

Article

Vertical Co-Evolution of Bacterial Communities and Sedimentary Geochemistry Across a Deep Regolith Profile in the Black Soil Critical Zone of Northeast China

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Abstract

Black soils are globally important for agricultural productivity, yet most studies of soil microbial communities are confined to the plow layer, leaving the deep regolith–bedrock interface largely unexplored. Here, we investigated bacterial diversity, community assembly, and co-occurrence networks along a 34-m regolith profile from the mollic epipedon to unweathered bedrock in the Songnen Plain, Northeast China. Bacterial alpha diversity exhibited a depth-dependent pattern with a secondary peak at 7.2–7.6 m, coinciding with buried organic carbon derived from paleolacustrine sedimentation. Community composition shifted significantly with depth, with *Acinetobacter* and *Achromobacter* enriching in deep layers as indicators of bio-weathering processes. Heterogeneous selection dominated community assembly, reinforced by strong dispersal limitation along the sedimentary gradient. Random forest and structural equation modeling identified depth as the primary driver, with pH, chemical weathering, and silt content mediating indirect effects. Network analysis revealed Chloroflexi as keystone taxa, with modules linked to methanogenic and nitrification functions in anaerobic deep strata. These findings demonstrate that sedimentary geochemical gradients and bacterial communities have co-evolved throughout the Black Soil Critical Zone, providing a mechanistic framework for whole-profile soil health management in one of the world’s most productive agricultural regions.

Keywords: black soil critical zone; deep regolith profile; bacterial community; co-evolution; biogeochemical cycling



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1. Introduction

The Earth’s Critical Zone (CZ) refers to an integration layer that spans from the top of the canopy down to the surface of fresh, unweathered bedrock, which can provide sustainable ecological services, energy, and resources for terrestrial life [1–4]. Bacterial communities that colonize the belowground portion of the Earth’s Critical Zone are the most abundant and diverse life forms of the biosphere in terrestrial ecosystems, responsible for various essential biogeochemical processes (i.e., element migration, carbon sequestration, biological weathering, and pollutant degradation) and interact with the hydrosphere, lithosphere, and atmosphere in the Earth’s surface system [5–7]. Despite many studies uncovering the

multifunctionality of soil microbiomes, most investigations focused solely on topsoil, with limited exploration of microbial diversity across the dozen-meter-thick regolith profile of CZ beneath the surface [8–11].

As a huge reservoir for unculturable bacterial taxonomic and functional diversity in the Earth's surface system, the regolith profile comprises soil and saprolite, which are often several meters deep and have been considered to be heterogeneous, with distinct niches supporting diverse microbial communities [12–15]. By using culture-independent sequencing technology and metagenomic analysis, recent studies demonstrated that bacterial communities in the regolith profile were significantly different from those in the topsoil [16–18]. Due to the limitations of oxygen and the depositional environment in the subsurface sediment, bacterial taxa abundant from topsoil to the unweathered bedrock have gradually shifted from a typical eutrophic group to oligotrophic species, which can better adapt to the oligotrophic conditions of the regolith profile [14]. Bacterial taxa living in this sedimentary environment of the regolith profile usually have special ecological functions, such as iron redox and nitrate reduction processes, which are closely related to biological weathering of minerals, pedogenesis, and the geomorphic evolution of the Earth's surface systems. In addition, current knowledge indicates that regolith of the CZ is usually recognized as a huge carbon pool, which is of paramount importance for global climate change. More than half of soil organic carbon accumulates below 1 m depth, and these deep carbon pools sustain microbial activity and root growth across pedogenic timescales [19]. Elucidating the subsurface bacterial biodiversity along deep regolith profiles and its interactions with ambient sedimentary environments is crucial for understanding microbially driven carbon pump mechanisms and the stability of the carbon pool of the CZ ecosystem.

Black soils, which are also classified as Mollisols, Isohumisols, Chernozems, and Phaeozems in different soil taxonomy systems, are the most fertile and productive soil resources around the world [19–23]. Black soils in China are mainly distributed in the Northeast China Plain, which covers nearly $55.6 \times 10^4 \text{ km}^2$ [24]. Due to the extensive anthropogenic farming in the past few decades, black soils in China are undergoing severe degradation processes, including water and wind erosion, acidification, and soil organic carbon loss, all of which are threatening long-term crop production, food security, and regional agroecosystem sustainability [25–28]. With the implementation of the Outline of Northeast Black Soil Protection Plan (2017–2030) and the Black Soil Protection Law of the People's Republic of China, the sustainable utilization and conservation of black soil resources have become a vital national strategic priority for maintaining grain production and safeguarding food security in China [29]. While prior studies have elucidated the coupling between soil microbiomes and black soil quality improvement, defined the connections between microbial survival strategies and carbon sequestration under varied tillage practices and fertilization regimes, and identified mechanisms supporting black soil health and multifunctionality, they are largely restricted to topsoil horizons [30–33]. However, surface soils are strongly influenced by short-term agricultural management practices and seasonal environmental fluctuations, which may obscure the long-term interactions between microbial communities and geological processes. Deep regolith profiles extending from soil horizons to weathered parent materials and bedrock interfaces preserve valuable records of sediment deposition, pedogenesis, and chemical weathering processes over geological timescales. Therefore, investigating microbial communities throughout deep regolith profiles provides a unique opportunity to uncover how bacterial diversity, community assembly, and functional potentials respond to persistent geochemical gradients during critical zone evolution. Despite this importance, microbial processes within the deep regolith of Black Soil Critical Zones remain poorly understood.

Here, we conducted a study to investigate belowground bacterial diversity, community composition, ecological interactions, and predicted functional potentials across a deep regolith profile in the Black Soil Critical Zone of the Northeast China Plain, a representative region of global black soil zones. By drilling borehole samples from surface soils to bedrock and applying high-throughput amplicon sequencing, we aimed to determine how bacterial communities vary along vertical geochemical gradients and how geological conditions influence microbial community assembly in deep regolith environments. We hypothesized that bacterial diversity, community composition, ecological networks, and predicted functional potentials would exhibit depth-dependent patterns associated with heterogeneous sedimentary and geochemical environments throughout the Black Soil Critical Zone. Given that bacterial communities contribute to biogeochemical cycling in subsurface ecosystems, this study provides new insights into the vertical organization of microbial communities from topsoil to bedrock and improves our understanding of microbial–geochemical interactions within critical zone ecosystems.

2. Materials and Methods

2.1. Study Area of the Black Soil Critical Zone and Sampling Collections

The field investigation site is established downstream of the KeYin River basin (between 126.7875 and 126.8730 E of longitude and 47.3227 and 47.3861 N of latitude), Heilongjiang Province, China. The study site is situated 32 km from the Hailun National Agroecosystem Field Station, Chinese Academy of Sciences. Based on the Köppen–Geiger climate classification, the study area had a typical temperate continental monsoon climate. The mean annual precipitation is about 560 mm, and the mean annual temperature is about 4.0 °C, with an annual accumulated active temperature (sum of daily mean temperatures ≥ 10 °C) of approximately 2650 °C. Soils in this region are mainly derived from Quaternary unconsolidated sediments and are classified as Mollisols, Isohumisols, Chernozems, and Phaeozems based on the Chinese Soil Taxonomy, USDA Soil Taxonomy, and World Reference Base for Soil Resources [20,22,23]. The soils here are characterized by a typical mollic epipedon with a higher base saturation and higher organic carbon concentration. The corresponding cropping type in the study area was maize (*Zea mays* L.) cropland in one-season planting.

By using the Truck-Mounted Rotary Drilling Rigs (108 mm inner diameter), we drilled a borehole from the surface soil down to the fresh weathered bedrock and obtained continuous regolith profile core samples. Borehole drilling samples were collected at 20 cm intervals in the 0–10 m layer, 50 cm intervals in the 10–20 m layer, and 1 m intervals in the 20–34 m deep regolith layer, totaling 31 samples along the profile. A total of 31 soil and regolith profile samples were collected from the topsoil to fresh bedrock at 3400 cm in this study. All specific sampling depths are listed in Table S1. We stripped the outer wall of the sediment core after sampling to prevent sample contamination by the drill cylinder inner wall (Figure 1).

2.2. Geochemical Properties Analyses

The regolith profile samples from the borehole were placed in sterile plastic bags and divided into two portions. One portion was air-dried and sieved before being used for the analysis of total organic carbon content, total nitrogen content, pH, and geochemical properties analysis, including mineral composition and grain size distribution. The moisture content of regolith profile samples was measured by weighing the fresh samples and then subtracting their weight after drying to a constant mass in an oven at 105 °C for 24 h. Briefly, total organic carbon content was determined using the Walkley–Black dichromate oxidation method, and total nitrogen content was measured with the Semi-Micro Kjeldahl method. pH

was measured with a glass electrode. Contents of mineral elements were determined using an ICP-AES (Optima 8000, PerkinElmer, Shelton, CT, USA). We further calculated weathering indices, including the chemical index of alteration (CIA, $[\text{Al}_2\text{O}_3/(\text{Al}_2\text{O}_3 + \text{CaO} + \text{Na}_2\text{O} + \text{K}_2\text{O})] \times 100$) and the base index (BA, $[(\text{CaO} + \text{Na}_2\text{O} + \text{K}_2\text{O})/\text{Al}_2\text{O}_3] \times 100$), to assess the regolith profile's relative degree of chemical weathering based on major-element analysis of K_2O , CaO , Na_2O , MgO , Al_2O_3 , Fe_2O_3 , MnO , P_2O_5 , SiO_2 , and TiO_2 . Mineral composition in subsurface sediments was analyzed using the X-ray diffraction method (XRD). The grain size characteristics were analyzed by a laser diffraction particle-size analyzer (Mastersizer 2000E, Worcestershire, UK). Before the analysis, ~ 0.2 g sample (diameter < 2 mm) was prepared by adding 30% H_2O_2 and 10% HCl to remove organic matter and carbonates, and the sample solution was ultrasonicated for 10 min before being transferred to the particle-size analyzer.

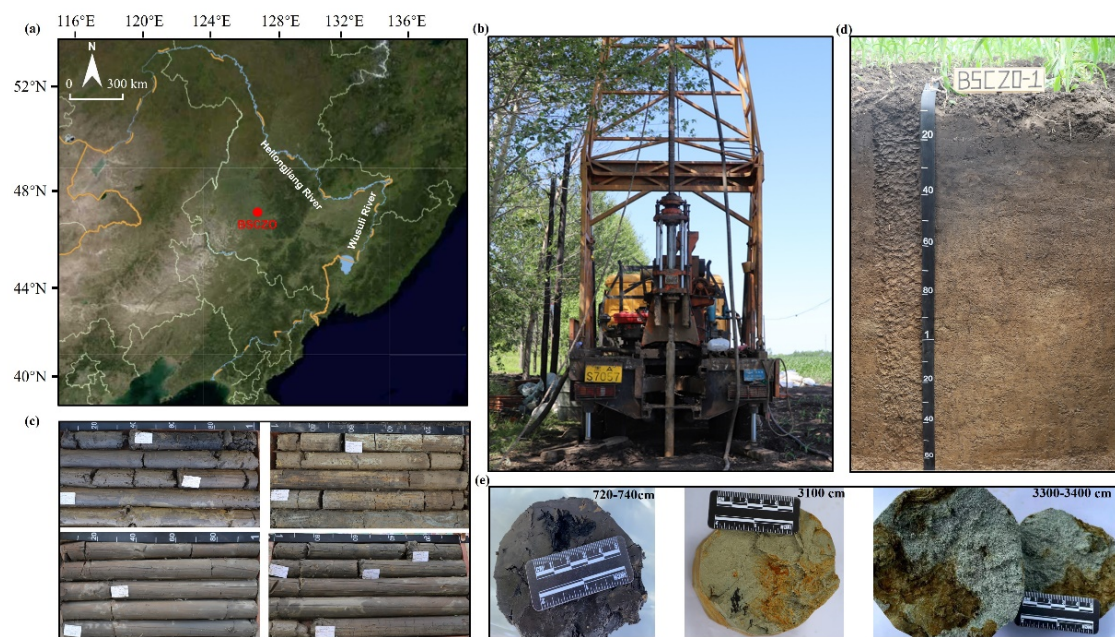


Figure 1. Location of the study site (a), instrument of Truck-Mounted Rotary Drilling Rigs for drilling borehole (b), borehole core samples of regolith (c), representative soil profile of the study site (d), micromorphology of the interval borehole samples at different regolith depths (e).

2.3. DNA Extraction, PCR Amplicon, and Sequencing

Regolith borehole samples collected for molecular analysis were immediately stored at -80 °C after sampling and were subjected to DNA extraction on the following day. DNA was extracted from 0.5 g of each well-mixed sample using the MP FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA), according to the manufacturer's instructions. The quality and quantity of extracted DNA were assessed using a NanoDrop 2000 Spectrophotometer (NanoDrop ND2000, Thermo Scientific, Wilmington, DE, USA).

Bacterial 16S rRNA gene amplicon libraries were generated using the PCR primers 515F (5'-GTGCCAGCMGCCGCGG-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3'). PCR reaction mixtures (20 μL) contained 4 μL of 5 $\times$ FastPfu Buffer, 0.8 μL of each primer (5 μM), 2 μL of 2.5 mM dNTPs, 10 ng of template DNA, and 0.4 μL of FastPfu Polymerase. The amplification conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 28 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, with a final extension at 72 °C for 10 min. PCR amplicons were separated by electrophoresis on 2.0% agarose gels and purified by gel extraction to remove potential non-specific amplification products and primer dimers before sequencing. Purified amplicons were pooled at equimolar concentrations and subjected to paired-end sequencing on an Illumina® MiSeq PE250 platform (MiSeq Control Software 2.5.0.5 and Real-Time Analysis software 1.18.54.0, Illumina Inc., San Diego, CA, USA).

Raw sequences were quality-filtered using standard quality-control procedures prior to downstream analysis in the QIIME pipeline (<http://qiime.org/install/index.html>, accessed on 11 May 2024). Paired-end reads were merged, and sequences containing ambiguous nucleotides or low-quality reads were removed. Chimeric sequences were identified and removed using the UCHIME algorithm. Detailed sequencing information for all samples, including sequencing reads, total bases, and average sequence lengths, is provided in Table S2. The remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using the UPARSE pipeline [34]. Considering the low-biomass characteristics of deep-regolith samples, potential contamination was carefully evaluated during sequence processing. Chloroplast and mitochondrial sequences were removed before downstream bacterial community analyses, and taxonomic assignments were screened to exclude potential non-target sequences. Taxonomic assignments were performed using the SILVA database (v138) with the RDP Classifier (v2.2) implemented in QIIME, using a confidence threshold of 70%. Phylogenetic trees were constructed from representative OTU sequences using the FastTree method, and Faith's phylogenetic diversity (PD) was calculated based on the resulting tree. Alpha diversity indices, including Shannon, Simpson, Chao1, ACE, and Faith's PD, were calculated using QIIME. To minimize biases caused by differences in library sizes among samples, OTU tables were rarefied to an equal sequencing depth before diversity analyses, while relative abundance normalization was applied for microbial community composition analyses. Rarefaction curves based on Shannon diversity indices are shown in Figure S1. Potential metabolic functions of bacteria in the subsurface regolith profile were predicted by Functional Annotation of Prokaryotic Taxa (FAPROTAX) [35]. All these obtained sequences of borehole samples from the Black Soil Critical Zone were deposited in the NCBI database with the accession number PRJNA1464389.

2.4. Statistical Analysis

Particle-size characteristics of regolith samples were divided into three categories, including clay (<2 µm), silt (2~50 µm), and sand (50~2000 µm). Bacterial relative abundance and alpha diversity of OTU richness, Shannon–Wiener diversity, and Simpson indices were evaluated for each depth of regolith borehole samples. To identify bacterial community composition and depth-distribution patterns, bacterial beta-diversity was ordinated by principal coordinate analysis (PCoA) and nonmetric multidimensional scaling (NMDS) analysis based on the Bray–Curtis distance matrix. PCoA was used to visualize overall differences in bacterial community composition among samples, whereas NMDS was used to evaluate the relative similarity patterns among samples based on rank distances. Bacterial co-occurrence networks of the Black Soil Critical Zone were constructed using the Molecular Ecological Network Analysis (MENA) pipeline based on the random matrix theory (RMT) threshold [36]. The network was constructed using the OTU abundance matrix after quality filtering and normalization. Pairwise correlations among OTUs were calculated, and significant associations exceeding the RMT-derived threshold were retained to construct the network. Positive and negative correlations were represented as positive and negative edges, respectively. Network visualization was performed using the Gephi platform (version 0.10.1) [37]. Topological properties, including the number of nodes, edges, and network modules, were calculated. Module eigengenes were calculated for each network module, and their relationships with regolith physicochemical and geochemical properties were evaluated using Spearman's correlation analysis ($p < 0.05$). Nodes were classified based on within-module connectivity (Z_i) and among-module connectivity (P_i) into peripheral nodes ($Z_i \leq 2.5$ and $P_i \leq 0.62$), connectors ($Z_i \leq 2.5$ and $P_i > 0.62$), module hubs ($Z_i > 2.5$ and $P_i \leq 0.62$), and network hubs ($Z_i > 2.5$ and $P_i > 0.62$). We further investigated assembly processes of bacterial communities in the regolith profile by using the

phylogenetic bin-based null model (iCAMP) [38]. iCAMP analysis categorizes community assembly processes into heterogeneous selection (HeS), homogeneous selection (HoS), dispersal limitation (DL), homogenizing dispersal (HD), and drift (DR). Bacterial diversity indices, community composition, iCAMP analysis, and ordination analyses were performed using the ‘vegan’, ‘iCAMP’, ‘spaa’, and ‘MicEco’ packages in the R platform. PERMANOVA was conducted using the adonis function implemented in the vegan package based on Bray–Curtis distance matrices with 999 permutations to evaluate the effects of regolith properties on bacterial community composition. The homogeneity of multivariate dispersions was assessed using the betadisper function before PERMANOVA [39]. The Sloan neutral community model was fitted using the MicEco package to evaluate the relative contribution of stochastic processes to bacterial community assembly.

Linear regression analyses were conducted to assess the relationships between bacterial alpha diversity and environmental variables. Random forest regression analysis was performed to identify the key geological properties controlling bacterial community composition. Bacterial community composition was used as the response variable, and regolith depth, pH, silt proportion, and weathering leaching index were used as predictor variables. The random forest model was constructed using the randomForest package with 999 trees, and variable importance was evaluated based on the percentage increase in mean squared error (IncMSE) [40,41]. Structural equation modeling (SEM) was conducted using IBM AMOS v23 to evaluate the direct and indirect relationships between regolith physicochemical and geochemical properties and bacterial community characteristics. Environmental variables included in the SEM were selected based on their ecological relevance and significant associations with bacterial diversity and community composition. The conceptual model hypothesized that regolith physicochemical and geochemical gradients directly influenced bacterial community characteristics, while some environmental variables could also exert indirect effects through their interactions. All variables were standardized before model construction, and multicollinearity among variables was examined prior to SEM analysis. Model parameters were estimated using maximum likelihood estimation with bootstrap resampling. Model fit was evaluated using χ^2/df , goodness-of-fit index (GFI), comparative fit index (CFI), and root mean square error of approximation (RMSEA). A satisfactory model fit was indicated by $\chi^2/\text{df} < 2$, GFI and CFI > 0.90 , and RMSEA < 0.08 .

3. Results

3.1. Geochemical Properties at Different Depths of Regolith Profile

The distribution of geochemical and physicochemical characteristics varied substantially across the regolith profile from the topsoil to the fresh unweathered bedrock (Figure 2). The mean values and standard deviations of these environmental variables at different depth intervals are summarized in Table S3. Briefly, the pH values ranged from 5.94 to 8.14 and exhibited a significant increasing trend with increasing regolith depth ($R^2 = 0.74$, $p < 0.001$). Moisture content varied between 21.0% and 34.4%, with the highest values observed at 540–600 cm depth, likely associated with groundwater distribution and water retention characteristics within the regolith profile. Total organic carbon (TOC) and total nitrogen (TN) concentrations ranged from 0.81–26.15 g kg^{−1} and 0.16–1.92 g kg^{−1}, respectively. Linear regression analysis demonstrated that TOC content showed a significant decreasing trend with increasing depth ($R^2 = 0.15$, $p < 0.05$). However, an evident accumulation of organic carbon was observed at 720–760 cm depth, suggesting localized organic carbon preservation within specific sedimentary layers. Particle-size distribution showed that silt (2–50 µm) was the dominant fraction across the regolith profile, accounting for 49.4–65.5% of total particle composition, followed by clay (< 2 µm) and sand (50–2000 µm). Regression analysis revealed that the silt fraction significantly decreased with increasing

depth ($R^2 = 0.69$, $p < 0.01$), whereas sand proportion increased significantly along the depth gradient ($R^2 = 0.16$, $p < 0.05$). The chemical index of alteration (CIA) ranged from 45.9 to 76.4, while the weathering leaching index (BA) ranged from 0.40 to 1.35 (Table S3). Clay mineral composition analysis showed that illite and illite–smectite mixed layers dominated the upper 2500 cm of the regolith profile, whereas smectite and kaolinite became enriched in samples below 3000 cm depth (Table S4).

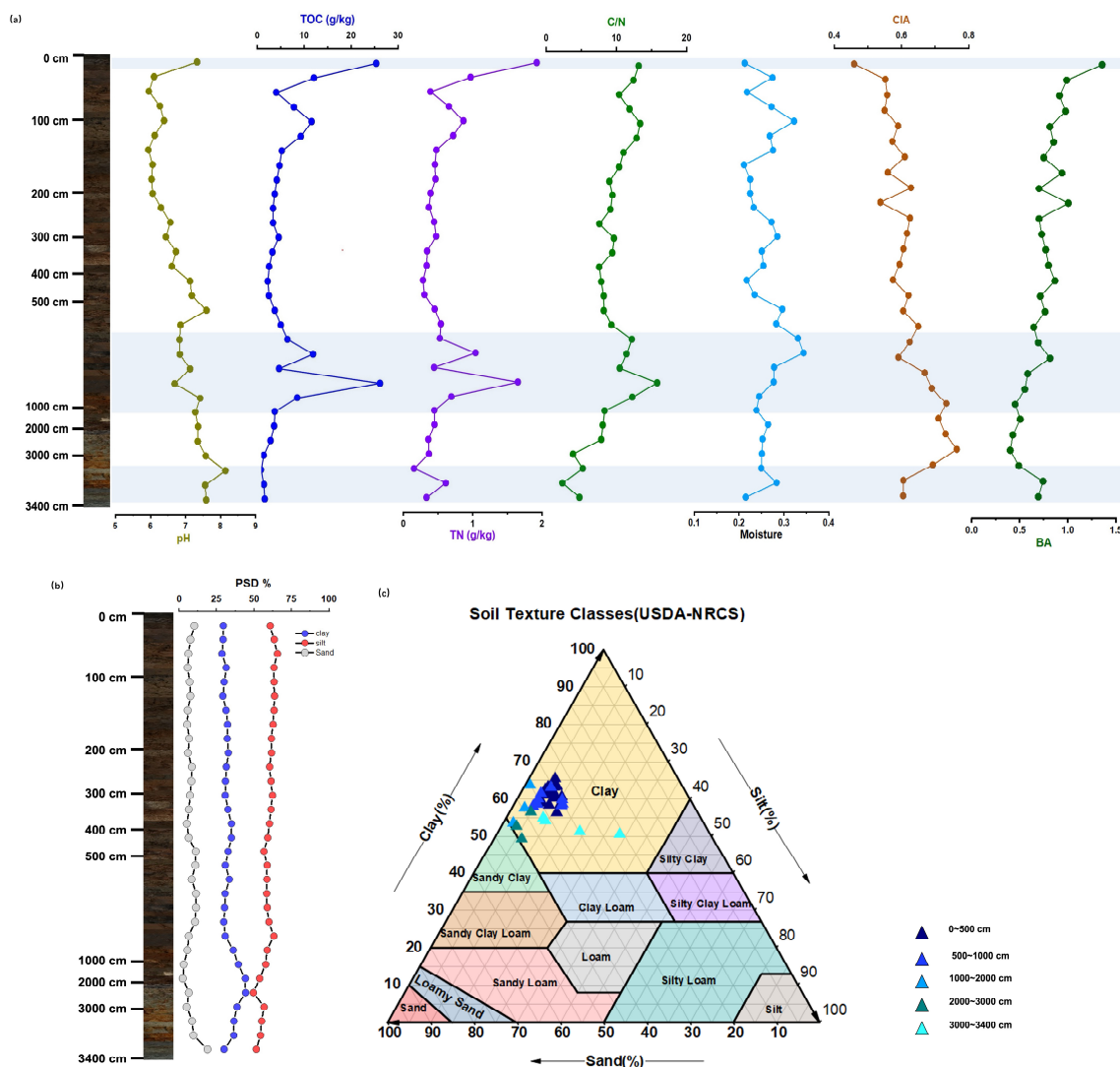


Figure 2. Vertical variations in physicochemical and geochemical properties and particle-size characteristics along the regolith profile. (a) Depth profiles of pH, total organic carbon (TOC), total nitrogen (TN), C/N ratio, moisture, chemical index of alteration (CIA), and base leaching index (BA). (b) Vertical variations in particle-size distribution (PSD), including clay, silt, and sand fractions. (c) Soil texture classification based on clay, silt, and sand proportions using the USDA-NRCS soil texture triangle; samples are grouped by depth intervals.

3.2. Bacterial Biodiversity, Community Composition, and Potential Functions in the Regolith Profile

After quality filtering and removal of ambiguous bases and low-quality reads, a total of 1,595,243 high-quality sequences representing 12,887 OTUs were obtained from the regolith profile based on 16S rRNA gene amplicon sequencing. Detailed sequencing information, including sequencing reads, total bases, and average sequence lengths, is provided in Table S2. Only 3.75% of OTUs were shared across the entire regolith profile, whereas deep layers near the bedrock contained a higher proportion of unique OTUs (8.86%), indicating

distinct bacterial assemblages in deep regolith environments. Bacterial alpha diversity indices, including Shannon diversity, Simpson index, OTU richness, and Faith's phylogenetic diversity (PD), are summarized in Table S5. Faith's PD ranged from 22.04 to 193.96, Simpson indices ranged from 0.01 to 0.50, and OTU richness varied from 178 to 3324 across samples. Shannon diversity ranged from 1.49 to 6.44 and exhibited a significant decreasing trend with increasing depth ($R^2 = 0.39$, $p < 0.05$) (Figure 3a).

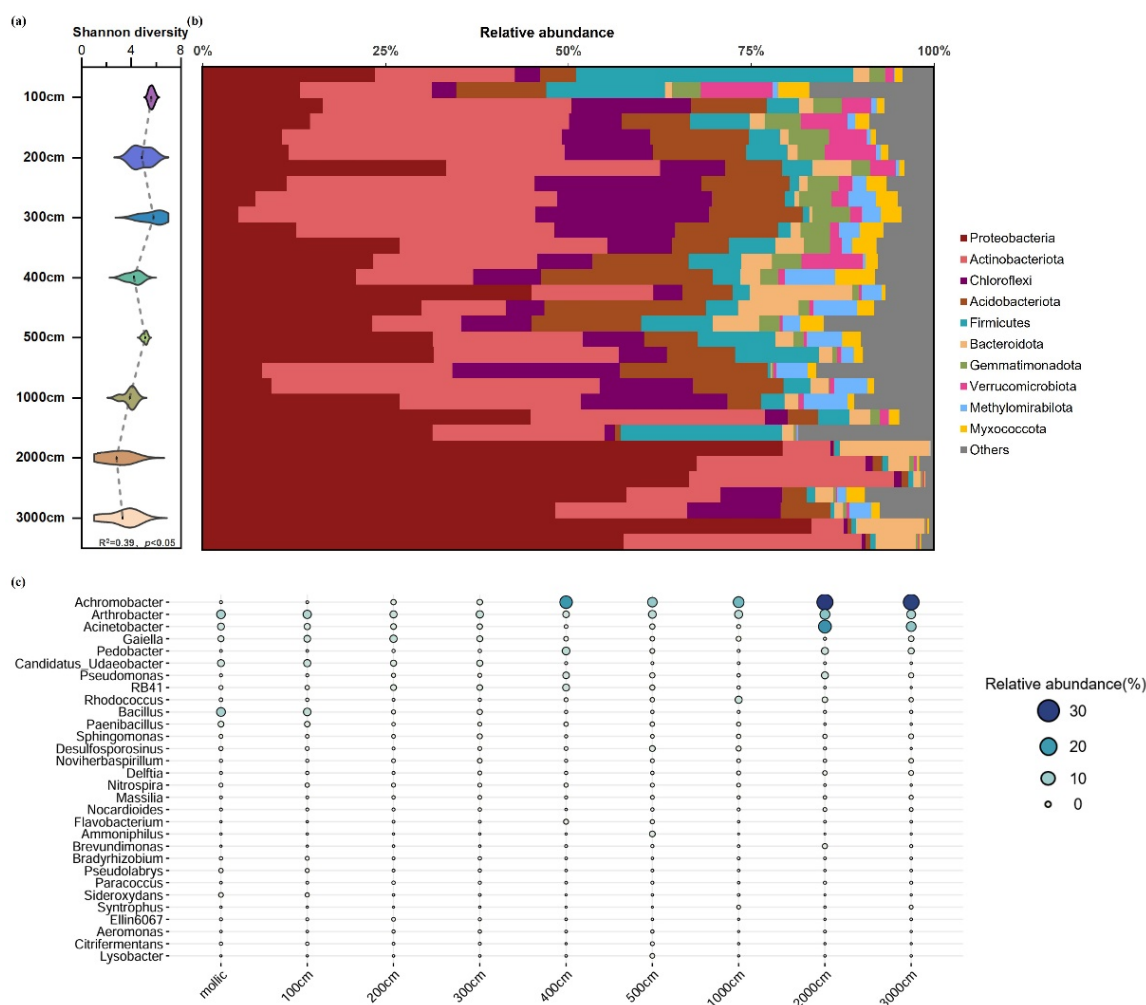


Figure 3. Bacterial diversity and community composition of the regolith profile. (a) Violin plot of bacterial Shannon diversity at different regolith depths ($n = 31$). (b) Bacterial community composition at the phylum level. (c) Heatmap of the relative abundance of dominant bacterial genera at different depths of the regolith profile.

Taxonomic analysis revealed that Proteobacteria, Actinobacteriota, Chloroflexi, and Acidobacteriota were the dominant phyla throughout the regolith profile, accounting for approximately 80% of total bacterial relative abundance (Figure 3b). Proteobacteria accounted for 4.8–83.2% of total abundance, followed by Actinobacteriota (4.3–44.9%), Chloroflexi (0.3–23.8%), and Acidobacteriota (0.2–23.5%). Proteobacteria showed significant enrichment in deeper layers (2000–3000 cm), whereas Actinobacteriota and Acidobacteriota were more abundant in shallow layers (0–300 cm). At the genus level, *Arthrobacter*, *Gaiella*, and *Bacillus* were enriched in shallow regolith layers, while *Pedobacter*, RB41, *Desulfosporosinus*, and *Ammoniphilus* were dominant at intermediate depths (300–1000 cm). In contrast, *Rhodococcus*, *Acinetobacter*, *Arthrobacter*, and *Achromobacter* were preferentially enriched in deeper layers (1000–3000 cm).

Ordination analyses based on Bray–Curtis dissimilarities revealed clear depth-associated patterns in bacterial community composition. NMDS analysis showed a reliable ordination result (stress = 0.14), and PCoA further confirmed the depth-dependent separation, with the first two axes explaining 25.66% and 17.36% of total community variation, respectively (Figure 4a,b). PERMANOVA analysis demonstrated that regolith depth significantly explained variation in bacterial community composition (pseudo- $F = 2.8$, $R^2 = 0.46$, $p < 0.001$). The Sloan neutral community model explained 48.33% of community variation ($R^2 = 0.4833$) with a migration rate ($m = 0.0233$), indicating that stochastic processes contributed partially but could not fully explain bacterial community assembly. The iCAMP framework further showed that deterministic processes dominated bacterial community assembly, with heterogeneous selection representing the major assembly mechanism, whereas dispersal limitation was the dominant stochastic process (Figure 4c,d). The Functional Annotation of Prokaryotic Taxa (FAPROTAX) approach was used to predict the potential ecological functions of bacterial communities. FAPROTAX analysis predicted a total of 72 potential functional groups across the regolith profile (Table S6; Figure S2). Chemoheterotrophy and aerobic chemoheterotrophy were the dominant predicted functions throughout the profile. Several nitrogen-cycling-related functions, including nitrate reduction, nitrate respiration, and nitrogen respiration, showed depth-dependent enrichment patterns. In addition, predicted functions associated with aromatic compound degradation and plastic degradation increased in deeper regolith layers, whereas cellulolysis and intracellular parasite-related functions exhibited decreasing trends with depth (Figure S3).

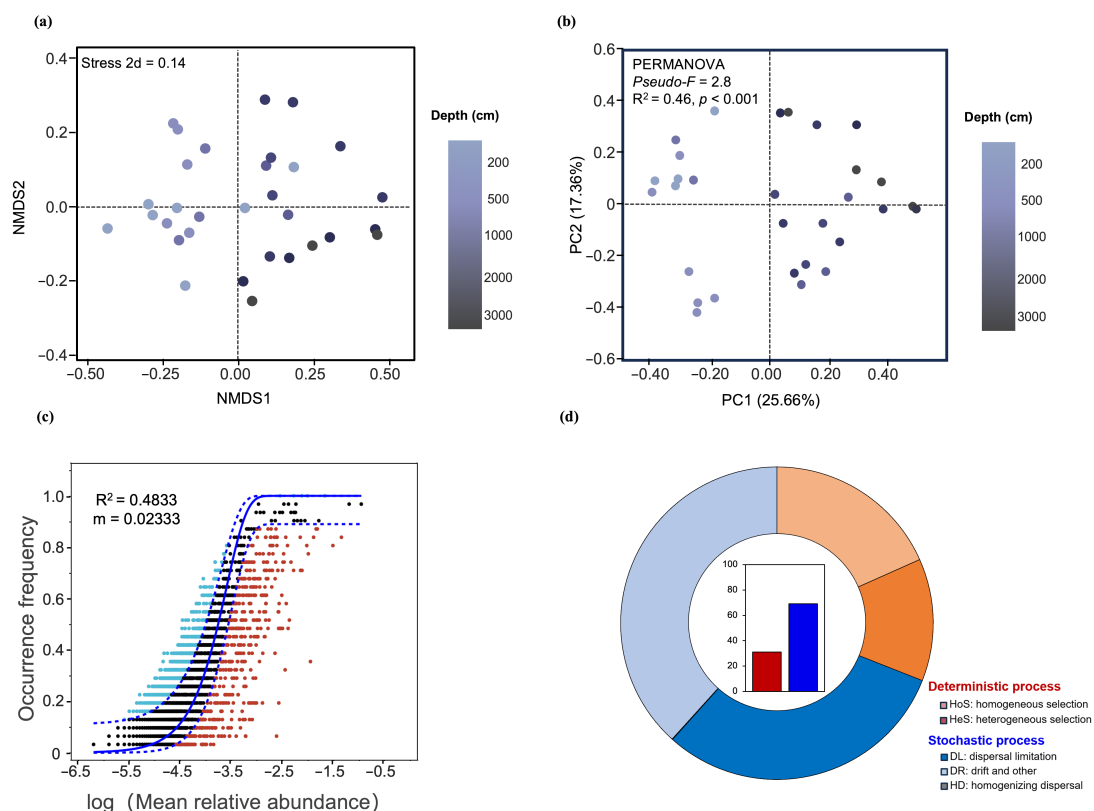


Figure 4. Bacterial community structure and assembly processes along the regolith profile. (a) Non-metric multidimensional scaling (NMDS) analysis of bacterial community composition based on Bray–Curtis dissimilarities. (b) Principal coordinate analysis (PCoA) of bacterial community composition based on Bray–Curtis dissimilarities. Samples in (a,b) are colored according to regolith depth. (c) Sloan neutral community model describing the relationship between mean relative abundance and occurrence frequency of bacterial OTUs. (d) Relative contributions of deterministic and stochastic processes to bacterial community assembly quantified using iCAMP.

3.3. Bacterial Co-Occurrence Networks in the Regolith Profile

To explore potential interactions among bacterial taxa along the regolith profile, a bacterial co-occurrence network was constructed based on OTU abundance patterns using the MENA pipeline. The resulting network consisted of 454 nodes and 1044 edges, including positive and negative associations among bacterial taxa (Figure 5a). The network showed a significant scale-free topology (power-law $R^2 = 0.89$), indicating non-random organization of bacterial associations. A total of six modules were identified within the bacterial network (Figure 5b). Module 1, module 3, and module 6 contained the largest proportions of network nodes, suggesting that these modules represented major interaction structures within the bacterial community. Node connectivity analysis revealed that OTU10453, affiliated with Chloroflexi and accounting for 0.46% of total relative abundance, showed the highest node degree and represented a highly connected taxon within the network. Based on within-module connectivity (Z_i) and among-module connectivity (P_i), OTUs were classified into four topological roles: peripheral nodes, connectors, module hubs, and network hubs. Connector taxa ($P_i > 0.62$ and $Z_i \leq 2.5$) were mainly affiliated with Thermoleophilia, Dehalococcoidia, Gammaproteobacteria, Acidobacteriae, Nitrospiria, and Anaerolineae, suggesting their potential importance in maintaining inter-module connectivity and network stability (Figure 5d).

Module eigengene analysis further revealed significant associations between bacterial network structures and regolith environmental gradients (Figure S4). Module 1 exhibited significant negative correlations with regolith depth ($r = -0.51$, $p < 0.01$) and pH ($r = -0.67$, $p < 0.001$), but it was positively correlated with silt proportion ($r = 0.49$, $p < 0.01$) and BA index ($r = 0.35$, $p < 0.05$). Module 3 showed similar environmental associations, with significant negative relationships with depth ($r = -0.67$, $p < 0.001$) and pH ($r = -0.68$, $p < 0.001$), and positive correlations with silt proportion ($r = 0.60$, $p < 0.001$) and BA index ($r = 0.45$, $p < 0.01$). Module 5 and module 6 were significantly associated with nutrient-related variables. Specifically, module 5 eigengene was positively correlated with TOC ($r = 0.46$, $p < 0.01$) and TN ($r = 0.47$, $p < 0.01$), whereas module 6 showed significant positive correlations with TOC ($r = 0.53$, $p < 0.001$) and TN ($r = 0.50$, $p < 0.001$). In addition, module 6 showed significant negative correlations with depth ($r = -0.63$, $p < 0.001$) and pH ($r = -0.40$, $p < 0.05$). These results indicate that bacterial interaction patterns were closely associated with vertical geochemical gradients and nutrient availability within the regolith profile.

3.4. Geochemical and Physicochemical Driving Forces in Shaping Bacterial Communities in the Regolith Profile

Random forest regression analysis was performed to identify the major environmental predictors explaining variation in bacterial community composition. The first axis of NMDS (NMDS1), representing the major variation in bacterial community composition, was used as the response variable. The random forest model explained 74.49% of the variation in NMDS1 scores ($R^2 = 0.7449$, $p < 0.001$) (Figure 6a). Among all environmental predictors, regolith depth was the most important variable, contributing the highest increase in mean squared error (IncMSE = 16.53%), followed by pH (IncMSE = 11.72%), silt proportion, clay content, and weathering-related indices. These results indicate that vertical geochemical gradients were the dominant factors shaping bacterial community composition throughout the regolith profile.

Structural equation modeling (SEM) was further applied to disentangle direct and indirect relationships between environmental variables and bacterial community characteristics. In the SEM framework, bacterial community composition was represented by NMDS1, whereas bacterial diversity was represented by the Shannon diversity index. The final model showed good goodness-of-fit ($\chi^2/df = 1.14$, RMSEA = 0.05, CFI = 0.99,

GFI = 0.99) (Figure 6b). The SEM results demonstrated that regolith depth exerted strong direct effects on bacterial community composition, while depth-associated changes in pH and chemical weathering indices indirectly influenced bacterial communities. pH showed a strong positive effect on bacterial community variation, whereas BA and CIA showed negative relationships with bacterial community composition. In addition, TOC and TN contributed positively to bacterial diversity, indicating that organic nutrient availability promoted microbial diversity within the regolith profile. Standardized total effects further demonstrated that pH, depth, TN, and CIA were among the major factors influencing bacterial community characteristics (Figure 6c). Together, these results suggest that vertical environmental gradients, particularly depth-associated geochemical changes, regulate bacterial community differentiation and diversity patterns in the deep regolith ecosystem.

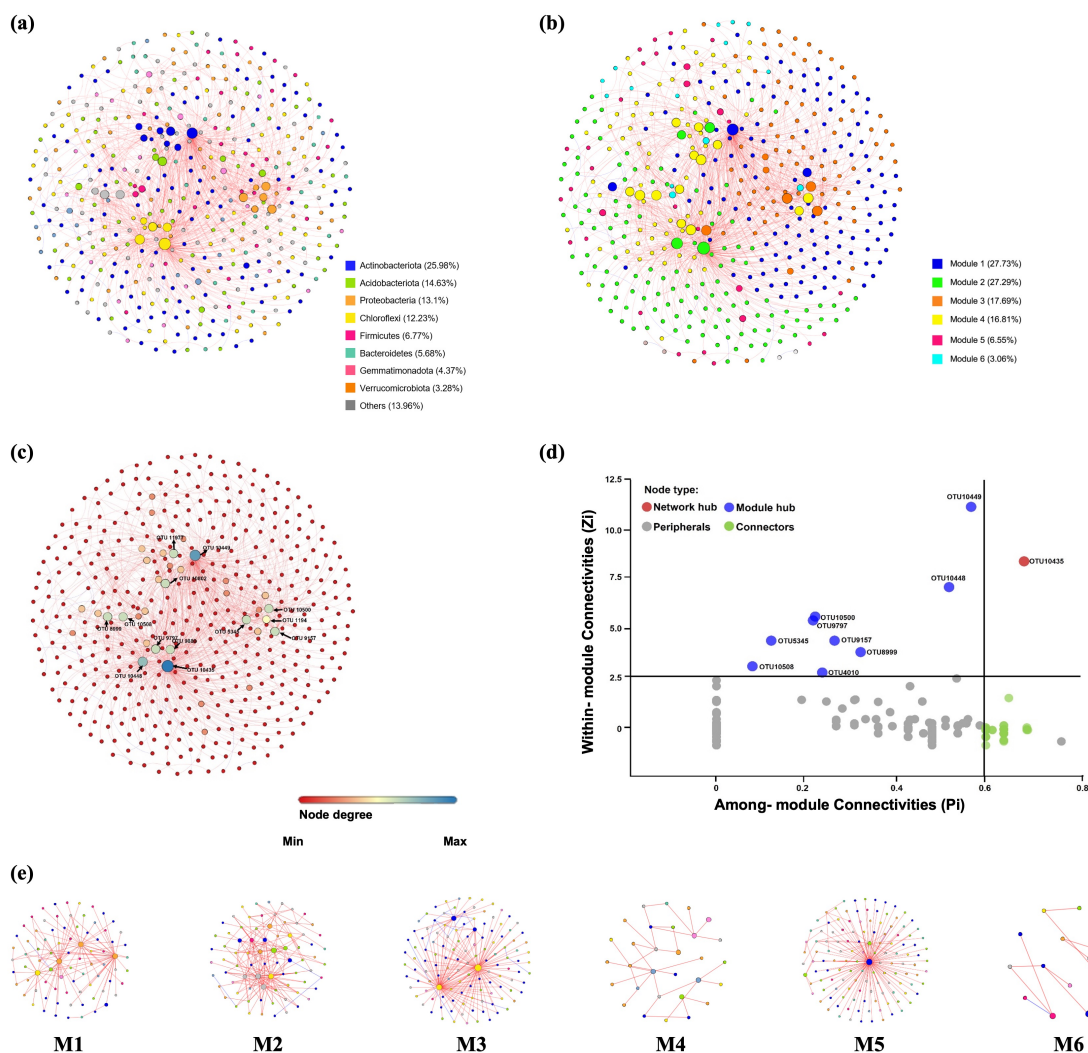


Figure 5. Bacterial co-occurrence networks based on OTUs in the regolith profile. (a) Overall bacterial co-occurrence network based on taxonomic affiliation. (b) Network modules identified from OTU interactions. (c) Node degree distribution of OTUs. (d) Zi–Pi plot showing the topological roles of OTUs, including peripheral nodes, connectors, module hubs, and network hubs. (e) Module-specific subnetworks of the six major modules (M1–M6) extracted from the bacterial co-occurrence network. Node size represents the relative abundance of OTUs. Node colors indicate phylum affiliation (a), module assignment (b), and node degree (c). Blue edges indicate positive associations, whereas red edges indicate negative associations among OTUs.

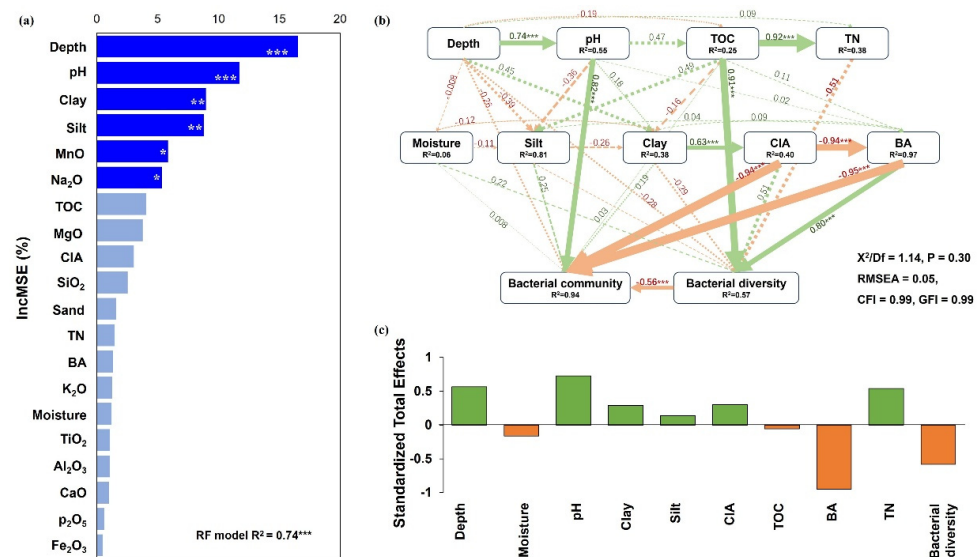


Figure 6. Potential geochemical drivers of bacterial community in the regolith profile. (a) Random forest regression model showing the relative importance of regolith physicochemical and geochemical properties in explaining bacterial community composition. (b) Structural equation model (SEM) illustrating direct and indirect relationships between regolith physicochemical and geochemical properties and bacterial community characteristics. Significant negative paths are shown as orange solid lines, whereas positive paths are shown as green solid lines. Dashed lines indicate non-significant relationships. Line width represents the strength of the standardized path coefficients. Numbers near the pathway arrows indicate standardized path coefficients. (c) Standardized total effects derived from the SEM shown above. Asterisks indicate statistical significance (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

4. Discussion

4.1. The Unseen Majority of Bacterial Diversity in the Black Soil Critical Zone

Black soils are among the most fertile soil resources worldwide, owing to their thick humus layer enriched in organic carbon and high agricultural productivity [41]. For decades, the black soils of the Northeast China Plain have suffered accelerated degradation, including water and wind erosion of sloping farmland, biodiversity loss, and food insecurity due to intensive regional crop management [27,42–44]. Extensive research has been conducted on belowground bacterial communities in the soil genetic humus layer and at the single-pedon scale of black soils, with findings contributing to a more comprehensive understanding of sustainable agricultural development in black soil regions worldwide from an ecological perspective [19,32,45]. In this study, we systematically explored bacterial diversity, community composition, potential multifunctionality, and ecological networks across a 34 m-deep vertical regolith profile in the typical Black Soil Critical Zone of the Northeast China Plain. Through borehole investigation, our results first extended the research scale of bacterial diversity in black soils from the top mollic epipedon to the unweathered bedrock interface, highlighting the mechanisms by which pedogenic evolution and depth of mollisols shape bacterial biodiversity of the deep biosphere in the black soil region. Despite the anaerobic, oligotrophic, and heterogeneous conditions prevalent in the deep regolith profile, our study revealed substantial diversity and abundance of generalist bacterial taxa inhabiting multiple strata of the regolith profile, spanning from the top of the humus layer down through the parent material and sedimentary layers to unweathered bedrock at critical zone scales.

In this study, we found that bacterial diversity in the regolith profile was depth-dependent, with the highest bacterial alpha diversity index occurring in the top mollic horizon (Figure 3a). This is consistent with a synthesis of previous studies, in which the accumulation of organic carbon in the humic epipedon of typical black soil provides

a nutrient-rich environment for soil microorganisms, recruiting a greater diversity of bacteria to participate in the carbon cycling and humification pedogenesis processes of the mollic epipedon [46–48]. Interestingly, a higher bacterial Shannon diversity index was observed at depths of 720~760 cm, which is likely attributed to the significant organic carbon accumulation and higher weathering degree at this depth, providing a favorable habitat for bacteria similar to the surface mollic layer (Figure 1e). Previous studies have suggested that organic carbon burial may occur in the deeper layers of black soil regions, likely associated with historical deposition processes or bioturbation of the Holocene mollisols [19,49]. In addition, the study area lies on the margin of the Early–Middle Pleistocene paleolake basin, where lacustrine sedimentation promotes organic carbon burial and consequently leads to organic carbon accumulation in deep layers [26,50,51]. Following a paleoclimatically driven lake-to-plain transition, meadowization processes under the warm climate of the Holocene promoted both the formation of extensive black soils and soil organic carbon accumulation in the Songnen Plain [52]. Building on this context, our work reveals for the first time that deep SOC burial within the evolution of the Songnen Plain governs the distribution patterns of microbial diversity in the subsurface of this Black Soil Critical Zone.

To minimize bias from field-based visual stratigraphy, the regolith profile was sampled at equidistant depth intervals; nevertheless, significant differences in bacterial community composition were observed across depths (Figure 3b). Across the regolith profile, bacterial genera *Acinetobacter*, *Gaiella*, *Pseudomonas*, *Rhodococcus*, *Bacillus*, *Delftia*, and *Massilia* were present throughout the entire regolith, a pattern consistent with their generalist lifestyle and global distribution in habitats ranging from soil and lakes to sediments and sewage [53–58]. Their persistent presence across depths underscores their resilience to environmental perturbations, adaptability to low organic carbon, and metabolic versatility in denitrification and heavy metal resistance [59]. We also found members of *Bacillus* and *Candidatus Udaeobacter* were predominant in the mollic epipedon, which are good predictors of agroecosystem multifunctionality in the Chinese mollisol region [60,61]. In contrast, members of *Acinetobacter* and *Achromobacter* demonstrated a gradual increase with depth of the regolith profile. The genus *Acinetobacter*, notable for its exceptional environmental adaptability, has been identified in diverse contaminated matrices, including groundwater and diesel oil-polluted soil, with studies confirming its biofilm-forming capacity as a key survival strategy in complex pollution scenarios [58,62,63]. *Achromobacter* has been reported to secrete indole-3-acetic acid (IAA) and exhibit phosphate-solubilizing ability, making it an indicator species involved in effective biological mineral weathering and soil formation [64]. The continuous enrichment of *Acinetobacter* and *Achromobacter* along the regolith depth gradient reveals that these two bacterial taxa adopt specialized life-history strategies to adapt to the selective pressures imposed by deep sedimentary environments. Their long-term colonization and evolution in drilling borehole samples are tightly coupled with the bio-weathering processes of sediments, thereby acting as crucial biological indicators for tracking sedimentary environmental evolution.

Beta diversity analyses revealed clear vertical differentiation of bacterial community structures along the regolith profile, as visualized by NMDS and further validated by PCoA. PERMANOVA results confirmed that depth was a significant driver of community variation, explaining 46% of the total variance, with distinct clustering between shallow (100~300 cm) and deep (400~3000 cm) assemblages. This stratified distribution pattern suggested divergent ecological pressures across the profile, prompting further investigation into the underlying community assembly mechanisms. The Sloan neutral community model provided a preliminary assessment of stochastic processes, yielding an R^2 of 0.483 and a low migration rate ($m = 0.023$). The moderate fit ($R^2 < 0.5$) indicated that stochastic processes (e.g., ecological drift, dispersal) alone could not fully explain the observed com-

munity structure, while the low migration rate implied strong dispersal limitation along the regolith profile. This pattern is likely driven by the heterogeneity of sediment grain size, porosity, and hydrological conditions formed during the regolith profile evolution, which hinders vertical microbial exchange between different layers [65,66]. We applied the iCAMP framework to further disentangle the relative contributions of deterministic and stochastic processes. Heterogeneous selection emerged as the dominant assembly mechanism, reflecting strong environmental filtering driven by pronounced vertical gradients in pH, organic carbon content, and the degree of chemical weathering. Complementarily, dispersal limitation (a stochastic process) also contributed substantially, consistent with the low migration rate from the neutral model and reinforcing that restricted microbial dispersal amplifies community divergence across the depth profile. FAPROTAX-based analysis suggested that bacterial communities in the deep regolith possessed predicted functional potential associated with biogeochemical cycling, particularly processes related to nitrogen cycling and organic matter transformation. These predicted functional profiles suggest that deep-regolith bacterial communities may participate in nutrient transformation and biogeochemical processes in subsurface environments. In a broader agricultural context, recent studies have also highlighted the importance of nitrogen-fixing bacteria and humic substances in regulating plant–microbe interactions and nutrient cycling, while field studies have demonstrated that *Bradyrhizobium*-based seed inoculation can improve soybean growth and productivity [67,68]. Although these studies were conducted primarily in cultivated surface soils and therefore cannot be directly extrapolated to deep regolith environments, they further illustrate the broader ecological and agricultural significance of microbially mediated nitrogen cycling.

Despite concerns about the robustness of microbial ecological networks inferred from high-throughput sequencing data using similarity or correlation metrics, network analysis remains invaluable for inferring potential inter-taxa relationships and elucidating the stability and environmental dynamics of microbial communities in diverse heterogeneous environments. In complex natural environments, such as soil, lakes, and marine sediments, associations among microbial species encompassing both competition and cooperation are pivotal for sustaining community stability and facilitating vital biogeochemical functions [69,70]. In this study, network analysis identified several distinct modules composed of species assemblages shaped by niche similarity or functional convergence within the regolith profile, and these modules further exhibited strong associations with historical geological evolutionary processes and contemporary biogeochemical dynamics of the regolith profile. OTU10453, affiliated with *Chloroflexi*, showed the highest node degree in the network. Previous studies have demonstrated that prolonged weathering significantly enhances the relative abundance of *Chloroflexi*, and their potential feast-or-famine organic carbon metabolisms may further underpin their role as keystone taxa within the regolith profile [71]. Moreover, taxa within modules 4#, 5#, and 6# were mainly affiliated with *Nitrospira* and *Dehalococcoidia*. *Nitrospira* are commonly associated with nitrification-related processes, particularly nitrite oxidation, whereas *Dehalococcoidia* are known for their involvement in organohalide respiration. These taxa may contribute to biogeochemical cycling through their predicted functional potentials in subsurface environments [72,73].

4.2. Co-Evolution of Black Soil Critical Zone and Microbial Communities: Sedimentary Mediation and Driver Disentanglement

Our results revealed pronounced vertical coupling between bacterial community characteristics and physicochemical and geochemical gradients across the deep regolith profile. This pattern highlights the importance of considering black soil ecosystems from a critical-zone perspective, extending beyond the biologically active surface soil to deeper weathered materials. The observed associations among microbial community structure,

diversity, predicted functional potentials, and environmental gradients are consistent with the broader concept that biosphere–geosphere interactions contribute to long-term critical-zone development. Rather than demonstrating direct evolutionary feedback, our findings provide evidence for persistent environment–microbe coupling across the regolith profile.

In this study, the sediment grain size characteristics, chemical weathering intensity, organic carbon distribution, and distinctive morphological features of the deep regolith profile observed in the Black Soil Critical Zone not only align well with the regional evolutionary framework of “aeolian parent material dominance, weak weathering regime, and accretionary pedogenesis” in the black soil region of the Songnen Plain but also offer critical physicochemical insights into deciphering the driving mechanisms of regional sedimentary processes on microbial diversity and community composition through depth-dependent differences in regolith profile geochemical properties [19,65,74]. Notably, the silt-dominated (2–50 μm) grain size of the regolith profile in the Black Soil Critical Zone validates the aeolian loess parent material origin documented in prior studies [50,65,75]. As the primary substrate for microbial habitats in regional black soils, silt constructs a capillary pore system that balances aeration and water retention, furnishing a stable physical microenvironment and sustained nutrient provision for microorganisms, which establishes the foundational prerequisite for maintaining microbial diversity [52]. The significant organic carbon accumulation at 7 m depth is congruent with the synchronous organic carbon sequestration during black soil accretionary pedogenesis reported previously [76]. The enriched organic carbon in this layer is presumably derived from aeolian herbaceous residues and potential lacustrine aquatic organic matter, providing ample carbon sources for microorganisms. This further explains the potential microbial diversity peak at this depth and underscores the positive regulatory role of organic carbon accumulation during sedimentary processes in shaping microbial communities.

Furthermore, the cyan-red reticulated morphology and relatively elevated Chemical Index of Alteration (CIA) values of the regolith profile at 31 m depth attest to the lacustrine sedimentary basement during the regression of the Songnen paleolake and redox-fluctuating conditions during paleoclimate oscillations [26,50]. This unique sedimentary context is likely to induce deep microenvironmental heterogeneity. Meanwhile, the presence of kaolinite in the clay mineral assemblage further corroborates the anomalous weathering intensity of this layer. Its combination with smectite and illite may modify deep pore architecture, organic carbon stability, and nutrient retention potential, thereby selecting for microbial taxa tolerant to extreme microenvironments and resulting in a distinct community composition compared to the shallow layer [69,77]. These findings not only validate the linkage mechanism by which regional sedimentary processes modulate microbial diversity through regulating physical habitats, nutrient supply, and chemical conditions but also provide rare clues for tracing the long-term shaping effects of paleoenvironmental changes on microbial communities via deep sedimentary archives, ultimately advancing the coupled understanding of the co-evolutionary relationships between sedimentary processes and microbial diversity patterns.

Vertical variations in bacterial diversity along regolith geochemical gradients revealed strong coupling between microbial communities and sedimentary environments within the Black Soil Critical Zone, suggesting potential long-term interactions between microbial communities and geological processes. Linear correlation analysis revealed that total organic carbon, silt fraction, and base leaching index promoted bacterial diversity by sustaining sufficient resource supply and favorable microhabitat conditions. In contrast, increased regolith depth, elevated pH, and intensified chemical weathering imposed strong environmental filtering that constrained bacterial community diversity. The consistent covariation between vertical geochemical gradients and bacterial spatial distribution pro-

vided macroscopic evidence for coupled environment–microbe evolution. Random forest modelling further identified regolith depth as the predominant driver of bacterial community stratification, with explanatory power far exceeding that of other geochemical variables. The robust model performance ($R^2 = 0.74$, $p < 0.001$) confirmed that depth-dependent geochemical variations dominated bacterial community differentiation across the regolith profile. These vertical distribution patterns of bacterial communities were fundamentally governed by the inherent physicochemical properties of black soils. The high organic carbon storage and cation exchange capacity of black soils provided abundant nutrients and stable microenvironments for shallow microbial communities [45,78]. Meanwhile, silt-dominated fine particles formed stable organic–mineral complexes that preserved organic carbon and diversified microhabitats, and strong base retention together with acid-buffering capacity further optimized microbial metabolic conditions [79,80]. Collectively, the intrinsic properties of black soils drove positive microbial responses to carbon accumulation, fine particle composition, and base status, whereas depth-induced geochemical degradation suppressed subsurface microbial diversity, forming a fundamental pattern of long-term environment–microbe coupling in sedimentary systems. Structural equation modelling further disentangled the direct and indirect relationships between environmental factors and bacterial communities. Depth directly imposes strong environmental filtering, while associated increases in pH and chemical weathering indirectly reduce bacterial diversity. Silt improves effective porosity and, together with organic carbon, supports higher diversity by enhancing substrate availability and microhabitat complexity. Sedimentary conditions of the regolith profile also strongly shape bacterial communities and interspecific interactions. Module eigengenes of bacterial co-occurrence networks mirror the diversity responses to geochemical gradients: organic carbon, total nitrogen, silt, and BA correlate positively with network module eigengenes, whereas depth, pH, and CIA correlate negatively with network topology. Within the Black Soil Critical Zone, vertical changes in sedimentary conditions impose distinct geochemical constraints that direct bacterial community assembly. Reciprocally, microbial colonization and predicted functional potentials may contribute to biogeochemical cycling and potentially influence long-term environment–microbe interactions within the regolith profile. The increasing enrichment of *Acinetobacter* and *Achromobacter* with depth suggests their potential ecological adaptation to deep sedimentary environments. Their distribution patterns may provide insights into microbial responses to long-term geological environmental changes. Collectively, our findings reveal a tightly coupled relationship between environmental gradients and microbial communities in the Black Soil Critical Zone, providing evidence for potential long-term environment–microbe interactions.

5. Conclusions

In summary, our study provides a holistic profile of bacterial diversity, community assembly, interspecific interactions, and functional potentials across a 34 m deep regolith profile from surface mollic epipedon to unweathered bedrock in the Black Soil Critical Zone, revealing distinct patterns of subsurface microbial distribution and their associations with critical zone processes. Bacterial diversity exhibits strong depth dependency shaped primarily by sediment depth and deep soil organic carbon burial linked to paleolake sedimentation and Holocene pedogenic evolution, challenging the conventional focus on only surface soil microbiomes in black soil research. The progressive enrichment of *Acinetobacter* and *Achromobacter* with depth highlights their specialized adaptive strategies and pivotal roles in sediment biological weathering, serving as valuable biological indicators for tracking paleoenvironmental and critical zone evolution. A *Chloroflexi*-affiliated OTU (OTU10453) showed a highly connected position in the bacterial co-occurrence network,

suggesting its potential importance in microbial network structure within the deep regolith profile. However, further genomic and functional analyses are required to confirm its ecological roles. Our findings expand the scope of black soil microbial ecology into the deep critical zone, emphasizing the potential importance of the deep biosphere in regulating terrestrial carbon and nutrient cycling. Given the importance of nitrogen cycling for black soil fertility and agricultural sustainability, our findings also provide a microbial ecological perspective for understanding nutrient transformation processes in agricultural landscapes. Previous studies have demonstrated that nitrogen-fixing bacteria and humic substances can regulate plant–microbe interactions and nutrient cycling in agricultural soils [67], while *Bradyrhizobium* inoculation can enhance soybean growth and productivity [68]. These findings highlight the broader relevance of microbial-mediated nutrient cycling for black soil ecosystem management. These results offer critical insights for predicting subsurface microbial responses to global warming and deep carbon dynamics and provide scientific support for the long-term conservation and sustainable management of black soil resources under global environmental change. This co-evolutionary framework also advances fundamental understanding of microbial geochemical feedback within terrestrial critical zones globally.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy16181841/s1>. Table S1. Specific borehole drilling samples in regolith profile of the black soil critical zone (n = 31). Table S2. Summary of sequencing data and quality control statistics of regolith samples in the black soil critical zone (n = 31). Table S3. Geochemical and physicochemical variables in regolith profile of the black soil critical zone (n = 31). Table S4. Clay mineral composition of the regolith profile of the black soil critical zone. Table S5. Bacterial alpha diversity in regolith profile of the black soil critical zone (n = 31). Table S6. Summary of FAPROTAX-predicted functional groups identified across regolith profile of the black soil critical zone. Figure S1. Rarefaction curves based on bacterial Shannon diversity indices of regolith profile in the black soil critical zone. Figure S2. Heatmap showing the relative abundance patterns of FAPROTAX-predicted bacterial functional groups across the regolith profile. Figure S3. Linear regression analyses showing significant relationships between regolith depth and FAPROTAX-predicted bacterial functional potentials. The analyses include carbon cycling-related functions, nitrogen cycling-related functions, and other predicted ecological functions. Regression statistics (R^2 and p values) are shown in each panel. Figure S4. Spearman correlation analysis between module eigengenes of bacterial ecological network and regolith physicochemical and geochemical properties of the black soil critical zone.

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