

Article

Asymmetric Roles of Ryegrass and Earthworms in Shaping Microbial Community Composition and Nutrient-Acquiring Enzyme Activities in Petroleum-Contaminated Soil

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Abstract

Petroleum contamination of the fragile loess soils of Northern Shaanxi, China, calls for low-cost biological remediation strategies. Combining plants with soil macrofauna is a promising option, but it remains unclear whether the two agents act on the same or on different components of the soil microbial system. In a 150-day soil-column experiment on a moderately contaminated loess soil (initial total petroleum hydrocarbon, TPH, 1.88 g kg⁻¹), we crossed ryegrass (*Lolium perenne*) and earthworms (*Metaphire tschiliensis*) in a 2 × 2 factorial design and measured bacterial and fungal community composition, five carbon (C)-, nitrogen (N)-, and phosphorus (P)-acquiring enzyme activities and coenzymatic stoichiometry. Ryegrass was the sole significant driver of hydrocarbon removal ($p < 0.001$; TPH reduced by 26.6% under the combined treatment), whereas earthworms had no significant main effect ($p = 0.168$). The two agents acted on distinct targets: earthworms restructured the bacterial community (PERMANOVA $R^2 = 0.253$, $p < 0.001$) without changing α -diversity and strongly raised NO₃⁻-N and N-acquiring NAG activity, whereas ryegrass shaped the fungal community ($R^2 = 0.105$, $p = 0.036$) and increased C-acquiring enzyme activities. A significant ryegrass × earthworm interaction emerged for β -1,4-glucosidase ($p = 0.017$) and for microbial C limitation ($p = 0.011$), which is consistent with the additional rhizosphere C demand being expressed only after earthworms had relieved N shortage. The relative abundance of the hydrocarbon-degrading guild was unrelated to residual TPH ($r = 0.19$, $p = 0.424$), indicating that the taxonomic abundance of potential degraders did not predict hydrocarbon removal. Vector angles (51.7–53.7°) fell between the conventional 45° and the recently proposed 55° threshold; available N:P ratios, substrate stoichiometry and the enzymatic response to earthworm-derived N are consistent with N rather than P limitation and suggest that the 45° threshold overestimates P limitation in alkaline soils. Within the conditions tested, plant establishment appears to be the priority for remediating petroleum-contaminated loess, and the combination of plants, earthworms and moderate N-containing organic amendments is a promising strategy that now requires direct experimental evaluation.

Keywords: earthworm; coenzymatic stoichiometry; hydrocarbon-degrading bacteria; microbial nutrient limitation; petroleum-contaminated soil; ryegrass



Academic Editor: Nikolaos Monokrousos

Received: 15 August 2026

Revised: 13 September 2026

Accepted: 20 September 2026

Published: 21 September 2026

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1. Introduction

Crude oil is among the most widespread organic contaminants of terrestrial ecosystems [1,2]. Northern Shaanxi, one of China's major energy bases, has accumulated a substantial legacy of contaminated and abandoned well sites in loess soils that are inherently infertile, alkaline, and erosion-prone, so that hydrocarbon input degrades physical, chemical, and biological soil quality simultaneously [1,3]. Physical and chemical treatments are effective but costly, so biological approaches are preferred where contamination is moderate and diffuse [2]. Because hydrocarbon transformation is tightly coupled to carbon (C) and nitrogen (N) dynamics, it can be redirected by biostimulation [4].

Phytoremediation with fast-growing grasses such as *Lolium perenne* is well documented: root exudates supply labile C, improve aeration, and stimulate rhizosphere microorganisms capable of co-metabolizing hydrocarbons, but their principal limitation is the slow rate of hydrocarbon dissipation [2]. Earthworms are classical ecosystem engineers whose burrowing, casting, and gut passage alter soil structure, enhance nutrient availability, and create hotspots of microbial activity and enzymatic turnover in the drilosphere and casts [5–10]. Combining the two agents in contaminated soils is promising: earthworms have been shown to enhance enzyme activity and reshape the microbial community during the phytoremediation of petroleum-contaminated soil [11]. That study, however, used freshly spiked contamination in a neutral, organic matter-rich agricultural soil over 60 days and quantified oxidative enzymes (catalase and polyphenol oxidase), which report general microbial activity rather than the demand for specific nutrients. It remains unclear whether this complementarity occurs in infertile alkaline soils with aged field contamination, and whether these two agents target redundant or complementary microbial processes. It also remains untested whether either agent modulates microbial nutrient limitation, which controls the transformation of exogenous substrates into microbial degradative activity. Prior work in conservation and saline–alkali systems reported asynchronous effects of organic inputs and earthworms on microbial-community composition and enzyme function [12,13].

Ecoenzymatic stoichiometry, which builds on the established use of microbial diversity and extracellular enzyme activities as functional indicators of soil quality [14,15], converts the activities of C-, N-, and phosphorus (P)-acquiring enzymes into estimates of microbial C limitation (vector length) and relative N versus P limitation (vector angle) [14,16]. A vector angle of 45° has traditionally separated N from P limitation, but Cui et al. [17] recently argued that this threshold overestimates P limitation and proposed a vector length of 0.61 and an angle of 55° as more reliable criteria. Whether these thresholds apply in extreme environments such as alkaline, hydrocarbon-loaded loess is untested, and our own work on saline–alkali soils suggested systematic deviation [18]. Uncertainty about the assumptions underlying enzymatic C:N:P stoichiometry reinforces the need to test these thresholds against independent evidence [19].

We therefore conducted a 150-day soil-column experiment with a full factorial ryegrass × earthworm design on a moderately petroleum-contaminated loess soil. The objectives were (i) to quantify the separate and combined effects of ryegrass and earthworms on bacterial and fungal diversity and community composition, on C/N/P-acquiring enzyme activities, and on microbial nutrient limitation; (ii) to test whether hydrocarbon removal is accompanied by enrichment of hydrocarbon-degrading taxa; and (iii) to identify the pathways linking the two biological agents to hydrocarbon removal. We hypothesized that (H1) the combined treatment would give the greatest TPH removal; (H2) earthworms and ryegrass would act on different components of the microbial system, producing asynchronous responses of community composition and enzymatic function; and (H3) the combined treatment would intensify microbial C limitation.

2. Materials and Methods

2.1. Soil, Plant, and Earthworm Material

Petroleum-contaminated soil was collected from the 0–20 cm layer of an abandoned oil-well site at Ganguyi Town, Baota District, Yan'an, Shaanxi Province, China (36°42′29.21″ N, 109°48′43.37″ E). The well was operated from 2007 to 2020 and had been abandoned for four years at the time of sampling. The region has a warm–temperate continental monsoon climate with a mean annual temperature of 9.4 °C and a mean annual precipitation of 507 mm. The soil is a loessial soil, classified as a Calcaric Cambisol (Siltic) in the World Reference Base for Soil Resources. It has a silt-loam texture according to the USDA texture classification (clay, 4.0%; silt, 68.5%; sand, 27.5%). The initial soil was alkaline (pH 8.82) and infertile, with an electrical conductivity of 122.9 $\mu\text{S cm}^{-1}$, organic C of 5.79 g kg^{−1}, total N of 0.40 g kg^{−1}, total P of 0.56 g kg^{−1}, and Olsen-P of 5.32 mg kg^{−1}; with a total petroleum hydrocarbon (TPH) content of 1.88 g kg^{−1}, it is classified as moderately contaminated. The contaminating oil derives from a single production well of the Yan'an oilfield and is a light, low-sulfur, paraffin-based crude. Because the well had been abandoned for four years before sampling, the residual hydrocarbons are weathered, and the volatile and more soluble aromatic fractions are largely depleted relative to fresh crude, leaving a residue dominated by long-chain saturates and polar compounds. Perennial ryegrass (*Lolium perenne* L.) and the anecic earthworm *Metaphire tschiliensis* were used. Mature, active earthworms (mean fresh weight of 3.2 ± 0.9 g) were collected on the Yangjialing campus of Yan'an University, rinsed with distilled water, and kept on moist filter paper in darkness for 24–48 h to void their guts before introduction.

2.2. Experimental Design

A 2 × 2 full factorial design was used, with ryegrass (presence/absence) and earthworms (presence/absence) as factors, giving four treatments: CK (neither), E (earthworms only), R (ryegrass only), and RE (both). Each treatment was replicated five times, for a total of 20 soil columns. Columns were PVC cylinders of 10.4 cm internal diameter and 15 cm height, packed to a soil height of 10 cm at a bulk density of 1.3 g cm^{−3} in layers, over a PVC drainage disc and filter paper. Column dimensions were selected to set the soil depth to the sampled 0–20 cm contaminated layer, where ryegrass roots and earthworm activity concentrate. The 5 cm headspace accommodated shoot bases and gauze covers to prevent earthworm escape, yielding ~1.1 kg soil per column—sufficient for the full suite of chemical, enzymatic, and molecular analyses in one destructive harvest. Earthworms were added at two individuals per column, and ryegrass was sown in seven evenly spaced clusters of four seeds per column. Soil water content was adjusted to field capacity (24.3%, w/w) before packing and maintained the gravimetrically throughout.

Ryegrass was sown on 4 May 2024; columns without ryegrass received an equivalent quantity of heat-killed seed. Seedlings were supplemented with full-spectrum LED lighting. After 85 days of pre-incubation, the earthworms were introduced into each of ten randomly selected columns (five with and five without ryegrass). Column tops were covered with gauze-wrapped wire mesh to prevent escape without shading the plants. Incubation continued for a further 65 days, giving a total duration of 150 days, and destructive sampling took place on 28 September 2024.

At harvest, earthworms were recovered by hand, counted, and released. All earthworms survived to harvest. Ryegrass established in all planted columns, yet growth was sparse, with total biomass < 1 g per column, consistent with low soil fertility and hydrocarbon loading. Aboveground and root biomass did not differ significantly between ryegrass-only and combined treatments ($p > 0.05$). The whole soil of each column was

mixed; a 50 g fresh subsample was stored at -80°C for DNA extraction and enzyme assays, and the remainder was air-dried and sieved to 1 mm and 0.25 mm for chemical analysis.

2.3. Soil Chemical Analyses

TPH was determined gravimetrically [20]. Briefly, 5.0 g of air-dried soil in a 50 mL centrifuge tube was sequentially extracted with 20 mL n-hexane, 20 mL dichloromethane, and 20 mL trichloromethane. Each extraction involved 5 min of shaking and 5 min of centrifugation at 4000 rpm, and then the three supernatants were pooled. The pooled extract was filtered through anhydrous Na_2SO_4 into a pre-weighed constant-mass beaker. Solvents were evaporated at 60°C to constant mass. After 30 min of cooling, the beaker was weighed, and TPH was calculated as the mass gain of the beaker per unit soil mass. Soil pH was measured at a water:soil ratio of 2.5:1, and electrical conductivity (EC) was measured with a conductivity meter. Organic C was determined by dichromate oxidation [21], total N by Kjeldahl digestion with an ammonia electrode [22] and total P colorimetrically after $\text{H}_2\text{SO}_4\text{--HClO}_4$ digestion [23]. Olsen-P was extracted with 0.5 M NaHCO_3 . Ammonium and nitrate N were extracted with 2 mol L^{-1} KCl and quantified on an AA3 continuous-flow analyzer; available N is reported as the sum of the two.

2.4. DNA Extraction, Sequencing, and Diversity Analysis

Total DNA was extracted with a DNeasy PowerSoil Kit (QIAGEN, Venlo, The Netherlands), following the manufacturer's instructions, and DNA quality and quantity were checked with a NanoDrop ND-1000 spectrophotometer and by agarose gel electrophoresis. The V3–V4 region of the bacterial 16S rRNA gene and the ITS2 region of the fungal rRNA gene were amplified, and paired-end sequencing was performed on an Illumina platform. Sequences were processed in QIIME 2 (quality filtering, denoising, and chimera removal) and resolved into amplicon sequence variants (ASVs); taxonomy was assigned against the SILVA (bacteria) and UNITE (fungi) databases. ASVs accounting for less than 0.001% of total reads were discarded, leaving 11,246 bacterial and 2867 fungal ASVs. Sequencing depth ranged from 34,028 to 80,775 reads per sample for bacteria and from 76,142 to 144,398 for fungi; libraries were rarefied to the smallest depth before α -diversity calculation, and relative abundances were used for compositional analyses. Observed ASVs, Chao 1, Shannon, and Simpson indices were calculated in QIIME 2.

2.5. Enzyme Activities and Coenzymatic Stoichiometry

Two C-acquiring enzymes (β -1,4-glucosidase, BG; β -D-cellobiosidase, CBH), two N-acquiring enzymes (β -1,4-N-acetylglucosaminidase, NAG; L-leucine aminopeptidase, LAP), and one P-acquiring enzyme (alkaline phosphatase, AP) were assayed fluorometrically using 4-methylumbelliferone and 7-amino-4-methylcoumarin substrates, following Sinsabaugh et al. [14] and Cui et al. [16]. Microbial nutrient limitation was quantified with the vector model:

$$x = (BG + CBH) / (BG + CBH + AP) \quad (1)$$

$$y = (BG + CBH) / (BG + CBH + NAG + LAP) \quad (2)$$

$$\text{Vector length} = \sqrt{(x^2 + y^2)} \quad (3)$$

$$\text{Vector angle} = \text{Degrees}(\text{Atan2}(x, y)) \quad (4)$$

Vector length increases with microbial C limitation. The vector angle expresses relative N versus P limitation; results are interpreted against both the conventional 45° threshold and the 55° threshold proposed by Cui et al. [17].

2.6. Statistical Analysis

All variables were checked for outliers, normality, and homogeneity of variance. Treatment effects were tested by two-way ANOVA with ryegrass, earthworms, and their interaction as fixed factors, and means were separated by Tukey's HSD test ($p < 0.05$). Community dissimilarity was calculated as Bray–Curtis distance on relative abundances and visualized by principal coordinates analysis; the effects of the two factors were tested by PERMANOVA with 4999 permutations. The influence of soil variables on community composition was assessed by distance-based redundancy analysis (db-RDA), with NO_3^- -N, TPH, and pH as constraining variables; retained after collinearity screening; and tested by 1999 permutations. Hydrocarbon-degrading taxa were identified by matching genus-level assignments against a curated list of 63 bacterial and 30 fungal genera reported in the literature to degrade petroleum hydrocarbons, and their summed relative abundance is referred to as the degrader guild. This assignment is taxonomic and literature-based: it identifies taxa whose described relatives degrade petroleum hydrocarbons. All amplicon data are compositional; therefore, all abundances reported here represent relative abundance. Relationships between individual genera and functional variables were examined with Spearman correlation. The magnitude of the two experimental effects was compared across all response variables using partial η^2 from the two-way ANOVA models, calculated as $\text{SS}_{\text{(effect)}}/(\text{SS}_{\text{(effect)}} + \text{SS}_{\text{(error)}})$, which places contaminant, chemical, community, and enzymatic responses on a common scale. Community composition was summarized for this purpose by the first PCoA axis of the corresponding Bray–Curtis matrix, and the copiotrophic guild as the summed relative abundance of *Aeromonas*, *Flavobacterium*, *Chitinibacter*, *Arenimonas*, and *Pseudomonas*. Analyses were performed in R 4.5.2 with the vegan, agricolae, and ggplot2 packages.

3. Results

3.1. Hydrocarbon Removal and Soil Chemical Properties

Ryegrass was the only significant driver of hydrocarbon removal ($p < 0.001$; Table 1). Residual TPH declined in the order of CK (1.88 g kg^{-1}) > E (1.79) > R (1.51) > RE (1.38), corresponding to a 26.6% reduction under RE relative to CK. Neither the earthworm main effect ($p = 0.168$) nor the interaction ($p = 0.814$) was significant.

Table 1. Effects of ryegrass (R) and earthworms (E) on total petroleum hydrocarbons (TPH) and soil chemical properties. Values are means $\pm$ standard error ($n = 5$). Different lowercase letters denote significant differences among treatments (Tukey's HSD, $p < 0.05$); the last three columns give p -values from two-way ANOVA. CK, control; E, earthworms; R, ryegrass; RE, ryegrass + earthworms.

Variable	CK	E	R	RE	R	E	R $\times$ E
TPH (g kg^{-1})	$1.88 \pm 0.08 \text{ a}$	$1.79 \pm 0.06 \text{ a}$	$1.51 \pm 0.12 \text{ b}$	$1.38 \pm 0.04 \text{ b}$	<0.001	0.168	0.814
pH	$8.81 \pm 0.03 \text{ b}$	$8.70 \pm 0.04 \text{ c}$	$8.96 \pm 0.02 \text{ a}$	$8.83 \pm 0.02 \text{ b}$	<0.001	<0.001	0.805
EC ($\mu\text{S cm}^{-1}$)	$131.0 \pm 8.7 \text{ b}$	$174.3 \pm 8.0 \text{ a}$	$109.7 \pm 6.6 \text{ b}$	$137.7 \pm 6.4 \text{ b}$	0.001	<0.001	0.322
Organic C (g kg^{-1})	$5.98 \pm 0.17 \text{ a}$	$6.13 \pm 0.35 \text{ a}$	$6.31 \pm 0.49 \text{ a}$	$6.15 \pm 0.10 \text{ a}$	0.575	0.989	0.631
Total N (g kg^{-1})	$0.419 \pm 0.007 \text{ a}$	$0.414 \pm 0.007 \text{ a}$	$0.386 \pm 0.003 \text{ b}$	$0.405 \pm 0.005 \text{ a}$	0.002	0.230	0.063
Total P (g kg^{-1})	$0.567 \pm 0.004 \text{ a}$	$0.571 \pm 0.004 \text{ a}$	$0.578 \pm 0.016 \text{ a}$	$0.560 \pm 0.002 \text{ a}$	0.997	0.449	0.225
C:N	$14.3 \pm 0.3 \text{ a}$	$14.8 \pm 0.7 \text{ a}$	$16.3 \pm 1.3 \text{ a}$	$15.2 \pm 0.3 \text{ a}$	0.112	0.669	0.290
C:P	$10.5 \pm 0.3 \text{ a}$	$10.7 \pm 0.6 \text{ a}$	$10.9 \pm 0.8 \text{ a}$	$11.0 \pm 0.2 \text{ a}$	0.562	0.820	0.906
NH_4^+ -N (mg kg^{-1})	$2.97 \pm 0.71 \text{ a}$	$2.45 \pm 1.28 \text{ a}$	$1.84 \pm 0.68 \text{ a}$	$2.10 \pm 0.32 \text{ a}$	0.382	0.875	0.638
NO_3^- -N (mg kg^{-1})	$1.76 \pm 0.35 \text{ c}$	$59.07 \pm 7.57 \text{ a}$	$0.27 \pm 0.07 \text{ c}$	$26.74 \pm 6.97 \text{ b}$	0.005	<0.001	0.009
Available N (mg kg^{-1})	$4.73 \pm 0.60 \text{ c}$	$61.52 \pm 8.51 \text{ a}$	$2.12 \pm 0.68 \text{ c}$	$28.84 \pm 7.18 \text{ b}$	0.006	<0.001	0.016
Olsen-P (mg kg^{-1})	$5.26 \pm 0.27 \text{ b}$	$6.94 \pm 0.22 \text{ a}$	$5.25 \pm 0.31 \text{ b}$	$5.90 \pm 0.34 \text{ a}$	0.084	<0.001	0.092

Earthworms exerted a strong influence on nutrient availability. NO_3^- -N increased from 1.76 mg kg^{-1} in CK to 59.07 mg kg^{-1} in E ($p < 0.001$), with a significant interaction ($p = 0.009$) reflecting plant uptake in RE (26.74 mg kg^{-1}). Available N followed the same

pattern, and Olsen-P was raised by earthworms from 5.26 to 6.94 mg kg⁻¹ ($p < 0.001$). Ryegrass raised pH (8.96 in R versus 8.81 in CK, $p < 0.001$) and lowered EC and total N, whereas earthworms lowered pH and raised EC (both $p < 0.001$). Organic C, total P, and the C:N and C:P ratios were unaffected by either factor ($p > 0.11$).

3.2. Microbial α -Diversity

Neither factor significantly affected any bacterial α -diversity index ($p \geq 0.199$ for all; Figure 1A,C,E,G). Bacterial richness was high and uniform across treatments (1899–2139 observed ASVs; Shannon 9.20–9.42). Fungal richness showed a marginal positive earthworm effect (observed ASVs $p = 0.054$; Chao 1 $p = 0.058$), the highest values occurring in RE (407 observed ASVs versus 328 in CK), but no effect reached significance (Figure 1B,D,F,H).

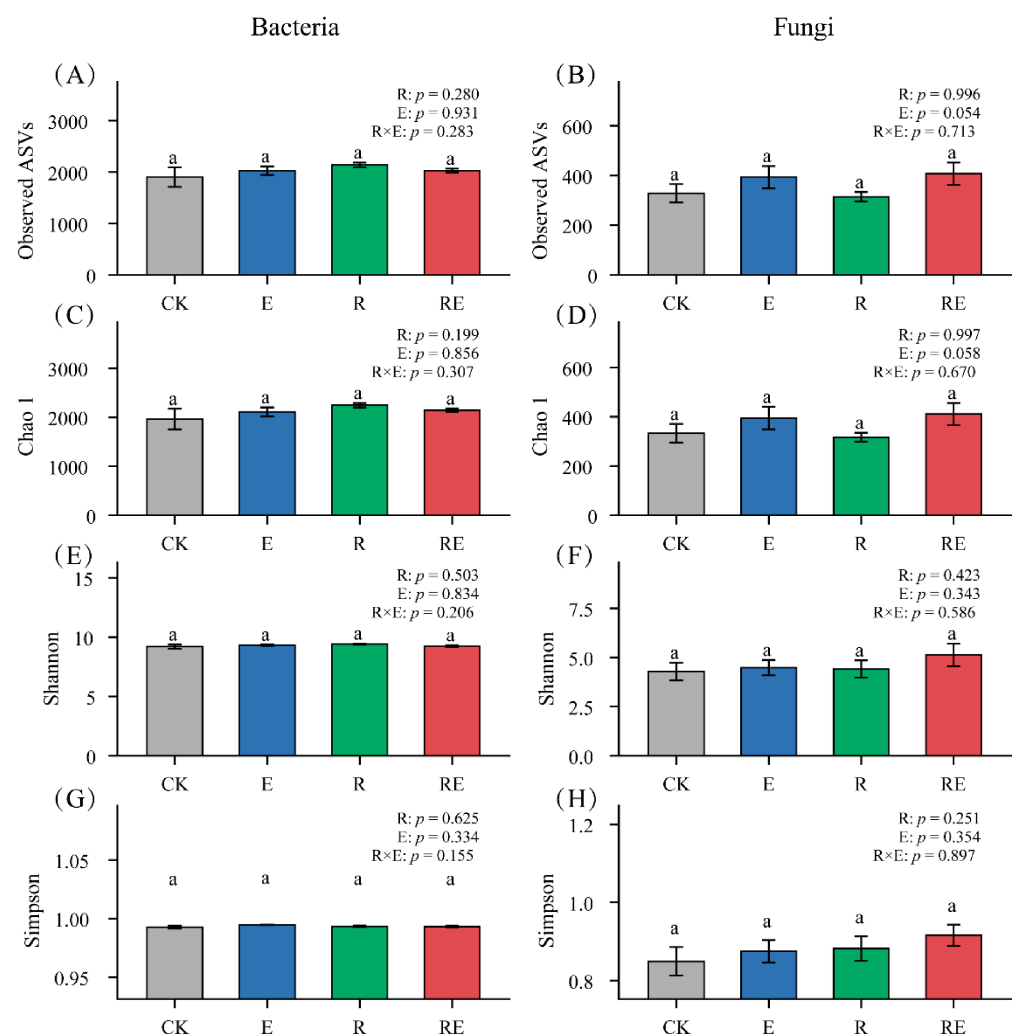


Figure 1. Alpha diversity (observed ASVs, Chao 1, Shannon, and Simpson indices; mean $\pm$ SE, $n = 5$) of soil bacteria (A,C,E,G) and fungi (B,D,F,H) under the four treatments. CK, control; E, earthworms; R, ryegrass; RE, ryegrass + earthworms. Identical letters indicate no significant difference (Tukey's HSD, $p < 0.05$); p -values from two-way ANOVA for the ryegrass (R) and earthworm (E) main effects and their interaction ($R \times E$) are given in each panel.

3.3. Community Composition

In contrast to α -diversity, community composition responded strongly, and in a factor-specific manner (Figure 2). For bacteria, earthworms explained 25.3% of compositional variation (PERMANOVA $p < 0.001$), while ryegrass had no detectable effect ($R^2 = 0.057$, $p = 0.262$). For fungi, the pattern was reversed: ryegrass was significant ($R^2 = 0.105$,

$p = 0.036$), whereas earthworms were only marginal ($R^2 = 0.093$, $p = 0.057$). Between 13% and 51% of fungal reads (maximum in CK) could not be assigned beyond the kingdom level. This large unassigned fraction is commonly observed for ITS amplicons obtained from degraded soils. Since the proportion of unassigned reads varied across treatments, relative abundances of the classified fungal phyla in Figure 2D should be compared only within treatments, not between treatments. The ordination and db-RDA analyses were performed at the ASV level and were thus unaffected by this limitation. For bacteria, earthworms increased the relative abundance of *Proteobacteria* from 26.3% to 36.7%, and *Bacteroidota* from 0.25% to 3.74% ($p < 0.001$), while reducing the relative abundances of *Acidobacteriota*, *Actinobacteriota*, *Chloroflexi*, and *Gemmatimonadota* ($p < 0.01$). This shift from typically oligotrophic to copiotrophic phyla reflects changes in relative abundance alone and does not demonstrate alterations in absolute abundance.

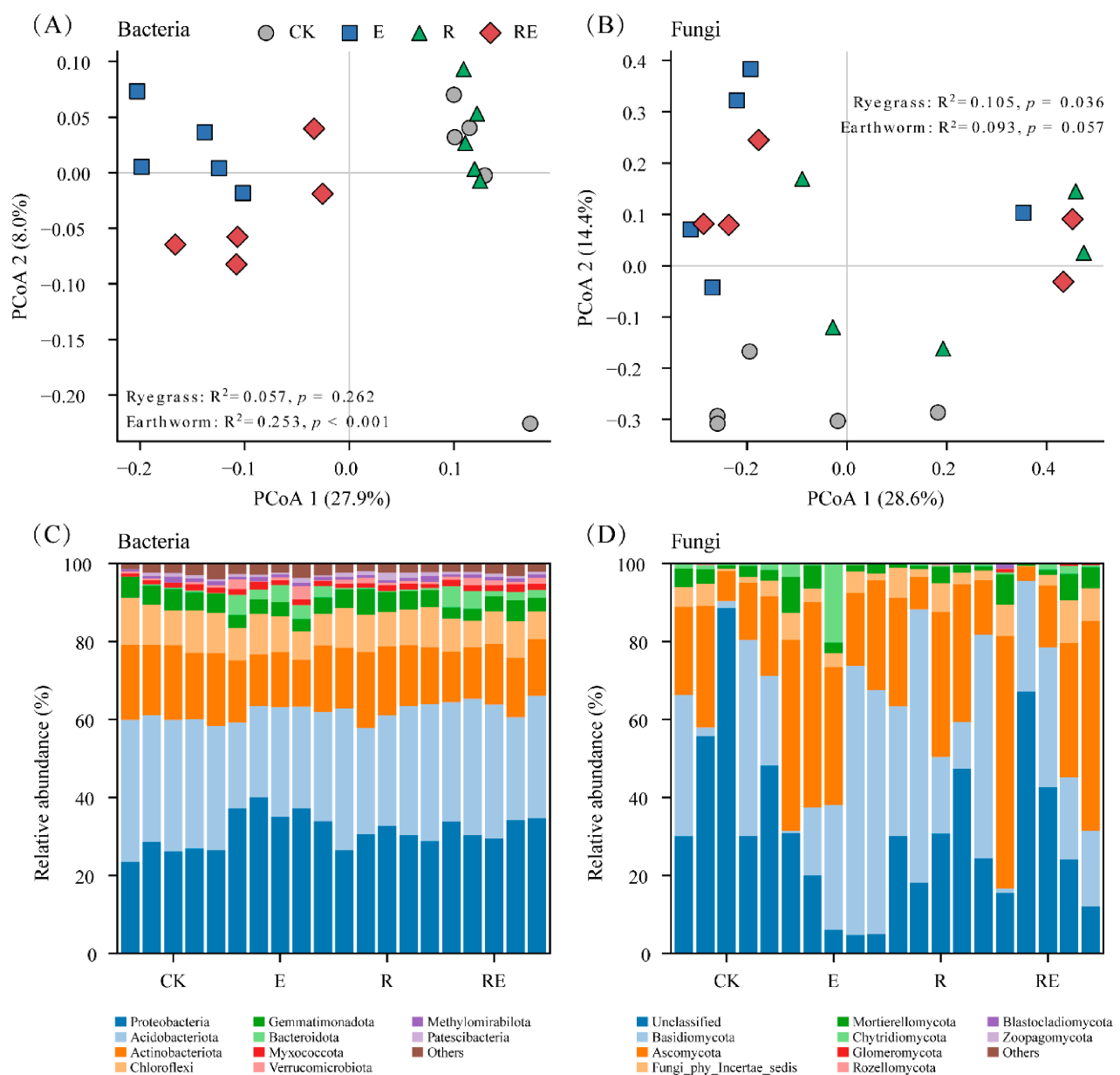


Figure 2. Composition of the soil bacterial and fungal communities. (A,B) Principal coordinates analysis based on Bray–Curtis dissimilarity, with PERMANOVA results for the rye grass and earthworm factors (4999 permutations). (C,D) Relative abundance of the ten most abundant phyla in each of the 20 columns, grouped by treatment.

Distance-based redundancy analysis confirmed this asymmetry (Figure 3). For bacteria, the constrained model explained a significant fraction of variation (adjusted $R^2 = 0.213$, $p < 0.001$), with NO_3^- -N being the strongest single constraint ($R^2 = 0.087$, $p < 0.001$), followed by TPH ($R^2 = 0.057$, $p = 0.020$); pH was not significant. For fungi, the model was weaker but significant (adjusted $R^2 = 0.115$, $p = 0.012$), with NO_3^- -N, again, being significant ($p = 0.032$), pH being marginal ($p = 0.059$), and TPH not being significant ($p = 0.279$). Nitrate, the variable most strongly controlled by earthworms, was thus the principal chemical correlate of community composition in both kingdoms, whereas hydrocarbon content mattered only for bacteria.

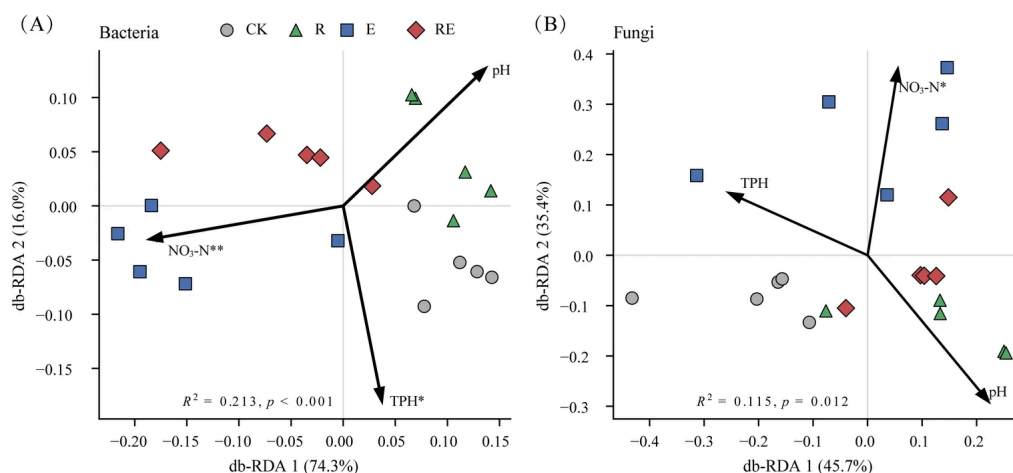


Figure 3. Distance-based redundancy analysis of the bacterial (A) and fungal (B) communities constrained by NO_3^- -N, TPH, and pH. Arrows are the constraining variables; asterisks denote the significance of each term in a marginal permutation test (*, $p < 0.05$; **, $p < 0.01$). Model statistics are given in each panel.

3.4. Extracellular Enzyme Activities

The five enzymes responded to different factors (Figure 4). BG was highest under RE (14.84 $\text{nmol g}^{-1} \text{h}^{-1}$, 64% above CK), with a significant ryegrass main effect ($p = 0.002$) and, importantly, a significant ryegrass $\times$ earthworm interaction ($p = 0.017$); earthworms alone slightly depressed BG but increased it markedly in the presence of ryegrass. CBH showed a ryegrass main effect only ($p = 0.020$). NAG was driven exclusively by earthworms, rising by 144% from 2.45 to 5.98 $\text{nmol g}^{-1} \text{h}^{-1}$ ($p < 0.001$), with no ryegrass effect ($p = 0.413$). LAP and AP were not significantly affected by either factor ($p \geq 0.062$), although absolute activities were high (LAP 71–81; AP 98–115 $\text{nmol g}^{-1} \text{h}^{-1}$).

3.5. Coenzymatic Stoichiometry and Microbial Nutrient Limitation

All samples clustered close to the origin of the enzymatic vector plane, with x between 0.076 and 0.124, and y between 0.10 and 0.16 (Figure 5A). Vector length, the index of microbial C limitation, was highest under RE (0.203) and lowest under E (0.128), with a significant ryegrass effect ($p = 0.008$) and a significant interaction ($p = 0.011$; Figure 5B). Vector angles ranged from 51.7° to 53.7° and were unaffected by either factor ($p \geq 0.419$; Figure 5C). They therefore exceeded the conventional 45° threshold, which would indicate P limitation, but they fell below the 55° threshold proposed by Cui et al. [17], which would indicate N limitation. All vector lengths were far below the proposed C-limitation threshold of 0.61.

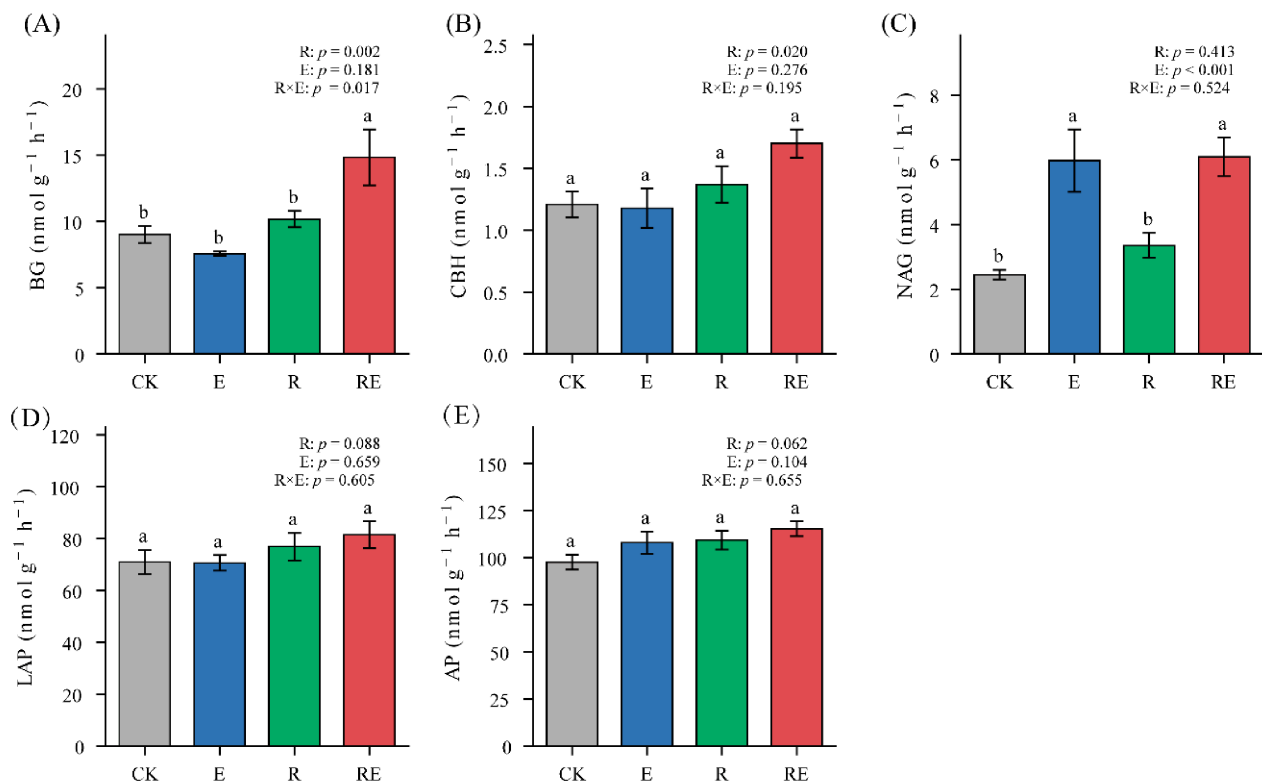


Figure 4. Activities (mean $\pm$ SE, $n = 5$) of β -1,4-glucosidase (BG (A)), β -D-cellobiosidase (CBH (B)), β -1,4-N-acetylglucosaminidase (NAG (C)), L-leucine aminopeptidase (LAP (D)), and alkaline phosphatase (AP (E)). Different lowercase letters indicate significant differences among treatments at $p < 0.05$.

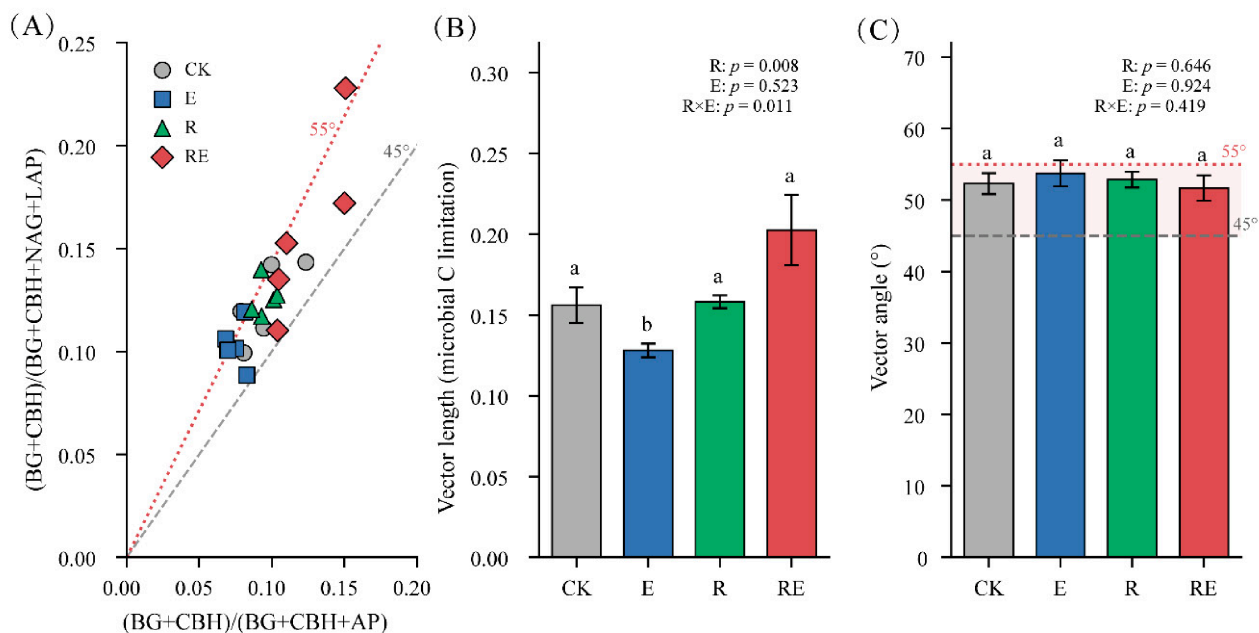


Figure 5. Ecoenzymatic stoichiometry. (A) Relative proportions of C versus P acquisition (x) and C versus N acquisition (y); the dashed grey line marks the conventional 45° threshold, and the dotted red line the 55° threshold of Cui et al. (2024) [17]. (B) Vector length, indexing microbial C limitation. (C) Vector angle; the shaded band delimits the interval between the two thresholds, within which the diagnosis of N versus P limitation is ambiguous. Different lowercase letters indicate significant differences among treatments at $p < 0.05$.

3.6. Hydrocarbon-Degrading Taxa

The bacterial-degrader guild comprised 537 ASVs and accounted for 6.9–8.3% of the community. Contrary to expectation, its total relative abundance was not increased by either treatment; earthworms, in fact, reduced it slightly ($p = 0.039$), and ryegrass had no effect ($p = 0.719$; Figure 6A). Critically, the relative abundance of the guild was uncorrelated with residual TPH across all 20 columns ($r = 0.190$, $p = 0.424$; Figure 6B). The fungal guild behaved similarly ($p \geq 0.237$).

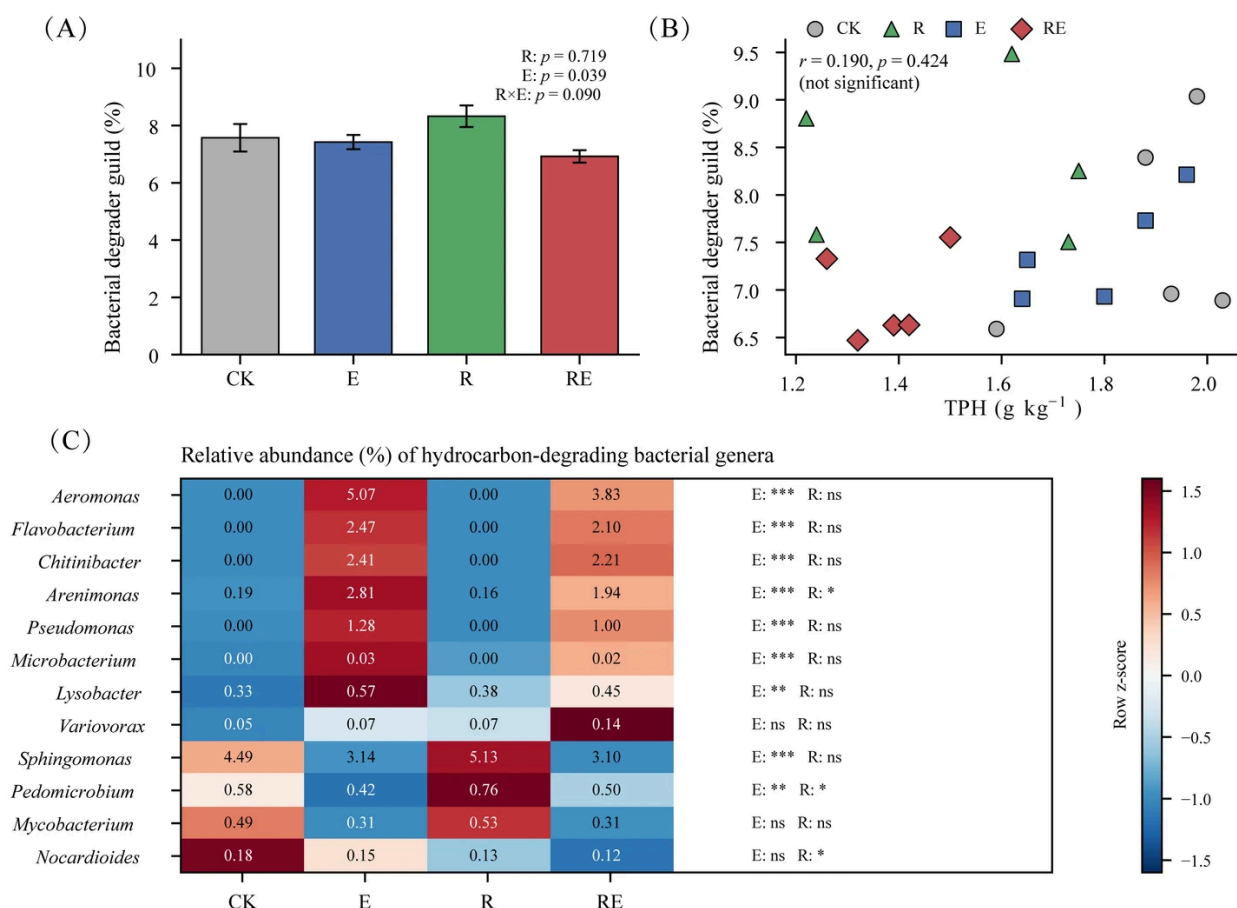


Figure 6. Hydrocarbon-degrading taxa. (A) Total relative abundance of the bacterial degrader guild. (B) Relationship between guild abundance and residual TPH across all 20 columns. (C) Relative abundance (%) of individual hydrocarbon-degrading bacterial genera; the fungal guild showed no significant treatment effect; cell color is the row z-score, and the annotation on the right gives the significance of the earthworm (E) and ryegrass (R) main effects (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant).

The composition of the guild, however, was transformed by earthworms (Figure 6C). Five copiotrophic genera that were essentially absent without earthworms—*Aeromonas* (0 to 5.07%), *Flavobacterium* (0 to 2.47%), *Chitinibacter* (0 to 2.41%), *Arenimonas* (0.19 to 2.81%), and *Pseudomonas* (0.003 to 1.28%)—together came to occupy about 14% of the bacterial community (all $p < 0.001$), while the oligotrophic *Sphingomonas* declined from 4.49% to 3.15% ($p < 0.001$). Ryegrass significantly reduced *Nocardioides* ($p = 0.014$) and increased *Altererythrobacter* ($p = 0.024$). *Variovorax* showed the highest abundance under RE (0.14%, about three times CK); however, this trend was not statistically significant ($p = 0.116$ –0.147).

3.7. Relative Magnitude of the Two Effects, and Linking Taxa, Nutrients, and Function

Placing all response variables on a common effect-size scale makes the asymmetry between the two agents explicit (Figure 7A). Ryegrass dominated hydrocarbon removal (partial $\eta^2 = 0.60$, $p < 0.001$) whereas the earthworm effect was small and non-significant ($\eta^2 = 0.12$). The pattern reversed completely for bacteria: earthworms explained 92% of the variation in bacterial community composition ($p < 0.001$), 89% in the copiotrophic guild, and 73% in both *Proteobacteria* and *Bacteroidota*, while ryegrass explained 1–14% and never reached significance. Bacterial α -diversity indices had effect sizes of 0.00–0.10 for both factors, and the largest value for any fungal index was 0.21 (Chao 1, earthworm effect, $p = 0.058$), so that the contrast between a near-complete restructuring of bacterial composition and the near-absence of any diversity response is visible within a single panel. Fungal composition leaned the other way, with ryegrass explaining 18% against 3% for earthworms, although only marginally ($p = 0.077$).

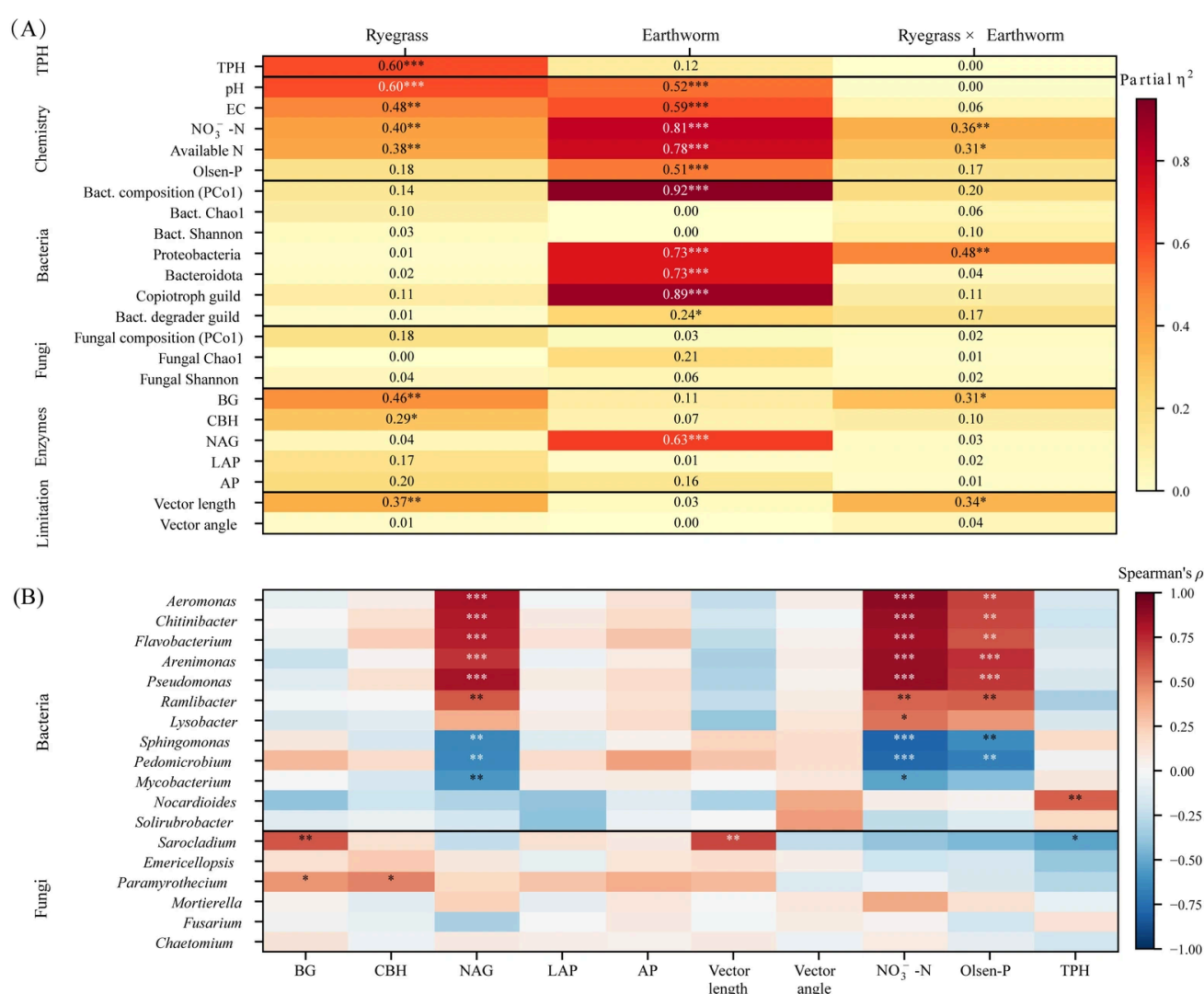


Figure 7. Relative magnitude and specificity of the two effects. (A) Partial η^2 for the ryegrass and earthworm main effects and their interaction on every response variable, grouped by category; asterisks give the significance of the corresponding term in the two-way ANOVA. Community composition is represented by the first PCoA axis of the Bray–Curtis matrix, and the copiotrophic guild by the summed relative abundance of the five earthworm-associated genera. (B) Spearman correlations between the relative abundance of selected bacterial and fungal genera and enzyme activities, vector indices, nutrient availability, and TPH. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

The enzymes divided along the same lines: NAG was almost entirely an earthworm effect ($\eta^2 = 0.63$, $p < 0.001$), and BG almost entirely a ryegrass effect ($\eta^2 = 0.46$, $p = 0.002$). The interaction term, negligible for most variables, was substantial and significant precisely where the two agents meet, BG ($\eta^2 = 0.31$, $p = 0.017$), microbial C limitation ($\eta^2 = 0.34$, $p = 0.011$), nitrate ($\eta^2 = 0.36$, $p = 0.009$), and *Proteobacteria* ($\eta^2 = 0.48$, $p = 0.001$), and was essentially zero for TPH ($\eta^2 = 0.004$).

Spearman correlations resolved two coherent blocks (Figure 7B). The earthworm-associated copiotrophs (*Aeromonas*, *Chitinibacter*, *Flavobacterium*, *Arenimonas*, *Pseudomonas*, and *Ramlibacter*) correlated strongly and positively with NO_3^- -N, Olsen-P, and NAG activity, whereas the oligotrophs (*Sphingomonas*, *Pedomicrobium*, and *Mycobacterium*) correlated negatively with the same variables. Neither block was related to C-acquiring enzymes or to vector length. Among fungi, *Sarocladium* was positively correlated with BG ($\rho = 0.618$, $p = 0.004$) and with vector length ($\rho = 0.657$, $p = 0.002$) and negatively with TPH ($r = -0.494$, $p = 0.027$).

4. Discussion

4.1. Ryegrass, Not Earthworms, Drove Hydrocarbon Removal

Hydrocarbon removal was attributable to ryegrass alone. Root exudation of organic acids and sugars supplies labile C, which primes co-metabolic degradation of hydrocarbons, improves rhizosphere aeration, and sustains a metabolically active microbial population. Earthworms are reported to accelerate hydrocarbon removal in other systems, and our inability to replicate this effect warrants discussion. The two most directly comparable studies are informative precisely because they differ from ours in specific, identifiable ways. Nobili et al. [24] reported 45.2–60.8% reductions in TPHs using *Eisenia fetida* and *Amyntas morrisi*, compared with a 16.1% reduction in treatments without earthworms. However, their experiment employed a substrate deliberately amended with lombricompost, rice hulls, and wheat stubble; the authors explicitly attributed earthworm survival and performance to this supplemental food source. Li et al. [11] likewise found that *Eisenia foetida* raised removal efficiency above single phytoremediation (for example, 43.4% versus 37.6% with *Impatiens balsamina*), yet reported that hydrocarbon concentration “decreased negligibly in the earthworm group” in the absence of plants—exactly the pattern we observed. In both studies, the earthworm benefits therefore required either an exogenous organic substrate or a plant; neither demonstrates hydrocarbon removal by earthworms acting alone. Vermiremediation with indigenous earthworm species has likewise been applied to engine oil-contaminated soil [3], but, again, in a substrate managed to support the earthworms.

Three explanations for the weaker earthworm effect in our system are plausible. First, our soil contained 1.88 g TPH kg^{-1} , above the 28-day LC_{50} of 916 mg measured TPH kg^{-1} reported for *Eisenia fetida* in diesel-contaminated soil, at which clitellar swelling and curling also appear [25]. Although aged field petroleum contamination has much lower bioavailability than freshly spiked diesel, and all earthworms were recovered alive, survival alone does not confirm an absence of physiological impacts. Since we did not measure individual biomass changes, cocoon production, or avoidance behavior, sublethal stress at this contaminant concentration remains a possibility that our dataset cannot test. We therefore present this as a plausible possibility rather than an established mechanism. Second, hydrophobic hydrocarbon fractions clog pores and degrade the habitat of an anecic species that requires stable burrows, and the small column volume further restricted burrowing. Third, and consistent with the comparison above, earthworms acting alone lack the continuous supply of root- or amendment-derived substrate needed to convert enhanced nutrient availability into hydrocarbon mineralization. H1 is therefore only partly

supported: the combined treatment gave the lowest residual TPH, but the improvement over ryegrass alone was not statistically significant, and no synergistic effect on TPH could be demonstrated.

4.2. Decoupling of Community Composition from Function

The most robust result of this study is the dissociation between community structure and ecosystem function. Microbial diversity is widely assumed to underpin ecosystem multifunctionality [26], yet, here, α -diversity was unresponsive to both agents, with only a marginal earthworm effect on fungal richness, while β -diversity responded strongly and specifically: earthworms restructured bacteria, and ryegrass restructured fungi. Comparable habitat- and context-dependent effects of earthworms on microbial communities have been reported previously [27–29]. Such divergence is expected where a disturbance selectively promotes a subset of taxa without eliminating others, so that richness is conserved while relative abundances shift. By contrast, in freshly organic matter-rich agricultural soil, *Eisenia foetida* raised operational taxonomic unit richness by roughly 40% and increased *Actinobacteria* while reducing *Proteobacteria* [11]. Our alkaline, infertile, and aged-petroleum-contaminated loess showed no richness response and the reverse phylum-level shift, with *Proteobacteria* rising from 26.3% to 36.7%. The direction of the earthworm effect on the bacterial community therefore appears to depend on the fertility and contamination history of the receiving soil rather than being a property of earthworm activity as such. The bacterial shift was a copiotroph–oligotroph transition driven by the sudden availability of labile C and N in casts, matching earlier reports that organic input activates dormant, fast-growing microorganisms [30]. The taxonomic detail corresponds closely to what Yang et al. [31] observed during earthworm gut passage and cast ageing: the principal beneficiaries there were *Bacteroidota* and, within them, *Flavobacteriales*, with an estimated generation time of about 2 h, together with *Gammaproteobacteria*, while *Alphaproteobacteria* declined. In our soil, *Bacteroidota* rose fifteen-fold (0.25% to 3.74%), *Flavobacterium* appeared from effectively zero to 2.47%, and the gammaproteobacterial genera *Aeromonas* and *Pseudomonas* rose from near zero to 5.07% and 1.28%. The fact that the same taxa respond in an alkaline, hydrocarbon-loaded loess and in a temperate agricultural mesocosm suggests a conserved, gut passage-driven selection rather than a site-specific response. Yang et al. [31] further showed that these shifts persist for at least 168 days, so the assemblage we recovered at harvest is unlikely to be a transient artefact of sampling date. In addition, casts are transient hotspots in which microbial biomass is built up and turned over rapidly, converting substrate into stabilized microbial necromass [32]. The fact that the fungi responded instead to the plant is consistent with their reliance on more complex, root-derived organic matter [33,34].

The degrader-guild analysis extends this decoupling to the functional group most directly relevant to remediation. The relative abundance of the guild neither increased under the treatments that removed most hydrocarbon nor correlated with residual TPH. This suggests the taxonomic abundance of putative degraders could not predict TPH dissipation and provides no evidence of increased per-degrader activity. We hypothesize that degradation arose from activation, rather than numerical expansion, of resident degraders; this hypothesis awaits further testing via catabolic gene expression (e.g., *alkB* and ring-hydroxylating dioxygenases), isotopic mineralization assays, or qPCR/biomass measurements to estimate absolute abundances. Two caveats apply. The guild is defined by the genus-level literature matches, and hydrocarbon catabolism is strain-specific, making genus identity only a functional proxy. Furthermore, compositional amplicon data mean that unchanged relative abundance does not exclude shifts in absolute abundance. The hydrolytic enzymes measured here index that activation only indirectly: BG was 46%

higher under RE than under R, yet residual TPH did not differ significantly between the two treatments (1.38 versus 1.51 g kg⁻¹, $p = 0.313$). A plausible explanation is that the rate-limiting step is a specific oxygenase-mediated oxidation, which no hydrolytic assay reports. Non-degradative sinks are unlikely to account for much: using ¹³C natural abundance, Liu et al. [4] showed that 84.7–88.5% of the hydrocarbon removed during bio-stimulation was mineralized rather than assimilated or humified, so root uptake and sorption can, at best, be minor terms. The most conservative interpretation is therefore that ryegrass promoted hydrocarbon removal via a pathway not indexed by the enzymes measured in this study. The behavior of *Nocardioide*s provides a complementary line of evidence for the same conclusion. *Nocardioide*s, with *Sphingomonas*, is among the principal genera mediating straight-chain alkane degradation in petroleum-contaminated profiles [35], and its proliferation coincided with the highest degradation rate during the N-stimulated stage of a stepwise remediation, alongside *Lysobacter* [36]. In our soil, however, *Nocardioide*s was most abundant where most hydrocarbon remained ($r = 0.611$, $p = 0.004$). The two observations are reconciled if abundance tracks substrate supply rather than realized degradation: *Nocardioide*s is enriched where alkanes are plentiful, which, at a 150-day endpoint, means the least remediated columns. Reports that equate degrader abundance with remediation potential should be treated with corresponding caution.

4.3. Complementary Control of C-, N-, and P-Acquiring Enzymes

The enzyme data reveal a clear functional division of labor: C-acquiring enzymes responded to ryegrass, reflecting the input of cellulosic root material and exudates; and NAG responded exclusively to earthworms. Its substrate, N-acetylglucosamine, derives from fungal chitin and from bacterial peptidoglycan, that is, largely from microbial necromass. Earthworm casts are precisely the compartment in which microbial biomass is built up and turned over most rapidly [32], so a rise in NAG is the expected enzymatic signature of cast formation. The strong enrichment of *Chitinibacter* provides a direct taxonomic correlate. Earthworms have previously been shown to raise soil enzyme activities, including dehydrogenase and urease, in diesel-contaminated soil; however, nitrification in that system was inhibited in a dose-dependent manner even in their presence [25]; the thirty-fold nitrate accumulation we observed indicates that nitrification was not similarly suppressed at our lower and aged contamination level. The pattern is also consistent with the finding that earthworms stimulate soil C mineralization chiefly by increasing substrate availability rather than by altering community structure [37]. Earthworms and roots also interact physically in building macroaggregates [38], and earthworm activity can simultaneously accelerate decomposition and promote stabilization of plant-derived C [39]. AP did not respond to either factor, which is expected in a calcareous soil of pH 8.8, where P availability is governed by calcium binding rather than by biological demand.

The interaction terms locate the point at which the two agents meet. A significant interaction arose only for BG and for vector length, that is, only within the C-acquisition pathway. Earthworms alone slightly depressed BG, and ryegrass alone raised it modestly; only in combination did BG increase sharply. This experiment reveals an association between earthworm addition, elevated NO₃⁻-N, and increased BG activity. We hypothesize that the extra C demand generated within the rhizosphere is allocated toward enzyme synthesis only once N limitation is alleviated by earthworm-derived nitrate—an interpretation consistent with, though not proven by, our dataset, since the design cannot establish the causal sequence directly. Fresh plant-derived C is a well-documented trigger of microbial activity in C-limited soils [40], and enzymatic responses to organic inputs depend strongly on the ambient substrate context [41]. This interpretation aligns with H2 and H3 and explains why the combined treatment, despite lacking synergistic effects on TPH, yielded the

highest C-acquiring enzyme activity and the strongest microbial C limitation. A factorial N-addition experiment would be required for direct validation of this hypothesis.

4.4. Nitrogen Rather than Phosphorus Limitation, and the Vector Thresholds

Vector angles of 51.7–53.7° fall between the conventional 45° and the revised 55° threshold, so that the diagnosis of nutrient limitation depends entirely on which criterion is adopted. Four independent lines of evidence are consistent with N rather than P limitation. First, the ratio of available N to Olsen-P was 0.90 in CK and only 0.43 under ryegrass, an order of magnitude below values typical of arable soils. Second, hydrocarbon contamination represents a massive input of C, with an extremely wide C:N ratio, and negligible N and P, a situation for which N supplementation is standard practice in bioremediation. The response is nevertheless dose-dependent: adjusting an oil-contaminated soil to a C:N ratio of 100:2 gave the highest TPH removal (32.6%), whereas a ten-fold-higher N dose reduced removal to 22.6% and inhibited earthworm growth by 60% [42]. Relief of N limitation, not N saturation, is what the present system appears to require. Third, in an alkaline soil, the assay conditions for alkaline phosphatase coincide with the soil pH, so measured AP activity (98–115 nmol g⁻¹ h⁻¹, an order of magnitude above BG) is systematically inflated relative to actual P demand; both x and y are consequently compressed toward the origin, and the angle becomes highly sensitive to small biases in the denominators. Fourth, the functional response is that expected under N limitation: supplying nitrate through earthworms unlocked a 45% increase in BG in the presence of ryegrass. If the system were P-limited, an N input should not have released C acquisition in this way.

Taken together, these observations provide evidence consistent with N limitation, or with co-limitation by N and C in which N dominates, and suggest that the 45° threshold overestimates P limitation under alkaline conditions. This interpretation rests on indirect, converging evidence rather than direct measurement, as no factorial N and P addition experiment was performed. This conclusion aligns in direction with Cui et al. [17] and with our own observations in saline-alkali soils, where vector lengths likewise remained far below 0.61 and angles clustered between 45° and 55° [18]. Systematic recalibration of these thresholds for extreme soils merits testing across a broader range of alkaline, hydrocarbon-contaminated soils. A factorial N and P amendment experiment represents the clear next step and is identified as a priority for future research.

4.5. Implications for the Remediation of Petroleum-Contaminated Loess

Three practical conclusions follow. First, plant establishment should be the priority: without ryegrass, earthworm introduction alone did not accelerate hydrocarbon removal despite substantially improving nutrient status. Second, earthworms are nevertheless valuable in combination, because they relieve the N constraint that otherwise limits rhizosphere carbon acquisition and because they introduce a fast-growing, copiotrophic bacterial assemblage. Third, because the combined treatment produced the strongest microbial C limitation, inputs of organic material containing both C and N are a logical next step to test. This recommendation requires two qualifications drawn from recent work. Organic amendment is not uniformly beneficial: in a stepwise remediation, adding organic fertilizer after an initial N stage reshaped the community, reduced the key degraders, and lowered the degradation rate from 65.3 to 25.0 mg kg⁻¹ d⁻¹ while raising earthworm mortality to 100% [36]. Excessive mineral N is likewise counterproductive and toxic to earthworms [42]. Where earthworms are part of the remediation system, a plausible design principle is therefore to dose the amendment so that it relieves N limitation while also serving as an earthworm food source, as in the composted substrate that sustained vermiremediation in Nobili et al. [24], rather than applying it at the highest rate the soil will accept. This

proposal should be evaluated in a dedicated factorial experiment, with plant, earthworm, and amendment treatments crossed, and with hydrocarbon fractions and ecotoxicological endpoints measured, before it is recommended for field use.

4.6. Limitations of This Study

Several limitations constrain the inferences drawn above. The experiment employed five biological replicates per treatment, and although doing so supports the detection of the large main effects reported herein, it also provides limited statistical power for small effect sizes. Coupled with the large number of response variables, genus-level comparisons, and correlation analyses, this introduces a multiple-testing issue; accordingly, these analyses are regarded as exploratory rather than confirmatory. This study investigated a single soil type with one moderate level of aged-petroleum contamination. Therefore, the findings cannot be extrapolated to fresh oil spills, acidic soils, high-organic-matter soils, or heavily contaminated sites without additional validation. The contaminant oil was characterized solely by bulk TPH concentration. Given that saturate, aromatic, resin, and asphaltene fractions differ markedly in bioavailability and degradation rates, the observed shifts cannot be assigned to any individual fraction.

Sampling was destructive and restricted to a single endpoint at day 150. Consequently, the experimental design cannot resolve the temporal sequence of the described processes: it cannot distinguish whether earthworms stimulated degradation at an early stage before the effect faded, whether ryegrass effects emerged early or late, or whether bacterial community restructuring preceded nitrate accumulation and whether enzymatic shifts occurred prior to TPH dissipation. All causal statements regarding pathways and temporal sequences must be interpreted with this constraint in mind, and time-series sampling represents the most valuable addition for future investigations. Hydrocarbon-degrading taxa were identified via taxonomic classification and published literature instead of direct functional evidence. No data on catabolic gene abundance, gene expression, or mineralization rates were acquired. Amplicon data are compositional, and microbial biomass or qPCR measurements were not collected; thus, relative abundances cannot be converted to absolute abundances. Earthworm performance was evaluated only based on survival rate. Nutrient limitation was inferred from the enzymatic vector model rather than verified via factorial N- and P-addition experiments. Finally, the experiment was conducted *ex situ* within 10 cm deep soil columns under controlled light, temperature, and moisture conditions. Specifically, the shallow columns restrict burrowing activity of anecic earthworms and may underestimate the functional contributions of this species under field conditions. While column experiments of this type effectively isolate mechanistic responses, they overestimate the level of environmental control achievable *in situ*. Field validation is required before these results can guide remediation design at contaminated sites.

5. Conclusions

In a moderately petroleum-contaminated loess soil, ryegrass and earthworms acted asymmetrically and complementarily. Ryegrass alone drove hydrocarbon removal and C-acquiring enzyme activity; earthworms alone restructured the bacterial community, raised nitrate availability more than thirty-fold, and drove N-acquiring enzyme activity, but they did not significantly reduce TPH. Microbial α -diversity was unresponsive throughout, and the relative abundance of hydrocarbon-degrading taxa was unrelated to residual contamination, showing that the taxonomic abundance of potential degraders did not predict hydrocarbon removal; activation of the resident degrader population is a plausible interpretation that remains to be tested directly. A significant interaction confined to β -1,4-glucosidase and microbial C limitation is consistent with rhizosphere C demand

being expressed only after earthworms had relieved N shortage. Converging chemical, stoichiometric, and functional evidence is consistent with N rather than P limitation, and it suggests that the conventional 45° vector-angle threshold overestimates P limitation in alkaline soils, although a factorial N- and P-addition experiment would be needed for confirmation. The original contribution of this study is that it showed that microbial community restructuring and hydrocarbon removal can be driven by different agents and are therefore not interchangeable indicators of remediation progress: earthworms reorganized the bacterial community and the soil N economy without measurably accelerating hydrocarbon loss, whereas ryegrass removed hydrocarbon without enlarging the degrader guild. Combining plants, earthworms, and N-containing organic amendments—with amendments applied at rates that alleviate N limitation and support earthworm survival—is accordingly proposed as a strategy for further experimental testing, not as a validated remediation recommendation.

Author Contributions: Conceptualization, X.L. and N.M.; Methodology, X.L., Y.G., Y.B., and N.M.; Formal Analysis, X.L. and Y.G.; Writing—Original Draft Preparation, X.L.; Writing—Review and Editing, X.L., Y.G., Y.B., and N.M.; Supervision, X.L. and N.M.; Funding Acquisition, X.L. and N.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Natural Science Foundation of China (42307408 and 42367042) and the Doctoral Research Startup Project of Yan'an University (YDBK2022-100 and YDBK2022-43).

Data Availability Statement: The datasets generated and analyzed in this study, including basic physicochemical properties and bacterial and fungal ASV abundances are openly available in Mendeley Data at [<https://doi.org/10.17632/j325jggwh8.1>]. All raw sequences were deposited in the NCBI Sequence Read Archive under accession number PRJNA1182408.

Acknowledgments: We acknowledge Doubao AI for support in grammatical revision and data analysis.

Conflicts of Interest: The authors declare no conflicts of interest.

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