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Long-term Nutrient Enrichment Differentiates Culturable Soil Bacterial Communities From Extracellular Enzyme Activities

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Abstract

Long-term nutrient enrichment can alter soil microbial communities, but whether persistent differences in community composition are accompanied by corresponding changes in extracellular enzyme activity remains unclear. We used a 75-year nutrient-manipulation experiment in a grassland in South Africa to examine how chronic nutrient enrichment is associated with culturable bacterial community composition and soil extracellular enzymes across soil depth and seasons. Nutrient enrichment was associated with a pronounced shift in bacterial community composition, whereas soil depth explained little compositional variation, and α-diversity showed no detectable response to treatment. In contrast, the multivariate extracellular enzyme profile was structured primarily by season and, to a lesser extent, soil depth, with no significant overall effect of long-term nutrient treatment. Bacterial community composition explained limited additional variation in the extracellular enzyme profile after accounting for nutrient treatment, season, soil depth, and soil physicochemical properties. These results indicate that long-term nutrient enrichment can be associated with differences in culturable bacterial community composition without producing a parallel shift in the overall soil extracellular enzyme profile. Culturable bacterial community composition and extracellular enzymes therefore showed different patterns, with culturable bacterial composition associated with long-term nutrient treatment and enzyme more strongly structured by contemporary seasonal variation.

Keywords: Long-term nutrient enrichment, Grassland soils, Culturable Soil bacterial communities, Extracellular Enzyme Activities, Seasonal variation, Ukulinga Grassland Nutrient Experiment

1. Introduction

Soil microbial communities mediate processes that regulate carbon and nutrient cycling, yet the extent to which soil bacterial community composition and microbial functioning retain the legacy of long-term nutrient enrichment remains unresolved. Long-term nutrient inputs can alter soil pH, nutrient availability, and organic matter dynamics, thereby modifying the environmental conditions experienced by soil microbial communities and the soil extracellular enzyme activities (EEAs) involved in biogeochemical processes [1-4]. These responses, however, need not integrate environmental variation over the same temporal scales. Long-term nutrient enrichment may leave persistent signatures in microbial community composition, whereas EEAs can remain responsive to contemporary environmental conditions such as temperature, moisture, and resource availability [5-8]. Thus, bacterial community composition and EEAs may integrate environmental history and contemporary environmental variation differently, potentially producing divergent responses to long-term nutrient enrichment [9,10].

Nitrogen (N) and phosphorus (P) enrichment can modify soil acidity and the availability and distribution of nutrients throughout the soil profile [11,12]. Such changes alter the physicochemical environment experienced by soil microorganisms, with soil pH being a particularly strong determinant of bacterial community composition [4,13]. Long-term fertilization experiments have demonstrated persistent changes in microbial community structure under sustained nutrient addition, although the magnitude and direction of these responses vary among ecosystems and depend on nutrient form, application rate, and duration [3,14-16]. Importantly, changes in microbial community composition do not necessarily translate into proportional changes in EEAs. Experimental studies have reported divergent responses of microbial communities and EEAs to nutrient addition, suggesting

that community composition and enzyme activity may integrate different aspects of the environmental conditions generated by long-term nutrient enrichment [1,7,8].

This distinction may be particularly important in grasslands, where strong seasonal variation is superimposed on persistent differences in soil resources. Soil enzyme activities can vary substantially between seasons as temperature, moisture, and substrate availability change [17-20]. while soil depth imposes additional gradients in organic carbon, nutrient availability, and microbial communities [11,21]. In South African grasslands, long-term nutrient enrichment has been associated with persistent changes in vegetation composition, productivity, soil chemistry, and nutrient cycling [22-24]. However, it remains unclear whether these long-term changes are reflected similarly in bacterial community composition and extracellular enzyme activity, particularly when seasonal and vertical variation in the soil environment are taken into account.

The Ukulinga Grassland Nutrient Experiment (UGNE) provides an unusual opportunity to address these questions. Established in the 1950s, the experiment has maintained replicated nutrient manipulations for approximately seventy-five years, allowing persistent nutrient legacies to be examined alongside contemporary seasonal and vertical variation [23.24]. The long duration of the experiment is particularly valuable because responses observed after decades of enrichment cannot readily be attributed to short-term nutrient pulses. Instead, they reflect the environmental conditions associated with sustained nutrient manipulation over multiple decades. Long-term fertilization experiments have shown that the form, magnitude, and duration of nutrient addition can influence the structure and functioning of soil microbial communities [15], but whether persistent differences in bacterial community composition are accompanied by corresponding differences in the present-day EEAs remains unclear.

Here, we used the 75-year nutrient-manipulation experiment at UGNE to test whether long-term nutrient enrichment is associated with coordinated or divergent responses in culturable bacterial community composition and soil EEA. We characterized culturable bacterial assemblages and five EEAs across nutrient treatments, three soil depths, and two contrasting seasons, together with measurements of soil physicochemical properties. First, we hypothesized that sustained nutrient enrichment alters bacterial community composition without necessarily reducing α-diversity, and that these compositional differences are associated with persistent changes in soil physicochemical conditions. Secondly, we hypothesized that the overall EEA profile is more strongly associated with contemporary environmental variation than with the legacy of long-term nutrient enrichment, while individual enzyme activities may show heterogeneous responses to nutrient treatment. Thirdly, we hypothesized that bacterial community composition and EEA activity represent partially independent dimensions of soil microbial functioning, such that bacterial community composition explains limited additional variation in the EEA profile after accounting for nutrient treatment, season, depth, and soil physicochemical conditions. Together, these analyses allowed us to evaluate whether 75 years of nutrient enrichment were associated with coordinated or divergent responses across soils physicochemical conditions, bacterial community composition, and EEA.

2. Materials and Methods

2.1. Site description

The study was conducted at the Ukulinga Grassland Nutrient Experiment (UGNE) near Pietermaritzburg, KwaZulu-Natal, South Africa (29°37′S, 30°16′E; 840 m a.s.l.; Fig 1). The UGNE is a long-term, *in situ* grassland experiment established in the 1950s to investigate

the effects of burning, mowing, and nutrient addition on grassland composition and productivity [25]. The experiment is located at the Ukulinga Research Farm (URF) of the University of KwaZulu-Natal (UKZN) and represents one of the longest-running nutrient manipulation experiments in southern Africa.

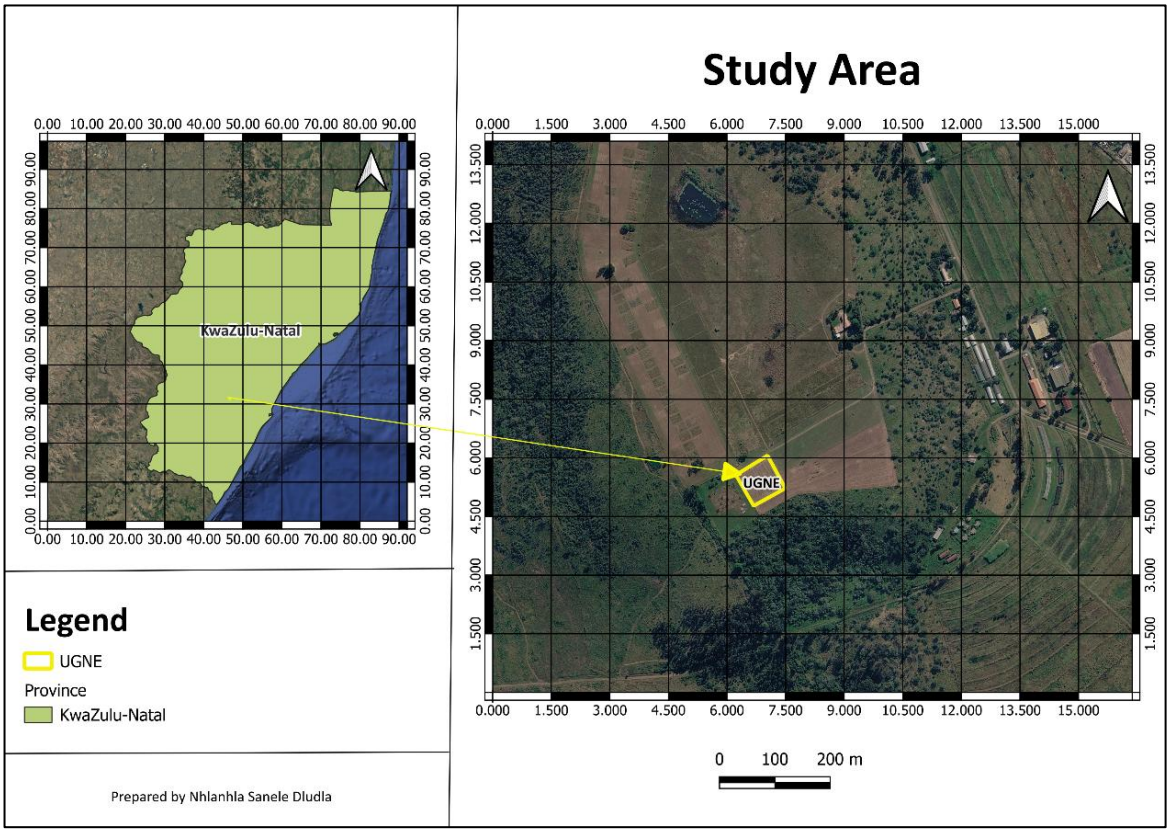


Figure 1 Location of the Ukulinga Grassland Nutrient Experiment (UGNE) at the Ukulinga Research Farm, University of KwaZulu-Natal, Pietermaritzburg, South Africa (29° 37′ S: 30° 16′ E, 840 m a.s.l). Base satellite imagery obtained from QGIS and Google Earth (accessed 06 October 2026) and modified by the authors

The experimental site is located in a grassland region characterized by seasonal variation in rainfall and temperature. The soils are developed on sandstone-derived parent material and have been classified within the South African soil classification system [26]. The long-term experimental treatments have generated persistent differences in soil nutrient availability

and soil chemical properties, providing an opportunity to examine the responses of soil bacterial communities and extracellular enzyme activities to prolonged nutrient enrichment.

2.2. *Climate data and climatic anomalies*

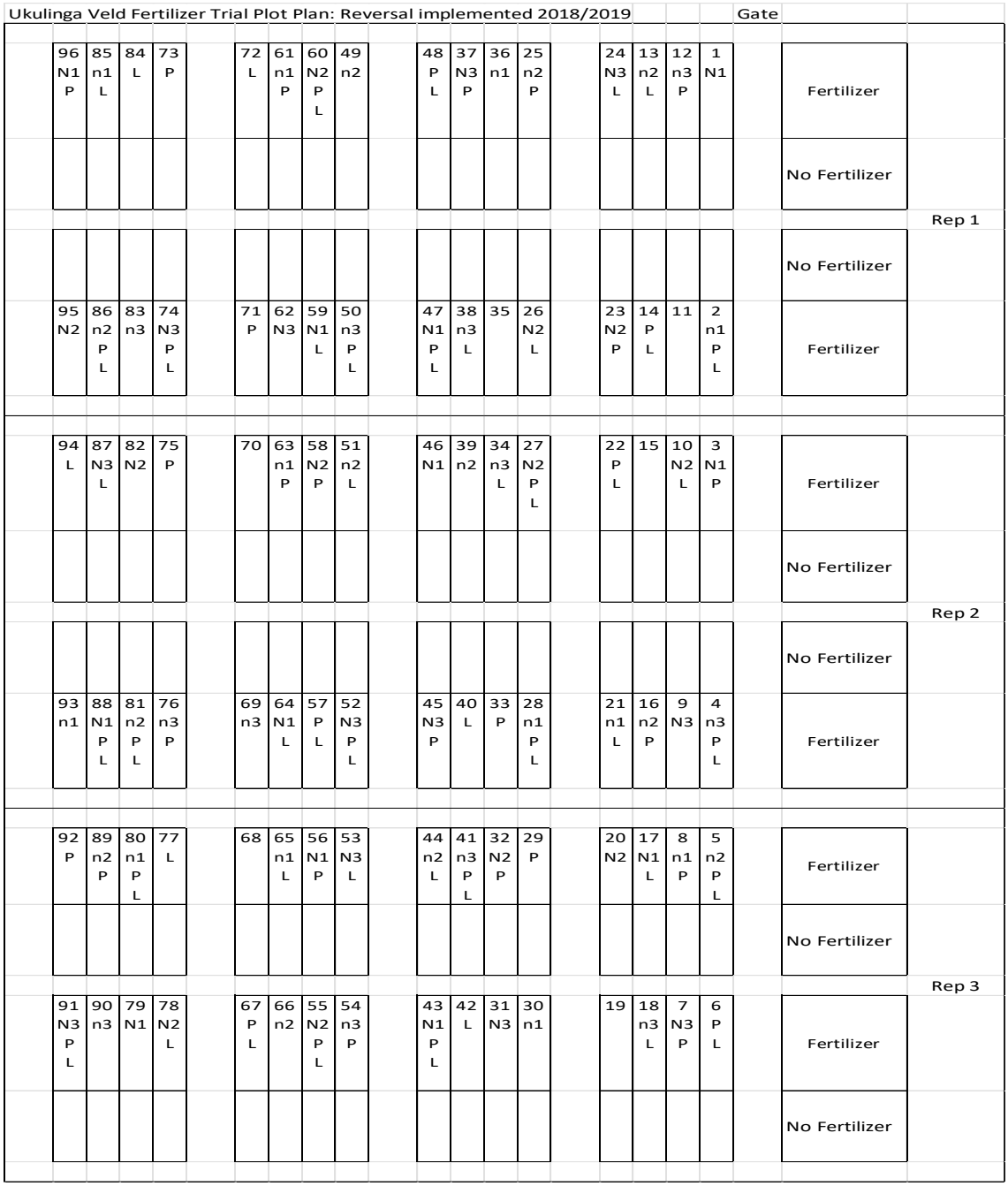
Monthly precipitation, air temperature and profile soil wetness data for 1981–2025 were obtained from the NASA Prediction of Worldwide Energy Resources (POWER) database for the study-site coordinates. Climatic conditions during the sampling periods were evaluated relative to the corresponding monthly climatology for 1981–2025, using standardized anomalies (z-scores).

2.3.Soil nutrient profiles under long-term nutrient enrichment

The UGNE uses a factorial experimental design comprising three replicate blocks, each containing 32 plots (4 x 2.7 m) arranged in a randomized layout with 1 m buffer zones between plots [27] (Fig 2). The experiment includes combinations of nitrogen (N), phosphorus (P), and lime (L). Nitrogen is supplied as either limestone ammonium nitrate (LAN; 28% N) or ammonium sulphate (AS; 21% N) at different application rates. Phosphorus is applied as superphosphate (11.3%), and lime is applied periodically. Potassium chloride is also applied periodically to maintain potassium availability [22,27].

For the present study, plots receiving ammonium sulphate in combination with phosphorus and lime (AS + PL) were selected to represent four nutrient-treatment levels: control (n0P0L0), low (n1PL), medium (n2PL), and high (n3PL). One plot of each treatment was selected within each of the three replicate blocks, resulting in 12 experimental plots. The

155 treatment levels therefore represented increasing long-term AS application rates under
156 otherwise comparable P and L additions.



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159 **Figure 2** Updated plot plan of the UGNE post 2018/2019. Original plots were split into two
160 subplots (4 m x 2.7 m) with one half receiving modified fertilizer treatment combinations
161 and the other half receiving no fertilizer treatment. N = 192. The yellow borders represent
162 selected sampling plots

Soil samples were collected during summer and winter in 2024 and 2025 from three depth intervals (0–10, 10–20, and 20–30 cm). At each depth, six to seven soil cores were collected from each plot using a 3.5 cm diameter soil corer and composited to obtain one representative sample per plot, season, and depth. Samples were homogenized by depth, sealed, transported to the laboratory, and stored at 4–6°C before analysis to minimize changes in soil properties.

Soil physicochemical properties and nutrient concentrations were determined by the Analytical Laboratory Services of the KwaZulu-Natal Department of Agriculture and Rural Development (KZN DARD). Organic C and clay content were estimated using near-infrared spectroscopy. Exchangeable Cu, K, Mn, P, and Zn were determined using the Ambic-2 extraction procedure, whereas exchangeable acidity, Ca, Mg, and pH were determined using KCl-based procedures, with soil pH measured in KCl [28]. The resulting soil dataset comprised measurements of pH, exchangeable acidity, total cations, Ca, K, Mg, Cu, Mn, Zn, available P, acid saturation, organic C, total N, and clay content for the sampled combinations of treatment, block, season, and soil depth.

2.4.Culturable soil bacterial community composition

Culturable bacteria were isolated from the same composited soil samples used for physicochemical analyses. Ten grams of homogenized soil from each sample were suspended in 100 mL of autoclaved distilled water and shaken for 10–15 min. A 1 mL aliquot of the resulting suspension was subsequently transferred to 9 mL of autoclaved distilled water and serially diluted three times. From each dilution, 100 µL was spread onto three culture media selected to recover bacteria with different putative nutrient-cycling capacities: Jensen's medium agar for N-fixing bacteria, Simmons citrate agar for N-cycling bacteria, and Pikovskaya's agar for P-solubilizing bacteria. Plates were incubated at 30°C

until visible bacterial growth occurred. Distinct colonies were selected on the basis of colony morphology, including shape, elevation, margin, colour, and texture, and were repeatedly sub cultured on the corresponding medium until pure cultures were obtained.

For molecular identification, pure bacterial colonies were lightly sampled using sterile autoclaved tips and transferred directly to PCR reactions. Each 25 µL PCR mixture contained 12.5 µL EmeraldAmp GT PCR Master Mix (Takara Bio), 11.5 µL distilled water, and 0.5 µL of each of the forward primer 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and reverse primer 1387R (5′-GGGCGGTGTGTACAAGGC-3′), targeting the bacterial 16S rRNA gene. PCR reactions were mixed by vortexing for 1 min and amplified using a T100 thermal cycler (Bio-Rad) following the PCR conditions described by (27).

Three microlitres of each PCR product were separated on a 1% (w/v) agarose gel at 120 V for 45 min. Amplicons were visualized using a Clare Chemical Research Dark Reader transilluminator (Inqaba Biotech), and images were captured using a ChemiDoc XRS+ System (Bio-Rad). PCR products showing the expected amplification pattern were submitted to Inqaba Biotechnical Industries (Pretoria, South Africa) for sequencing.

The resulting 16S sequences were edited to remove ambiguous bases and chimeric sequences, and noisy sequence ends were manually trimmed using R version 4.5.2 and RStudio [29]. Edited sequences were compared with sequences in the National Center for Biotechnology Information (NCBI) GenBank database using BLASTN (accessed 6 January 2026) to identify the bacterial isolates.

For downstream community analyses, sequence records were first paired by isolate and sample. An isolate was retained when the two sequence records generated for the isolate yielded a concordant genus-level identification. Isolates lacking a reliable genus-level identification or showing discordant genus assignments between the two sequence records

were excluded from the community matrix. This procedure resulted in 135 isolates assigned to 40 genera and represented 69 plot x season x soil-depth community samples.

Because the culture media were used as selective recovery media rather than as independent biological replicates, bacterial community composition was summarized at the sample level. For each plot x season x depth combination, the occurrence of each identified bacterial genus across the recovered isolates was converted to presence–absence. The resulting sample-by-genus matrix was used for analyses of culturable bacterial community composition.

2.5.Soil extracellular enzyme activity profiles

Five extracellular enzyme activities associated with C, N, and P cycling were determined: β-glucosidase, β-glucosaminidase, acid phosphatase, alkaline phosphatase, and nitrate reductase. Soil, substrate, standard, and buffer solutions were prepared following the procedures described by [30].

For preparation of the soil water used in the assays, the solution was shaken at 115 rpm for 2 h and refrigerated for 48 h before use. Diluted buffer and standard solutions were prepared by mixing 5 and 10 mL, respectively, of the corresponding stock solutions with 90 and 95 mL of distilled water. For acid phosphatase assays, the diluted standard and buffer solutions were adjusted to pH 5 before use.

β-glucosidase, β-glucosaminidase, acid phosphatase, and alkaline phosphatase activities were determined using fluorometric microplate assays following [30]. Fluorescence was measured using a microplate reader (Apex Scientific) at 410 nm. Measurements were generally recorded at 10-min intervals, although the interval was adjusted when required according to the enzyme concentration of individual samples.

Nitrate reductase activity was determined using a modified procedure based on [31]. A solution containing 0.9 mM 2,4-dinitrophenol and 25 mM potassium nitrate (KNO_3) was used as the substrate solution. The nitrite (NO_2^-) released during the reaction was quantified colorimetrically at 520 nm using a UV-1800 spectrophotometer (Shimadzu). Enzyme activity was expressed as μmol h^{-1} g^{-1} and calculated using a standard curve prepared from KNO_3 concentrations of 5, 10, 15, 20, 25, and 30 mM. The complete dataset was available for acid phosphatase, alkaline phosphatase, nitrate reductase, β-glucosidase, and β-glucosaminidase, with 72 observations for each enzyme.

2.6. Statistical analyses

All statistical analyses were conducted in R version 4.5.2 [29]. Statistical significance was evaluated at $\alpha = 0.05$. Gaussian generalized estimating equation (GEE) models were used to evaluate the effects of nutrient treatment, season, soil depth, and their interactions on soil physicochemical properties and individual EEAs. Nutrient treatment, season, and soil depth were specified as fixed effects, and plot was used as the clustering unit to account for repeated sampling of the same plots across seasons and soil depths. Robust covariance estimates and an exchangeable working correlation structure were used. For the enzyme activity analyses, experimental block was additionally included as a fixed effect. Enzyme activities were $\log_{10}$ transformed before analyses. Overall effects were evaluated using Wald χ^2 tests, and model-based pairwise contrasts were used to examine significant effects, with P values adjusted using the Benjamini–Hochberg procedure within each response x simple-effect family.

Culturable bacterial community composition was represented by the presence–absence of bacterial genera in each plot x season x soil-depth sample. Community dissimilarities were calculated using the Jaccard index and visualized by principal coordinates analysis (PCoA).

Differences in community composition associated with nutrient treatment, season, and soil depth, were evaluated using permutational multivariate analysis of variance (PERMANOVA), with permutations restricted according to the experimental design to account for repeated sampling plots across seasons and soil depths. The relative contribution of each factor was expressed using pseudo-F statistics and R^2. Homogeneity of multivariate dispersion was assessed using PERMDISP to determine whether significant PERMANOVA results could be attributed to differences in within-group dispersion. Indicator genera were identified using indicator-value analysis, with statistical significance assessed using the same restricted permutation structure for nutrient treatment, season, and soil depth.

Multivariate variation in extracellular enzyme activity was characterized using principal component analysis (PCA) based on standardized activities of nitrate reductase, β-glucosidase, and β-glucosaminidase, alkaline phosphatase, and acid phosphatase. Differences in the multivariate enzyme profile among nutrient treatments, seasons, and soil depths were evaluated using permutational multivariate analysis of variance (PERMANOVA), with permutations restricted according to the experimental design.

Bacterial community composition was represented by the first six genus-level PCoA axes based on Jaccard dissimilarities. Enzyme activities were $\log_{10}$ transformed and standardized before multivariate analysis. Soil physicochemical variables were standardized before multivariate analysis. Soil physicochemical variables were standardized and summarized using the first five principal components, which together explained 86.6% of the total variance (Table S6). The additional contribution of bacterial community composition to variation in the multivariate enzyme profile was evaluated after accounting for nutrient treatment, season, soil depth, soil physicochemical conditions using restricted Freedman–Lane permutations.

3. Results

3.1. Soil nutrient profiles under long-term nutrient enrichment

Long-term AS + PL enrichment was associated with marked differences in several soil physicochemical properties, although responses varied among treatments and soil depths (Fig 3; Table 1; Table S1). Soil pH differed among treatments and showed significant treatment x depth and treatment x season x depth interactions (Table 1 and Table S1). Across all observations, mean pH was lowest under the High AS + PL treatment (4.07), whereas the Low and Medium treatments had higher mean values (5.22 and 5.12, respectively) than the Control (4.64) (Fig 3; Table 1; Table S1). Exchangeable acidity also varied among treatments and showed significant treatment x depth and higher-order interactions (Table 1 and Table S1), with the highest overall values under Low and High AS + PL (1.69 and 1.58, respectively), compared with 0.17 in the Control and 0.12 under Medium AS + PL (Fig 3).

Organic C showed strong vertical stratification, decreasing from 5.16% in the 0–10 cm layer to 4.56% and 4.31% in the 10–20 and 20–30 cm layers, respectively (Fig 3; Table 1; Table S1). This depth pattern differed among nutrient treatments, as indicated by significant treatment x depth and treatment x season x depth interactions (Table 1 and Table S1). In contrast, clay content was not significantly affected by treatment but increased with depth, from 34.17% in the 0–10 cm layer to 39.67% in the 20–30 cm layer (Fig 3; Table 1; Table S1).

Several exchangeable and available nutrients also differed among treatments (Table 1 and Table S1). Mean Ca concentration was highest under Medium AS + PL (16.63) and lowest in the Control (9.49), while Mg was lower under High AS + PL (2.74) than under the other treatments (4.44–4.75) (Fig 3; Table 1; Table S1). Total N increased from 0.313% in the

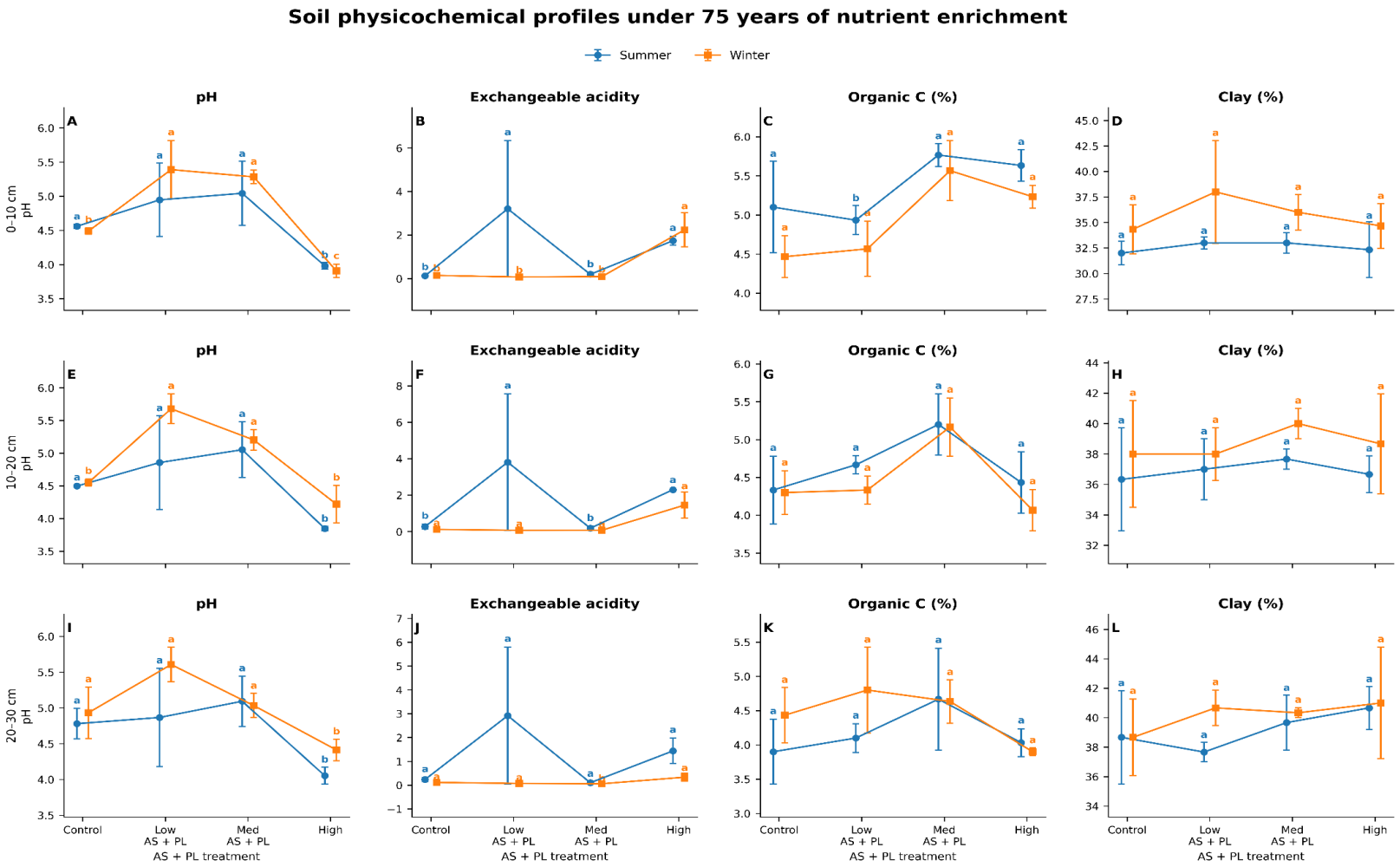

Figure 3 Soil physicochemical profiles under 75 years of nutrient enrichment. Mean (± SE) soil pH, exchangeable acidity, organic carbon (C), and clay content across four nutrient treatments (Control, Low AS + PL, Medium AS + PL, and High AS + PL), two seasons (summer and winter), and three soil depths (0–10, 10–20, and 20–30 cm). Points represent treatment means, and error bars indicate standard errors (n = 3 plots per treatment × season × depth combination)

Control to 0.378% under Medium AS + PL, whereas available P was substantially higher in all fertilized treatments than in the Control, with the highest mean under Low AS + PL (59.71) (Fig 3; Table 1; Table S1). K showed the opposite pattern, with the highest mean in the Control (0.611) and lower values under the fertilized treatments (0.281–0.445) (Fig 3; Table 1; Table S1). For these nutrients, significant treatment x depth and/or treatment x season x depth interactions indicated that treatment differences were not consistent across the soil profile (Table 1 and Table S1).

Seasonal differences were also evident for several soil properties (Table 1 and Table S1). Across treatments and depths, exchangeable acidity was higher in summer than winter (1.38 vs. 0.40), whereas pH was slightly higher in winter (4.89 vs. 4.63) (Fig 3). Clay content was also higher in winter (38.19%) than summer (36.22%) (Fig 3). More pronounced seasonal contrasts occurred for K and several nutrient concentrations, as reflected by significant season-related interactions (Table 1 and Table S1). Overall, the soil profiles showed persistent differences associated with long-term nutrient enrichment superimposed on substantial vertical and seasonal variation (Fig 3; Table 1; Table S1).

Table 1 Effects of long-term nutrient enrichment, season, and soil depth on soil physicochemical properties**.** Wald χ^2 statistics, degrees of freedom (in parentheses), and associated *P* values are reported for the main effects of treatment, season, and depth, and for their two-way and three-way interactions. Treatment effects correspond to differences among the four nutrient-enrichment treatments. Significant effects ($P < 0.05$) indicate that the corresponding factor or interaction was associated with variation in the soil property considered. Detailed post hoc pairwise comparisons, including effect estimates, 95% confidence intervals, and Benjamini–Hochberg-adjusted *P* values, are provided in the Supplementary Material

Response	**Treatment**	**Season**	**Depth**	**Treatment × Season**	**Treatment × Depth**	**Season × Depth**	**Treatment × Season × Depth**
Soil pH	150.101 (3); P < 0.0001	2.423 (1); P = 0.1196	501958.491 (2); P < 0.0001	1.537 (3); P = 0.6738	−22178974456436.180 (8); P = 1.0000	12.635 (2); P = 0.0018	28.840 (6); P < 0.0001
Exchangeable acidity	92.914 (3); P < 0.0001	1.051 (1); P = 0.3053	5.020 (2); P = 0.0813	3.728 (3); P = 0.2924	210.527 (8); P < 0.0001	7.283 (2); P = 0.0262	36.152 (6); P < 0.0001
Organic C	20.340 (3); P = 0.0001	1.666 (1); P = 0.1968	381.615 (2); P < 0.0001	1.020 (3); P = 0.7963	216.650 (8); P < 0.0001	25.853 (2); P < 0.0001	29.352 (6); P < 0.0001

Clay	0.998 (3); P = 0.8017	2.625 (1); P = 0.1052	347.280 (2); P < 0.0001	0.577 (3); P = 0.9017	1212.597 (8); P < 0.0001	7.561 (2); P = 0.0228	4.327 (6); P = 0.6325
Ca	70.407 (3); P < 0.0001	0.391 (1); P = 0.5317	10.074 (2); P = 0.0065	2.432 (3); P = 0.4877	131.676 (8); P < 0.0001	22.048 (2); P < 0.0001	58.908 (6); P < 0.0001
Mg	21.129 (3); P < 0.0001	1.150 (1); P = 0.2836	1.417 (2); P = 0.4924	7.820 (3); P = 0.0499	129.939 (8); P < 0.0001	2.901 (2); P = 0.2345	58.759 (6); P < 0.0001
Mn	8.931 (3); P = 0.0302	0.346 (1); P = 0.5562	3926.346 (2); P < 0.0001	6.077 (3); P = 0.1079	1653.078 (8); P < 0.0001	31.918 (2); P < 0.0001	62.355 (6); P < 0.0001
Zn	2.186 (3); P = 0.5348	2.298 (1); P = 0.1295	13.366 (2); P = 0.0013	2.633 (3); P = 0.4517	310.496 (8); P < 0.0001	8.422 (2); P = 0.0148	66.983 (6); P < 0.0001
N	14.693 (3); P = 0.0021	0.742 (1); P = 0.3889	105.324 (2); P < 0.0001	0.828 (3); P = 0.8427	1297.970 (8); P < 0.0001	14.754 (2); P = 0.0006	29.933 (6); P < 0.0001
P	78.770 (3); P < 0.0001	1.158 (1); P = 0.2819	1609.655 (2); P < 0.0001	5.008 (3); P = 0.1712	897.643 (8); P < 0.0001	150.841 (2); P < 0.0001	60.446 (6); P < 0.0001
K	69.951 (3); P < 0.0001	261.535 (1); P < 0.0001	2595.826 (2); P < 0.0001	49.998 (3); P < 0.0001	4652.393 (8); P < 0.0001	4153.681 (2); P < 0.0001	43.546 (6); P < 0.0001

Values are Wald χ^2 (degrees of freedom), followed by the corresponding P value.

3.2.Culturable soil bacterial community composition

Long-term nutrient enrichment was associated with pronounced differences in the composition of the culturable soil bacterial community, whereas soil depth showed little evidence of compositional differentiation. After quality filtering of the paired sequence records, 135 isolates with concordant genus-level identification were retained, representing 40 genera across 69 plot x season x depth community samples. Jaccard-based PCoA separated communities primarily along the first two axes, which accounted for 21.8% and 9.3% of the variation, respectively (Fig 4). Nutrient treatment explained the largest proportion of compositional variation (pseudo-F = 1.64, R^2 = 0.381, v = 0.005), while season accounted for a smaller but significant component (pseudo-F = 1.47, R^2 = 0.063, p = 0.016). In contrast, soil depth explained little variation in community composition (R^2 = 0.018, p = 0.917).

The treatment effect remained significant when the experimental block structure was accounted for (pseudo-F = 1.68, R^2 = 0.381, p = 0.023), supporting the association between long-term nutrient treatment and bacterial community composition. There was no evidence for a treatment x season interaction ($p \approx 0.13$), indicating that the compositional differences associated with nutrient treatment did not show a detectable seasonal dependence. Multivariate dispersion did not differ among nutrient treatments (p = 0.73) or between seasons (p = 0.67), indicating that the treatment and seasonal effects were not attributable to differences in within-group dispersion.

Only two genera showed significant associations with experimental groups after accounting for the restricted permutation structure. *Paenarthrobacter* was associated with the Medium AS + PL treatment (IndVal = 1.00, p = 0.018), whereas *Burkholderia* was associated with summer samples (IndVal = 0.714, p = 0.013; Table 2). No genus showed a significant association with soil depth. Together, these results indicate that long-term nutrient treatment

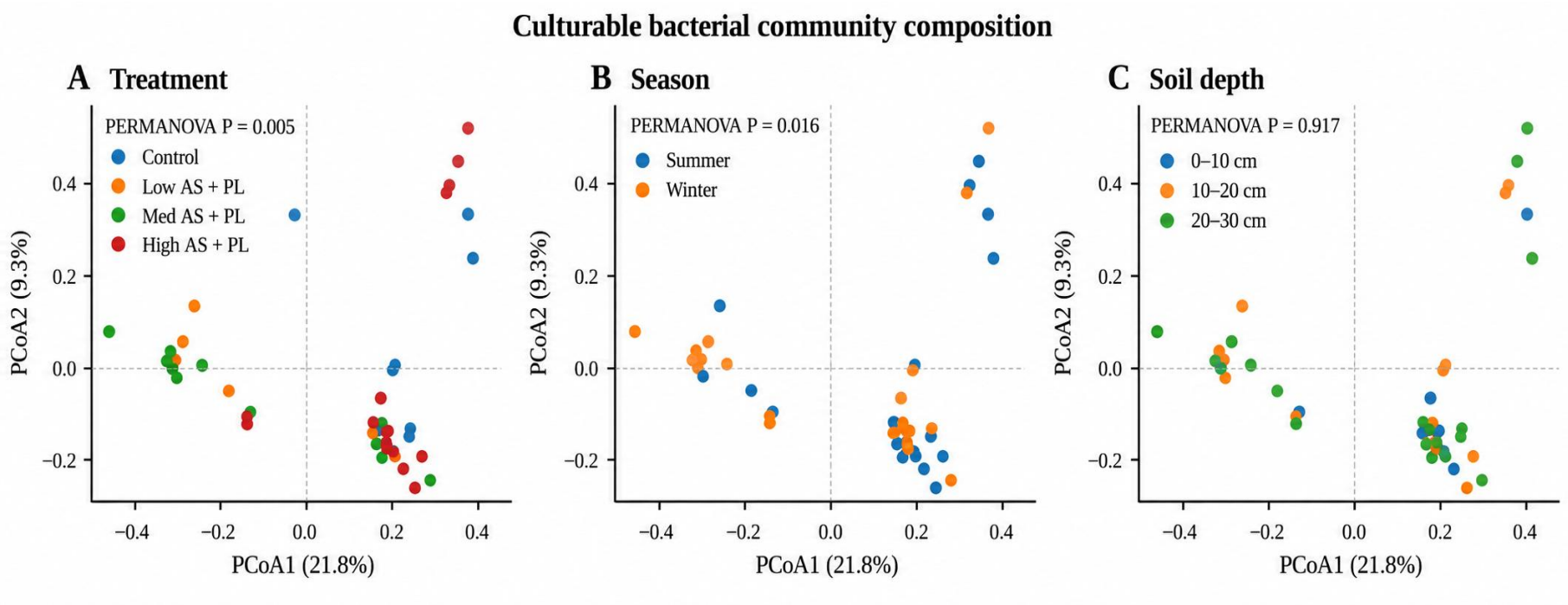

Figure 4 Principal coordinates analysis (PCoA) based on Jaccard dissimilarities of genus-level presence–absence data from culturable bacterial isolates, shown separately by (A) nutrient treatment, (B) season, and (C) soil depth. The first and second principal coordinates explained 21.8% and 9.3% of the total variation, respectively. Each point represents a plot × season × depth community sample. Only isolates with concordant genus-level identification between the two sequencing reads were included in the community matrix

was associated with a persistent shift in the composition of the culturable bacterial assemblage, while seasonal variation accounted for a smaller component of compositional variability and vertical stratification across the sampled soil profile was comparatively limited.

Table 2 Indicator genera showing significant associations with nutrient treatment or season based on indicator-value analysis. IndVal values and permutation P values are reported. Only significant associations are shown

Genus	Associated factor/group	IndVal	P
Paenarthrobacter	Medium AS + PL	1.000	**0.018**
Burkholderia	Summer	0.714	**0.013**

3.3.Soil extracellular enzyme activity profiles

Extracellular enzyme activities showed heterogeneous responses to long-term nutrient treatment, season, and soil depth, with the relative importance of these factors differing among enzymes (Table 3; Fig 5). Nitrate reductase activity showed significant main effects of nutrient treatment and season, together with strong treatment x depth and treatment x season x depth interactions (Table 3; Fig 5A). Mean nitrate reductase activity was higher in summer than in winter, and treatment differences varied markedly across soil depths, indicating a context-dependent response to long-term nutrient enrichment (Table S3). β-glucosidase activity was strongly structured by season and showed a significant effect of soil depth, but no significant main effect of nutrient treatment or treatment x season interaction (Table 3; Fig 5B). β-glucosaminidase showed no significant main effects of

nutrient treatment, season, or depth, although a significant season x depth interaction was detected (Table 3; Fig 5C).

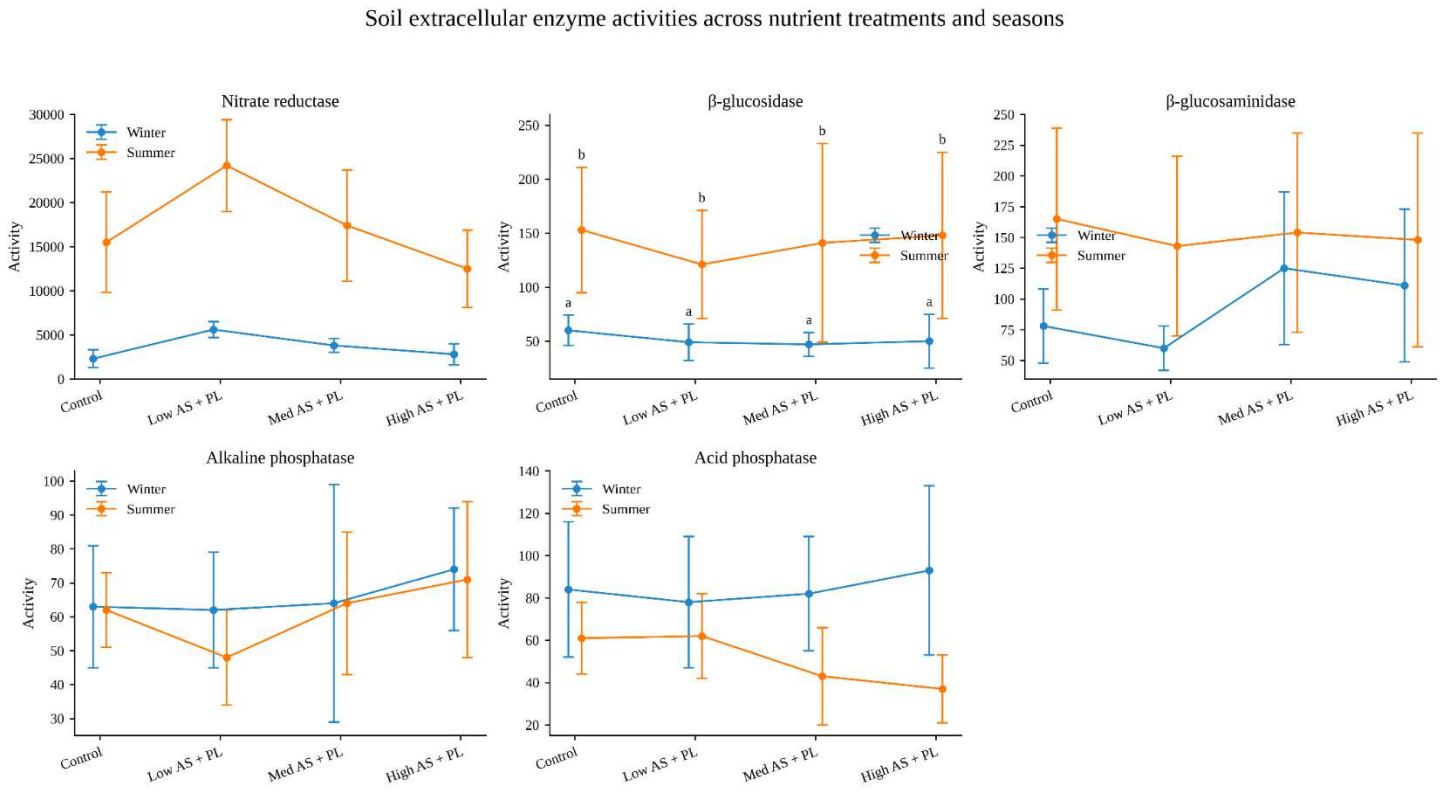

Figure 5 Soil extracellular enzyme activities under long-term nutrient enrichment. Mean (± SE) activities of (A) nitrate reductase, (B) β-glucosidase, (C) β-glucosaminidase, (D) alkaline phosphatase, and (E) acid phosphatase across nutrient treatments, seasons, and soil depths. Nutrient treatments were Control, Low AS + PL, Medium AS + PL, and High AS + PL. Soil depths were 0–10, 10–20, and 20–30 cm. Enzyme activities were log10-transformed for statistical analysis

Alkaline phosphatase showed significant effects of nutrient treatment and season, as well as significant treatment x season and treatment x depth interactions (Table 3; Fig 5D). These interactions indicate that differences among nutrient treatments were not consistent across seasons or soil depths. In contrast, acid phosphatase showed no significant main effects or interactions involving nutrient treatment, season, or soil depth (Table 3; Fig 5E). Thus, the

five enzyme activities did not exhibit a uniform response to long-term nutrient enrichment, with treatment effects being particularly evident for nitrate reductase and alkaline phosphatase and strongly dependent on environmental context (Table S3).

Table 3 Univariate effects of nutrient treatment, season, and soil depth on soil extracellular enzyme activities. Results are shown for nitrate reductase, β-glucosidase, β-glucosaminidase, alkaline phosphatase, and acid phosphatase based on the complete factorial dataset (N = 72). Wald χ^2 statistics and P values are reported for the main effects and their interactions. Activities were $\log_{10}$ transformed before analysis. Gaussian generalized estimating equation (GEE) models included nutrient treatment, season, soil depth, all interactions, and experimental block as fixed effects, with plot as the clustering unit and an exchangeable working correlation structure. Wald χ^2 statistics and P values are reported

Enzyme	Effect	df	Wald χ^2	P
Nitrate reductase	Treatment	3	8.715	**0.033**
Nitrate reductase	Season	1	8.331	**0.004**
Nitrate reductase	Depth	2	2.425	0.297
Nitrate reductase	Treatment × season	3	2.361	0.501
Nitrate reductase	Treatment × depth	6	211.271	**<0.001**
Nitrate reductase	Season × depth	2	1.051	0.591
Nitrate reductase	Treatment × season × depth	6	58.258	**<0.001**
β-glucosidase	Treatment	3	3.797	0.284
β-glucosidase	Season	1	83.286	**<0.001**
β-glucosidase	Depth	2	6.984	**0.030**
β-glucosidase	Treatment × season	3	5.419	0.144
β-glucosidase	Treatment × depth	6	11.493	0.074
β-glucosidase	Season × depth	2	3.183	0.204
β-glucosidase	Treatment × season × depth	6	11.531	0.073

β-glucosaminidase	Treatment	3	0.246	0.970
β-glucosaminidase	Season	1	3.772	0.052
β-glucosaminidase	Depth	2	0.613	0.736
β-glucosaminidase	Treatment × season	3	0.987	0.804
β-glucosaminidase	Treatment × depth	6	6.967	0.324
β-glucosaminidase	Season × depth	2	12.208	**0.002**
β-glucosaminidase	Treatment × season × depth	6	7.642	0.265
Alkaline phosphatase	Treatment	3	15.786	**0.001**
Alkaline phosphatase	Season	1	16.922	**<0.001**
Alkaline phosphatase	Depth	2	1.621	0.445
Alkaline phosphatase	Treatment × season	3	28.647	**<0.001**
Alkaline phosphatase	Treatment × depth	6	16.330	**0.012**
Alkaline phosphatase	Season × depth	2	4.717	0.095
Alkaline phosphatase	Treatment × season × depth	6	10.595	0.102
Acid phosphatase	Treatment	3	0.331	0.954
Acid phosphatase	Season	1	0.905	0.341
Acid phosphatase	Depth	2	1.095	0.578
Acid phosphatase	Treatment × season	3	0.304	0.959
Acid phosphatase	Treatment × depth	6	11.566	0.072
Acid phosphatase	Season × depth	2	0.819	0.664
Acid phosphatase	Treatment × season × depth	6	7.840	0.250

Consistent with these univariate patterns, the multivariate extracellular enzyme profile was structured primarily by season (pseudo-F = 16.84, $R^2 = 0.191$, p = 0.0001), whereas nutrient treatment explained a small and non-significant proportion of variation ($R^2 = 0.046$, pseudo-

F = 1.36, p = 0.745; Table 4). Soil depth accounted for a smaller but significant component of multivariate variation (R^2 = 0.027, pseudo-F = 1.19, p = 0.021; Table 4). The first two principal components of the five-enzyme activity profile accounted for 62.2% of the total variance, with β-glucosidase and β-glucosaminidase contributing positively to PC1, whereas acid phosphatase showed a negative loading; alkaline phosphatase contributed most strongly to PC2 (Table S4). Overall, these results indicate that although individual enzyme activities showed treatment- and depth-dependent responses, variation in the overall enzyme activity profile was more strongly associated with season than with long-term nutrient treatment (Table 4; Fig 5).

Table 4 Multivariate effects of nutrient treatment, season, and soil depth on the soil extracellular enzyme activity profile. Results are from restricted-permutation PERMANOVA based on Euclidean distances among log_{10} transformed and standardized activities of nitrate reductase, β-glucosidase, β-glucosaminidase, alkaline phosphatase, and acid phosphatase. Treatment permutations respected the experimental block structure; season and depth were tested using restricted Freedman–Lane permutations. R^2 denotes the proportion of total multivariate variation attributable to each sequential model term

Model term	df	SS	R^2	Pseudo-F	P
Treatment	3	16.5876	0.0461	1.3558	0.7449
Season	1	68.6588	0.1907	16.8355	**0.0001**
Depth	2	9.6692	0.0269	1.1855	**0.0208**
Residual	65	265.0844	—	—	—

3.4. Integration of bacterial community composition, soil physicochemical conditions, and extracellular enzyme activity

The integrative analysis indicated that variation in the soil extracellular enzyme activity profile was associated with both environmental conditions and bacterial community composition, although the contribution of bacterial composition was not statistically significant after accounting for the environmental variables (Table 5; Fig 6). Across the 71 matched plot x season x depth observations, the environmental model, including nutrient treatment, season, soil depth, and the first five principal components of the soil physicochemical variables, accounted for 35.6% of the variation in the five-enzyme activity profile. The bacterial community was represented by the first six axes of the Jaccard-based PCoA and did not explain significant additional variation after accounting for the environmental component (pseudo-F = 1.07, partial R^2 = 0.108, p = 0.374; Table 5).

Variance partitioning further indicated that the environmental component uniquely accounted for 33.7% of the total variation in enzyme activity, whereas bacterial community composition uniquely accounted for 7.0%. A small proportion of variation was shared between the environmental and bacterial components (1.9%), while 57.4% remained unexplained (Fig 6). Thus, although bacterial community composition contributed to the overall partitioning of enzyme-profile variation, its unique contribution was not statistically supported after accounting for nutrient treatment, season, soil depth, and soil physicochemical conditions.

Table 5. Association between culturable bacterial community composition and the multivariate extracellular enzyme activity profile. Results of the integrative multivariate analysis testing the additional contribution of bacterial community composition to variation in the five-enzyme activity profile after accounting for nutrient treatment, season, soil depth, and soil physicochemical conditions. Bacterial community composition was represented by the first six axes of the genus-level Jaccard PCoA, and soil physicochemical

conditions by the first five principal components. Significance was assessed using restricted Freedman–Lane permutations within experimental plots.

Model term	df	Pseudo-F	Partial R^2	P-value
Environmental model	11	—	0.356	—
Bacterial community composition (Jaccard PCoA axes 1–6)	6	1.072	0.108	0.374

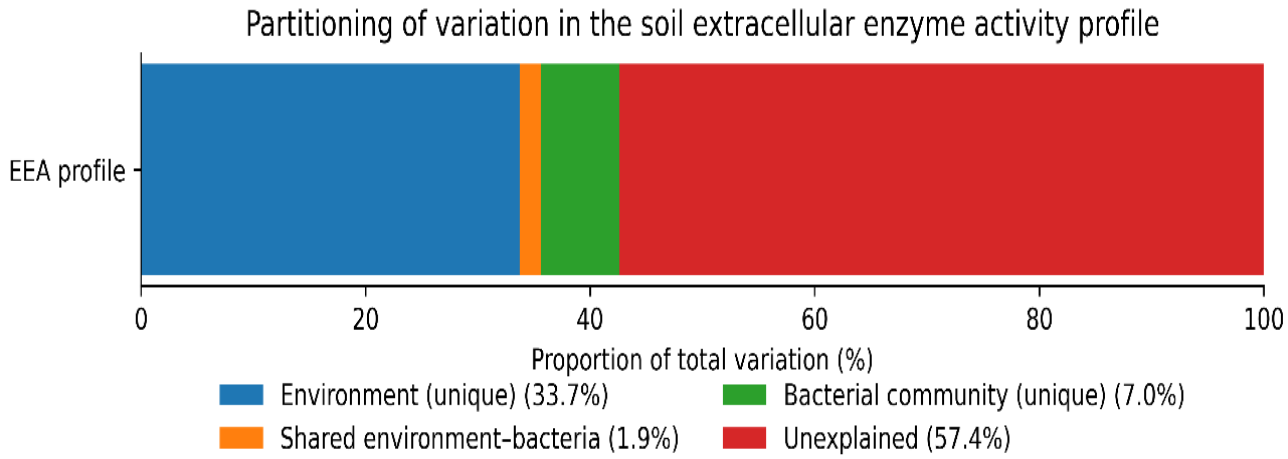

Figure 6 Proportion of variation in the multivariate profile of five soil extracellular enzyme activities partitioned into the unique contribution of environmental variables, the variation shared between environmental variables and bacterial community composition, the unique contribution of bacterial community composition, and unexplained variation. The environmental component comprised nutrient treatment, season, soil depth, and the first five principal components of soil physicochemical properties. Bacterial community composition was represented by the first six axes of the Jaccard-based PCoA

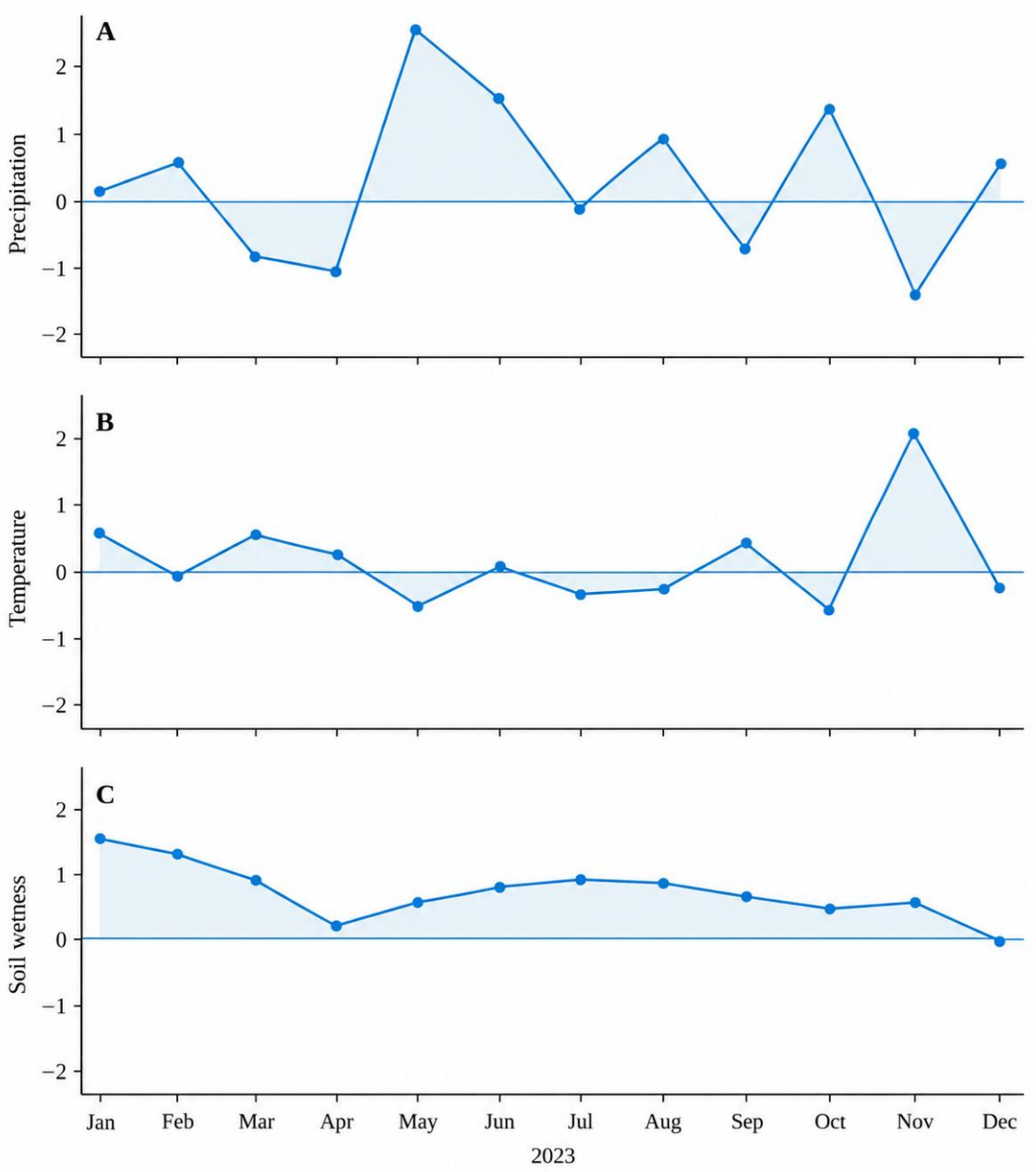

Figure 7 Monthly standardized anomalies (*z*-scores) relative to the 1981–2025 climatological period for (A) precipitation, (B) air temperature, and (C) profile soil wetness. Values above and below zero indicate conditions respectively above and below the 1981–2025 monthly reference values. Climate data were obtained from the NASA Prediction of Worldwide Energy Resources (POWER) database

3.4.Climatic context of the sampling period

Climatic conditions during 2023 were not characterized by an anomalously dry annual regime (Fig 7). Mean daily precipitation in 2023 was 3.84 mm d^{-1}, corresponding to a positive anomaly of 0.30 mm d^{-1} relative to the 1981–2025 mean (z = 0.45), while mean annual temperature was 17.56 °C (z = 0.25) and profile soil wetness was 0.67 (z = 0.86) (Table S5; Fig 7). Thus, the sampling year was slightly wetter than the long-term mean but did not represent an extreme annual precipitation anomaly. Monthly climate conditions showed pronounced variability throughout 2023, with alternating positive and negative anomalies in precipitation and temperature and generally positive anomalies in profile soil wetness (Fig 7). Overall, the climatic context of the sampling period was characterized primarily by seasonal hydroclimatic variability rather than an extreme annual precipitation anomaly (Fig 7; Table S5).

4. Discussion

The 75-year nutrient manipulation at Ukulinga provides a rare opportunity for examining how long-term nutrient enrichment is reflected in belowground microbial processes under contemporary environmental variation. Long-term AS + PL enrichment was associated with pronounced differences in soil physicochemical conditions, including pH, exchangeable acidity, and the availability of several macro- and micronutrients. At the same time, seasonal and vertical variation remained superimposed on these treatment differences. Such long-term experiments are particularly valuable for detecting environmental legacies that may develop over timescales that are difficult to capture in short-term fertilization experiments [15,25]. This experimental setting, therefore, allowed us to examine whether sustained nutrient enrichment was associated with persistent differences in bacterial

community composition and whether these differences were accompanied by corresponding patterns in soil EEAs.

Our first hypothesis predicted that sustained nutrient enrichment would be associated with shifts in bacterial community composition without necessarily reducing α-diversity. Nutrient treatment explained the largest component of variation in the composition of the culturable bacterial assemblage, whereas soil depth explained little compositional variation, and α-diversity did not differ detectably among treatments, seasons, or depths. The treatment effect remained significant after accounting for experimental block structure and was not attributable to differences in multivariate dispersion, indicating that long-term nutrient enrichments was associated with a shift in bacterial community composition rather than simply greater heterogeneity among fertilized plots. This pattern is consistent with evidence that long-term fertilization can restructure soil microbial communities without producing consistent changes in diversity, with responses depending on the duration and form of nutrient addition and on environmental context [14-16]. The combination of a strong compositional response and stable α-diversity suggests that nutrient enrichment altered which taxa to alter which taxa co-occurred without reducing the number or evenness of recovered taxa. Changes in soil pH and nutrient availability provide plausible environmental correlates of this compositional restructuring, given the strong sensitivity of bacterial communities to soil chemical conditions [4,13,32]. Because our analyses targeted culturable isolates, however, these results describe a treatment-associated reorganization of the culturable bacterial assemblage rather than the response of the entire soil bacterial community.

The second hypothesis predicted that overall EEA profiles would be more strongly associated with contemporary environmental variation than with the legacy of nutrient enrichment. Consistent with this prediction, season accounted for the largest proportion of variation in the multivariate EEA profile, whereas nutrient treatment explained a smaller and non-significant proportion of overall variation. Individual enzymes responded differently to nutrient enrichment and season, and treatment effects on nitrate reductase and alkaline phosphatase depended on soil depth and/or season. Soil depth contributed a small but significant component of variation in the multivariate EEA profile, although this effect was much weaker than the dominant seasonal signal.

The climatic context of the sampling period is important when interpreting this seasonal pattern. The 2023 sampling year was not characterized by an anomalously dry conditions, as annual precipitation and profile soil wetness were close to or slightly above their long-term means. However, precipitation, temperature and soil wetness varied substantially among months, indicating pronounced within-year hydroclimatic variation. These conditions provide context for interpreting season as a contemporary environmental gradient, although the available climate data do not allow the individual contributions of temperature, precipitation, and soil wetness to the observed enzyme responses to be separated statistically.

Extracellular enzymes represent distinct biochemical functions and may respond differently to resource availability, pH, temperature, moisture and substrate supply [1,33]. Accordingly, seasonal shifts in soil conditions can modify enzyme activity even when long-term differences in soil chemistry persist. Similar seasonal regulation of extracellular enzyme activity has been reported in grasslands and other terrestrial ecosystems,

570 emphasizing the importance of contemporary environmental conditions in shaping
571 microbial process es [17,19,20].
572
573 Our third hypothesis predicted that bacterial community composition and EEA activity
574 would represent partially independent response dimensions, such that bacterial composition
575 would explain limited additional variation in enzyme activity after environmental
576 covariates were considered. Consistent with this prediction, bacterial community
577 composition explained little additional variation in the multivariate EEA profile (pseudo-F
578 = 1.07, partial R^2 = 0.108, p = 0. 374; Table 5). These results suggest that bacterial
579 community composition and the measured extracellular enzyme activity were structured at
580 different environmental scales [34]. This interpretation is consistent with evidence that soil
581 enzyme activity can track abiotic conditions more closely than community composition,
582 and with studies reporting inconsistent responses of microbial community structure and
583 enzyme activity to nutrient addition [7,8]. The lack of a statistically significant bacterial
584 community effect should not be interpreted as evidence that bacteria are functionally
585 irrelevant. Rather, it indicates that the taxonomic composition of the culturable fraction and
586 the measured enzyme activities provide complementary information about microbial
587 processes and need not vary synchronously across environmental gradients.
588
589 The ecological significance of this contrasting pattern lies in the different temporal and
590 spatial scales represented by the two response dimensions. The 75-year nutrient
591 manipulation was reflected primarily in bacterial community composition, whereas
592 extracellular enzyme activities were more strongly associated with contemporary
593 environmental variation. These results indicate that the environmental drivers of bacterial
594 community composition and extracellular activity need not operate at the same temporal or

spatial scales. Long-term fertilization can alter soil chemical conditions and microbial community structure, while enzyme activities may remain responsive to current resource availability and environmental conditions [15,3]. Furthermore, the absence of significant treatment x season interactions suggests that long-term nutrient enrichment did not modify the seasonal pattern of enzyme activity. This distinction may help explain why microbial community surveys and process-based measurements can yield apparently different responses to chronic nutrient enrichment.

Together, these findings indicate that long-term nutrient enrichment reorganized the soil environment and the composition of culturable bacterial assemblages without producing parallel changes in the overall EEA profile. More broadly, bacterial community composition and EEAs reflected different dimensions of environmental variation: long-term nutrient enrichment was associated with differences in community composition, whereas enzyme activity was more strongly structured by contemporary seasonal variation. The value of the UGNE therefore lies not only in its exceptional duration, but also in its capacity to reveal contrasting environmental patterns among different dimensions of microbial processes, a distinction that is difficult to resolve in short-term experiments.

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