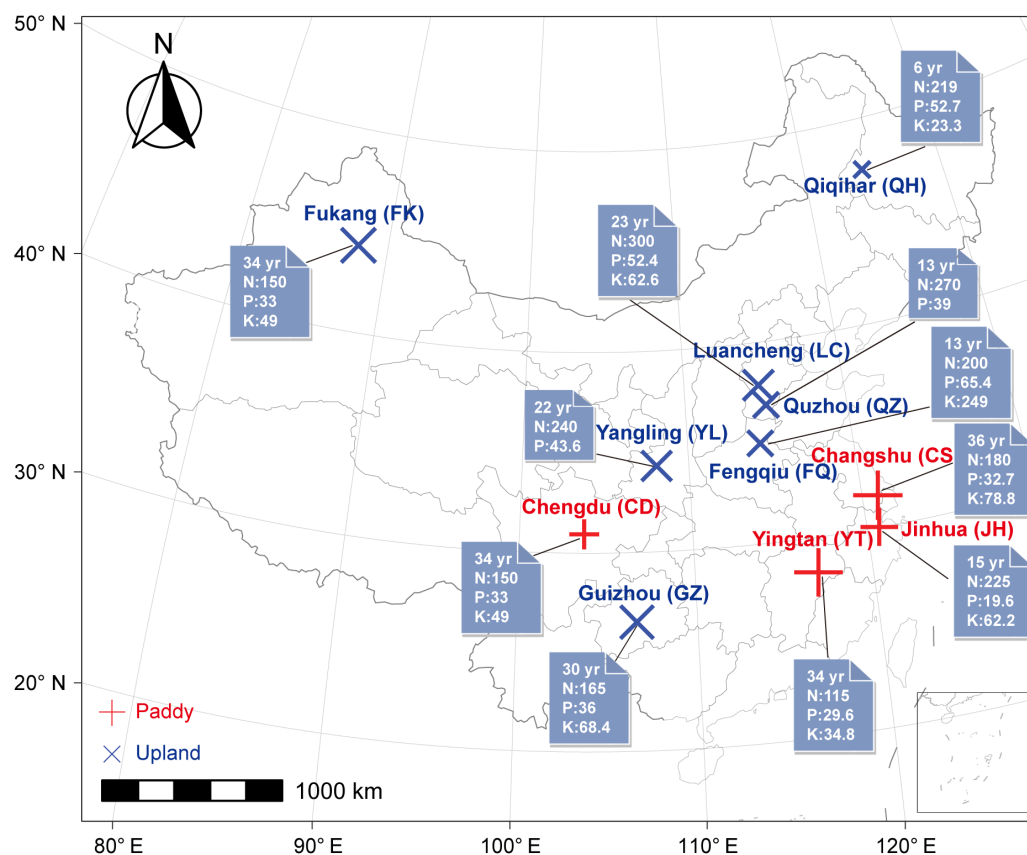


Supplementary Fig. 4. Effects of long-term mineral fertilization on microbial taxa and functional guilds. Overall effects are shown as diamonds, subgroup-level estimates as circles, with point size proportional to precision (1/standard error). Other legend details are as described in Supplementary Fig. 1. Viral phyla and functional

guilds were not subjected to subgroup analysis because of their limited representation in the literature and their primary presence in our field-sampled dataset. Silhouettes are from PhyloPic (<http://phylopic.org>). Source data are provided as a Source Data file.



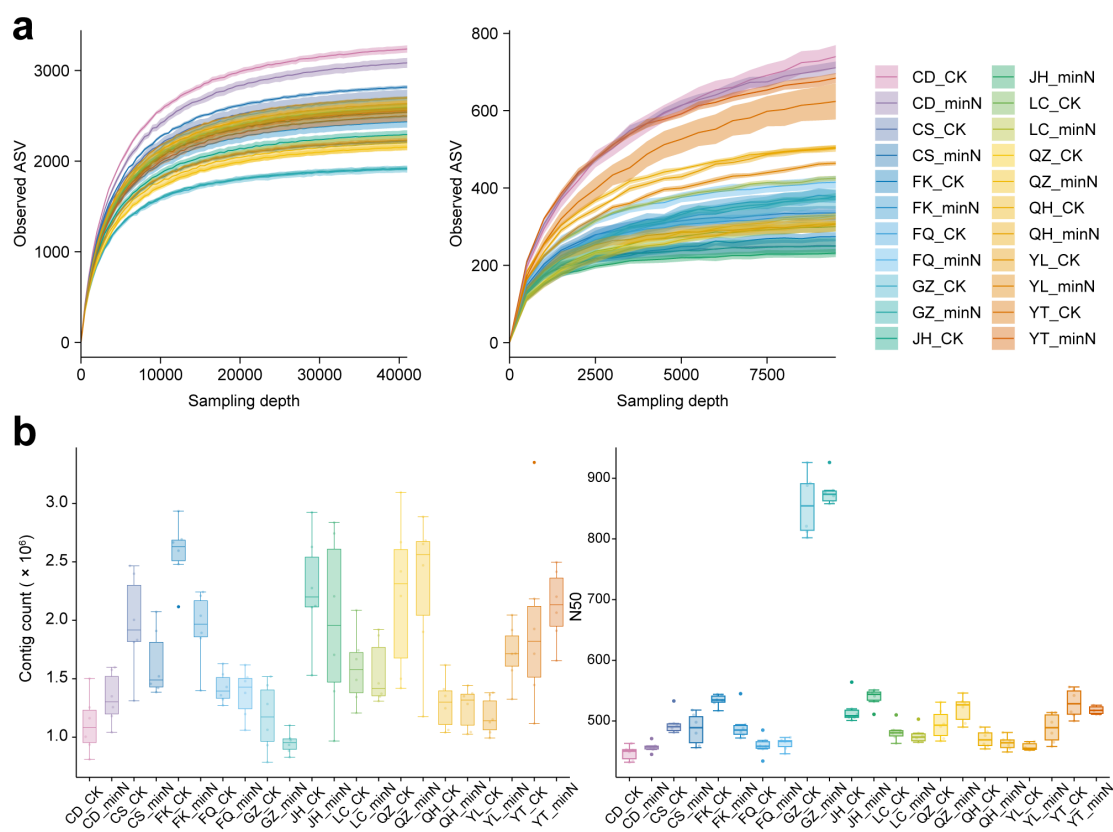
Supplementary Fig. 5. Microbial community centroid shifts in response to long-term mineral fertilization. Effect sizes represent centroid shifts between fertilized and control groups, quantified as Euclidean distances based on the first two axes of unconstrained ordination derived from dissimilarity matrices. Given that centroid shifts are inherently non-negative and may exhibit skewed distributions, a nonparametric bootstrap approach was applied to obtain unbiased estimates of both effect sizes and their sampling variances (see Methods). For each study, sample identities were resampled with replacement within fertilized and control groups (1,000 iterations), and the mean distance between group centroids was used as the effect size. The standard deviation of bootstrap replicates was used to estimate the standard error for subsequent multilevel meta-analysis. Source data are provided as a Source Data file.



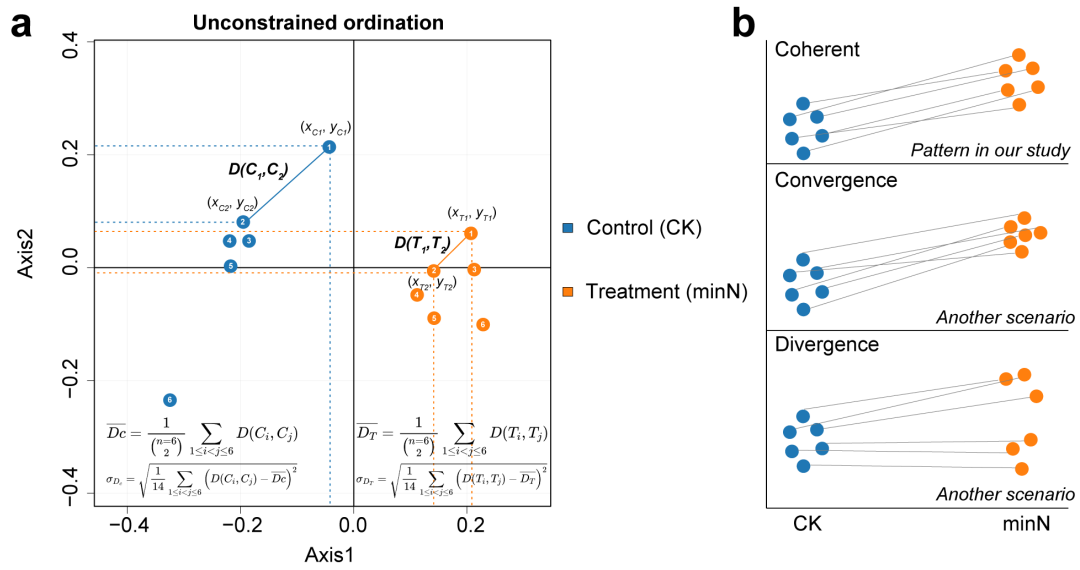
Supplementary Fig. 6. Geographic distribution of the 11 long-term fertilization experiments (LTEs) included in our targeted field sampling across China. Symbols indicate upland (blue crosses) and paddy (red crosses) agroecosystems. Labels show fertilization duration (in years) and average annual application rates of nitrogen (N), phosphorus (P), and potassium (K), expressed as elemental nutrients (kg ha⁻¹ yr⁻¹). Associated metadata are provided in the Source Data. Source data are provided as a Source Data file.



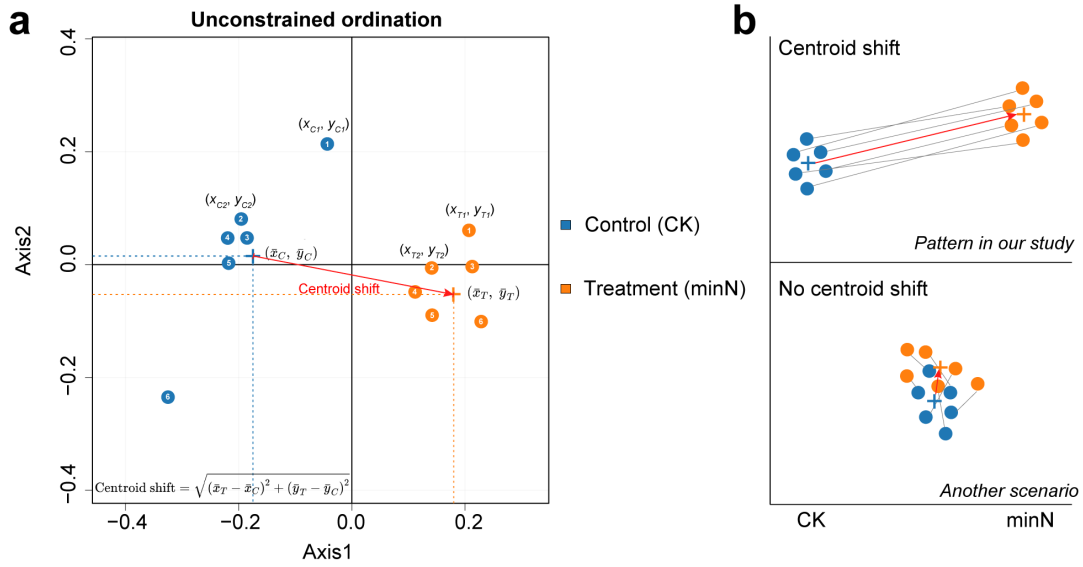
Supplementary Fig. 7. Coverage of variables across the global dataset compiled from systematic literature curation and targeted field sampling. Columns correspond to individual studies. Color intensity reflects the number of paired observations ($n = 1,751$ in total) recorded for each variable within each study. Our sampling complemented literature data by improving coverage of underrepresented variables. Source data are provided as a Source Data file.



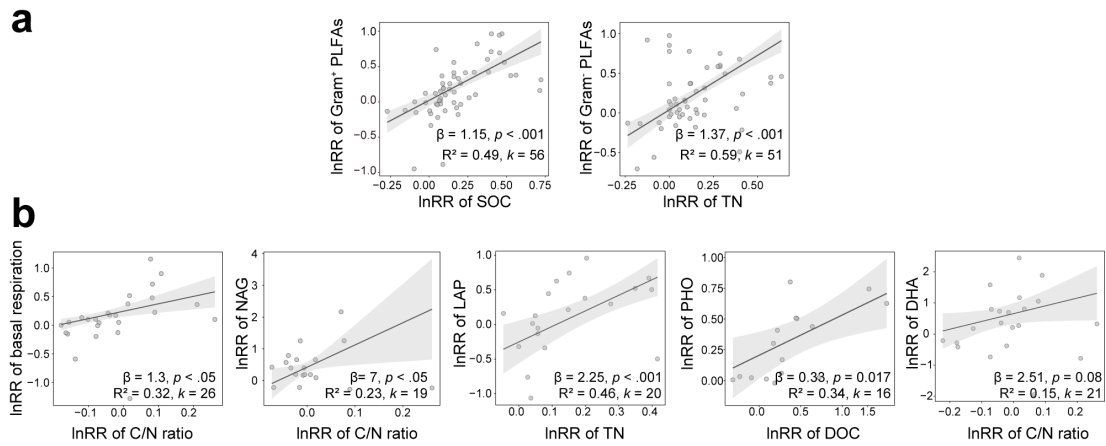
Supplementary Fig. 8. Sequencing depth and assembly quality of amplicon and metagenomic data. (a) Rarefaction curves showing observed amplicon sequence variants (ASVs) as a function of sequencing depth for 16S rRNA (left) and ITS (right) datasets across our sampling sites. (b) Boxplots showing the number of assembled contigs (left) and N50 values (right) derived from metagenomic sequencing. N50 represents the contig length above which 50% of the total assembly length is contained. Sample site abbreviations are defined in Supplementary Fig. 6. Source data are provided as a Source Data file.



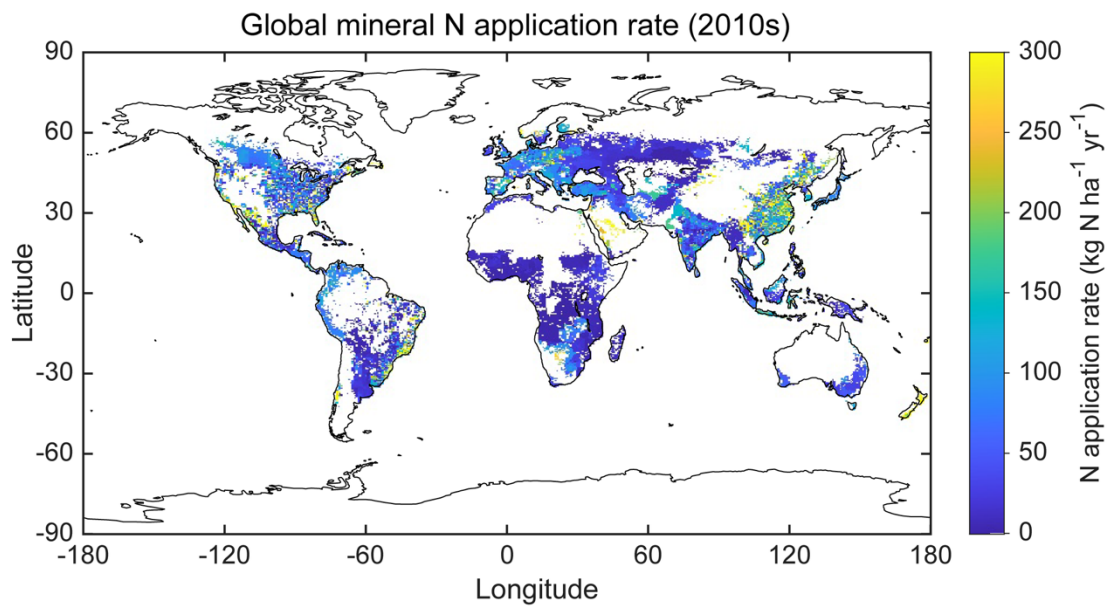
Supplementary Fig. 9. Conceptual illustration of β -dispersion based on unconstrained ordination. (a) Two example groups illustrating the calculation of β -dispersion, defined as the average pairwise Euclidean distance among samples within a group ($\overline{D_C}$, $\overline{D_T}$). This metric quantifies within-group variability in microbial community. Coordinates are derived from unconstrained ordination of dissimilarity matrices. (b) Possible β -dispersion scenarios: coherent dispersion (as observed in our study), reduced dispersion (convergence), and increased dispersion (divergence).



Supplementary Fig. 10. Conceptual illustration of centroid shift based on unconstrained ordination. (a) Centroid shift quantifies the difference in microbial community composition between groups as the Euclidean distance between group centroids in unconstrained ordination space. Sample coordinates are derived from dissimilarity-based ordination, and group centroids $(\bar{x}_C, \bar{y}_C)$ and $(\bar{x}_T, \bar{y}_T)$ are calculated as the mean position of all samples within each group. (b) Possible centroid shift scenarios: clear shift in community centroid (as observed in our study) and no shift in centroid position.

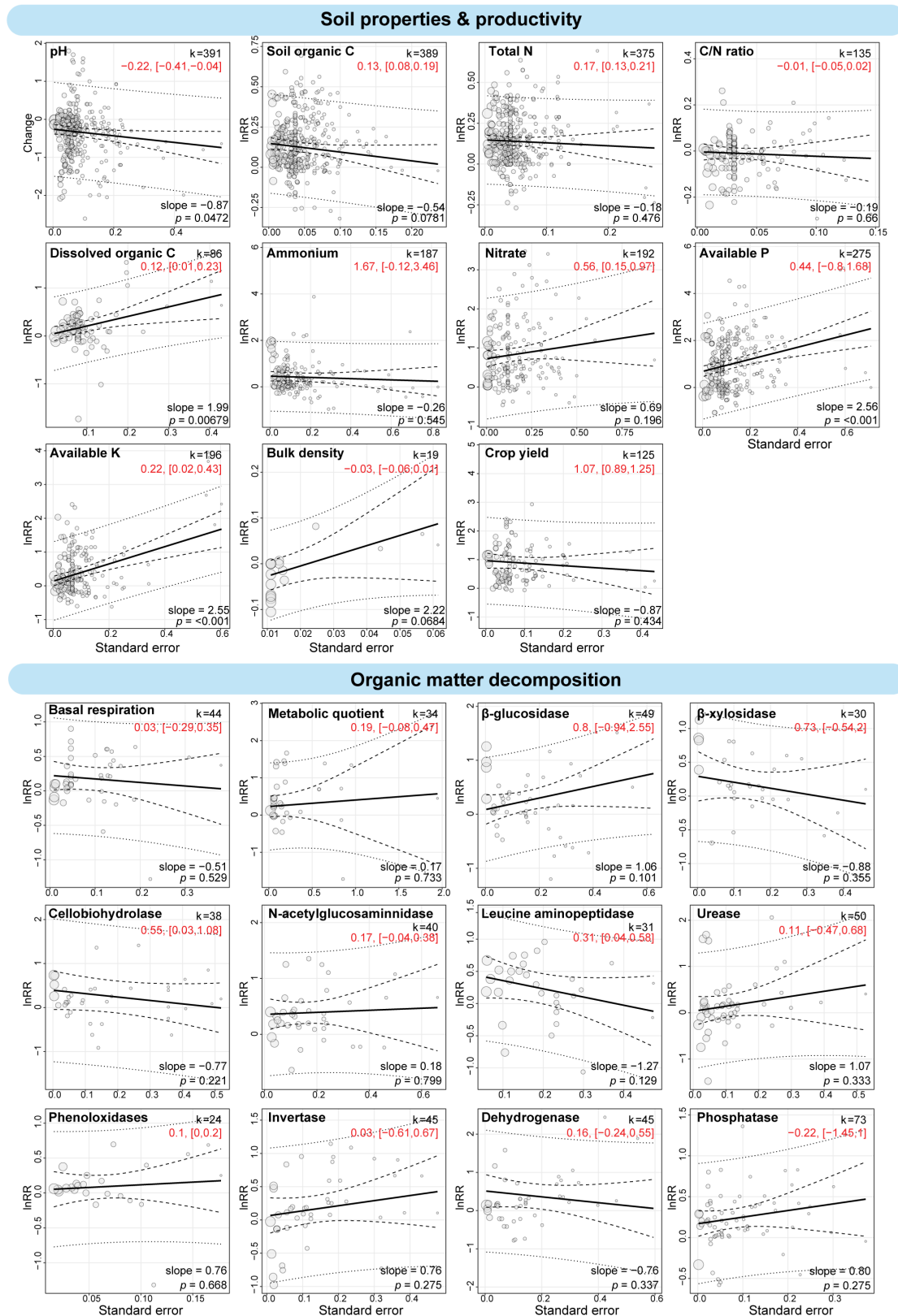


Supplementary Fig. 11. Soil physicochemical properties explain increases in microbial biomass and activity under long-term mineral fertilization. Inverse-variance weighted linear regressions were used to examine associations between changes in soil properties and microbial responses, based on log response ratios (lnRR) derived from meta-analysis. β indicates regression slope, R^2 denotes coefficient of determination, and k is the number of effect sizes. Source data are provided as a Source Data file.



Supplementary Fig. 12. Global distribution of average annual mineral nitrogen application rates in the 2010s. The map shows the spatial distribution of mineral nitrogen (N) inputs to croplands, averaged over 2011–2020, at a resolution of 5 arc-minutes. Data were obtained from the National Tibetan Plateau/Third Pole Environment Data Center¹⁹. Color scale represents N application rates in kg N ha⁻¹ yr⁻¹.

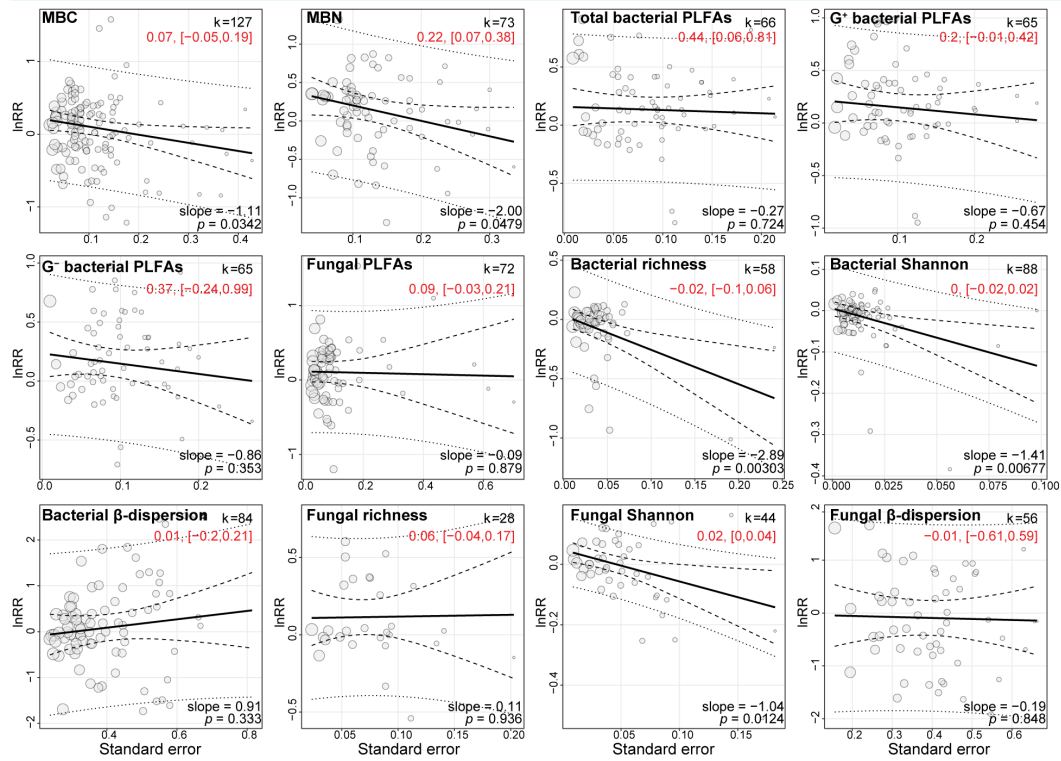
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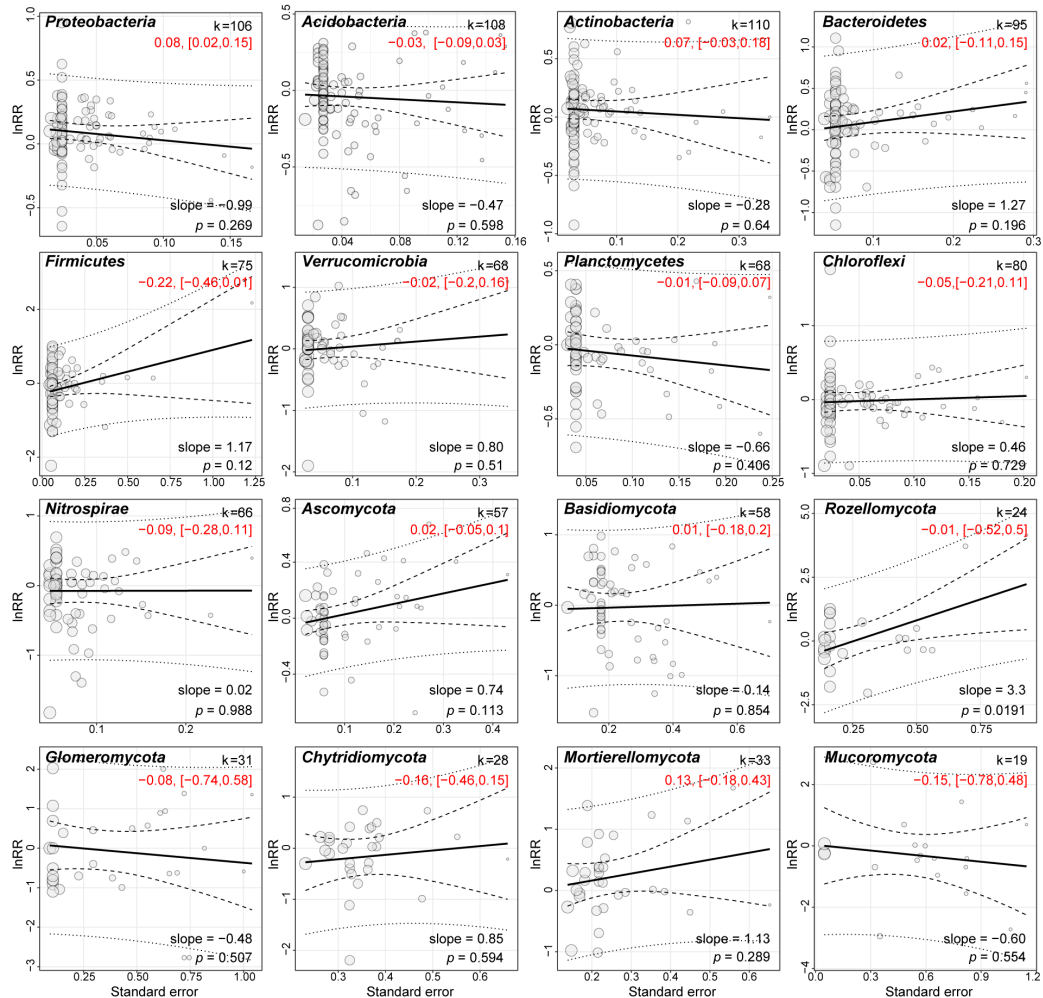
Supplementary Fig. 13. Assessment of publication bias. Funnel plots were used to evaluate potential publication bias in effect size estimates (lnRR) using Egger's regression test. Each point represents an individual effect size (k), plotted against its

standard error. The solid black line denotes the fitted regression line, with dashed lines indicating the 95% confidence intervals. A significant slope ($p < 0.05$) indicates potential publication bias. Values in parentheses show the bias-corrected overall effect sizes and 95% confidence intervals estimated using the two-step method²⁰. Source data are provided as a Source Data file.

Microbial biomass & Microbial diversity



Microbial taxa



Supplementary Fig. 14. Assessment of publication bias. Egger's regression plots and bias-corrected estimates shown as in Supplementary Fig. 13. Source data are provided as a Source Data file.

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