

Article

Ferrate-Modified Biochar Boosts Ryegrass Phytoremediation of Petroleum and Zinc Co-Contaminated Soils

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Abstract

Phytoremediation is widely acknowledged as a viable method for soil remediation; however, its efficacy remains limited in soils co-polluted with petroleum hydrocarbons and heavy metals. To overcome this constraint, the present study explored an innovative approach utilizing ferrate-modified biochar (FeBC) to augment phytoremediation efficiency. Experimental findings revealed that ferrate treatment markedly modified the physico-chemical characteristics of biochar, yielding thinner, smoother-surfaced structures with pronounced iron enrichment. At a 5% application rate alongside ryegrass cultivation, FeBC exhibited superior remediation performance, achieving 52.0% degradation of petroleum hydrocarbons (notably within the meso-aggregate fraction) and a 19.2% decline in zinc bioavailability via immobilization, thereby reducing zinc uptake in ryegrass tissues. Furthermore, FeBC amendment induced significant shifts in rhizosphere soil biochemistry and microbial ecology, characterized by diminished catalase activity but elevated urease and alkaline phosphatase activities. Phospholipid fatty acid profiling indicated a substantial rise in bacterial biomass (encompassing both Gram-positive and Gram-negative groups), particularly in meso- and micro-aggregates, whereas soil bacterial α -diversity declined markedly, accompanied by distinct compositional changes across aggregate size fractions. These results offer mechanistic insights into the synergistic interaction between FeBC and ryegrass in co-contaminated soil rehabilitation, the aggregate-dependent distribution of remediation effects, and microbial community adaptations to FeBC treatment. Collectively, this study advances the understanding of ferrate-modified biochar's role in phytoremediation enhancement and clarifies its operational mechanisms in petroleum-zinc co-contaminated soil systems.

Keywords: biochar modification; soil remediation; mixed contamination; microbial community



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1. Introduction

Petroleum hydrocarbon contamination represents a pressing environmental issue stemming from anthropogenic activities such as industrial discharges, transportation spills, and improper waste disposal [1,2]. The ecological ramifications of such pollution are extensive, affecting soil physicochemical properties (e.g., reduced porosity and hydraulic conductivity), disrupting biogeochemical cycles by impairing microbial activity, and inducing phytotoxicity that diminishes plant productivity and ecosystem functionality [3,4]. Additionally, enhanced contaminant mobility through the vadose zone elevates groundwater contamination risks, necessitating remediation approaches that mitigate both pollutant

concentrations and ecological disturbances. A further complication arises from the frequent co-occurrence of petroleum hydrocarbons and heavy metals (e.g., Zn, Pb, Cd) in contaminated soils. These metals, either inherent in petroleum products or introduced via industrial effluents, agricultural runoff, and mining activities [1], create synergistic pollution effects that exacerbate environmental degradation. The coexistence of organic and inorganic pollutants presents a multifaceted challenge, demanding integrated remediation strategies capable of concurrently addressing both contaminant types to restore soil health and ecosystem stability.

Numerous remediation technologies have been developed to address soil contamination from petroleum hydrocarbons and heavy metals, including physical methods like excavation and soil vapor extraction, chemical approaches such as *in situ* oxidation, and biological solutions like bioremediation. Among these, phytoremediation has emerged as an economically viable and ecologically sustainable bioremediation strategy that utilizes plants to extract, detoxify, or metabolize soil pollutants [5,6]. Research has demonstrated that the rhizosphere effect plays a pivotal role in determining the efficacy of phytoremediation, particularly through the activity and composition of root-associated microbial communities which significantly influence contaminant transformation processes [6,7]. However, despite growing recognition of the rhizosphere microbiome's importance in phytoremediation systems, significant knowledge gaps persist regarding the precise identification of key microbial taxa and their specific functional contributions to contaminant degradation and metal immobilization processes in polluted soils.

Biochar, a carbon-rich material produced through the oxygen-limited pyrolysis of biomass, possesses unique physicochemical characteristics that render it particularly effective for soil remediation applications, including an extensive surface area, highly porous architecture, pH stabilization capacity, and chemical stability [8,9]. This versatile amendment demonstrates remarkable efficacy in treating diverse soil contaminants ranging from petroleum hydrocarbons and heavy metals to various organic pollutants, either as a standalone treatment or when integrated with complementary remediation strategies like phytoremediation. When employed in phytoremediation systems, biochar enhances the remediation process through multiple mechanisms: it serves as a supplemental carbon and nutrient source for plant growth, modifies soil physicochemical properties to create more favorable growing conditions, and mitigates pollutant toxicity to protect vegetation [6]. The synergistic combination of biochar and phytoremediation represents a particularly promising approach for addressing complex soil contamination scenarios while simultaneously improving soil quality and ecosystem functions.

Biochar modification represents a strategic approach to enhance biochar's functional properties for targeted applications through various physical, chemical, or biological treatment methods [9]. Among chemical modification techniques, ferrate treatment offers unique advantages due to its strong oxidizing properties, where ferrate ions react with biochar surfaces to form iron oxide nanoparticles that fundamentally alter the material's characteristics [10,11]. This ferrate-mediated modification process significantly enhances biochar's remediation potential by creating nanostructured surfaces with increased specific surface area and porosity, while simultaneously improving both chemical reactivity and structural stability. Despite these promising physicochemical improvements, current understanding of ferrate-modified biochar's environmental applications remains incomplete, particularly regarding its complex interactions with rhizosphere microorganisms and plant root systems during soil remediation processes, highlighting critical knowledge gaps that require further investigation to fully exploit this modified material's potential in contaminated soil rehabilitation.

Soil aggregates constitute fundamental structural units of soil systems that critically influence plant root development, carbon sequestration dynamics, and various ecosystem functions [12,13]. These aggregates, which represent the primary microhabitats for soil microorganisms [14,15], are classified into distinct size fractions that differentially regulate soil physicochemical processes [16,17]. Microbial-mediated aggregate formation initiates at the microscale through extracellular polysaccharide production, which binds primary soil particles ($\sim 1\ \mu\text{m}$ diameter) into stable micro-aggregates that can develop into larger structures up to 0.2 mm [18]. At macro scales ($>0.2\ \text{mm}$), organo-mineral complexes facilitate the physical association of binding agents including fine roots and fungal hyphae [19]. The heterogeneous distribution of pollutants across aggregate fractions arises from microenvironmental variations in pore architecture, substrate chemistry, and microbial community composition [20,21]. While research has established that biochar amendments can enhance aggregate stability, elevate organic matter content within aggregates, and stimulate native microbial activity, significant knowledge gaps persist regarding biochar's fraction-specific impacts on pollutant (organic contaminants and heavy metals) transformation and removal during phytoremediation processes, particularly across different aggregate size classes where pollutants naturally exhibit distinct concentration gradients and chemical speciation patterns.

This investigation systematically evaluated the remediation potential of potassium ferrate-modified biochar coupled with ryegrass cultivation in petroleum-zinc co-contaminated soils. Through comprehensive analysis, we examined aggregate-specific pollutant distribution patterns, phospholipid fatty acid (PLFA) biomarkers, and microbial community composition across different soil aggregate fractions. The study was guided by two key hypotheses: (1) ferrate-modified biochar would significantly enhance ryegrass-mediated contaminant removal through synergistic interactions, and (2) distinct rhizosphere microbial communities would develop in different aggregate size fractions with characteristic compositional shifts directly correlated with localized pollutant concentrations and speciation.

2. Materials and Methods

2.1. Biochar Synthesis and Physicochemical Characterization

The ferrate-modified biochar (FeBC) was synthesized through a three-stage process involving chemical impregnation and thermal activation [22]. Initially, 5 g of corncob-derived pristine biochar (CBC; physicochemical characteristics detailed in Supplementary Table S1) was homogenized with 1 g potassium ferrate (K_2FeO_4) in 150 mL deionized water using magnetic stirring (300 rpm) for 48 h at ambient temperature ($25 \pm 2\ ^\circ\text{C}$). The slurry was then oven-dried at $65\ ^\circ\text{C}$ for 24 h to obtain a ferrate-impregnated precursor. This intermediate product underwent pyrolysis in a quartz tube furnace under continuous N_2 flow ($200\ \text{mL min}^{-1}$), employing a controlled thermal regime: (i) heating from ambient to $800\ ^\circ\text{C}$ at $5\ ^\circ\text{C min}^{-1}$, (ii) maintaining isothermal conditions for 2 h, and (iii) natural cooling to $<50\ ^\circ\text{C}$. Post-pyrolysis, the material was sequentially washed with deionized water ($18.2\ \text{M}\Omega\cdot\text{cm}$) until achieving neutral pH (7.0 ± 0.5) in the effluent, followed by final drying at $65\ ^\circ\text{C}$ for 12 h to yield the FeBC product. Moreover, the following types of biochar were used in this experiment. CT: CBC; CS5: 10 g CBC + 2 g K_2FeO_4 ; CS2: 10 g CBC + 5 g K_2FeO_4 ; CS1: 10 g CBC + 10 g K_2FeO_4 ; CS0.5: 10 g CBC + 20 g K_2FeO_4 . Batch adsorption experiment design was provided in Supplementary Materials Method X1. Based on the results of batch adsorption experiments (Supplementary Figure S1), CS5 was chosen for the following experiment and named by FeBC.

The analytical methods for characterizing biochar's surface morphology, elemental composition, mineral structure, surface functional groups, and adsorption properties are detailed in Supplementary Materials Method X2.

2.2. Pot Experiment Design

The pot experiment was systematically designed with two distinct phases: (1) soil conditioning and (2) phytoremediation assessment. Contaminated soil samples were collected from the rhizosphere zone (0–20 cm depth) adjacent to a decommissioned petroleum extraction site in Dongying (37°43' N, 118°67' E), a region characterized by historically intensive hydrocarbon exploitation activities in Shandong Province, China. Prior to experimental setup, composite soil samples were thoroughly homogenized and characterized for baseline physicochemical parameters including texture classification, organic matter content, and hydrocarbon concentrations, with complete analytical data archived in Supplementary Table S2. After obtaining clean soil samples, blocks and plant roots were removed, and petroleum hydrocarbons were added and thoroughly mixed to achieve a total petroleum hydrocarbon concentration of 1500 mg kg⁻¹. Additionally, 1 mol L⁻¹ zinc chloride solution was incorporated into the soil to attain a total zinc content of 500 mg kg⁻¹. The polluted soil was allowed to age for four weeks before use. Following initial characterization, soil samples underwent standardized preparation through sequential mechanical sieving (using a 2 mm stainless steel mesh) and thorough homogenization to ensure particle size uniformity. The processed soil substrate was then allocated into sterilized polyethylene containers (600 mL capacity).

The experimental design incorporated five distinct treatment groups: control (CT), phytoremediation alone (P), and three phytoremediation-biochar combinations with varying biochar application rates (PBC1, PBC3, PBC5), each replicated nine times (n = 9) in a completely randomized design (experimental setup illustrated in Supplementary Figure S2). Three biochar-amended treatments were established with differential application rates: PBC1, PBC3, and PBC5 received 10, 30, and 50 g kg⁻¹ ferrate-modified biochar (FeBC) by soil dry weight (equivalent to 1%, 3%, and 5% w/w, respectively), each in combination with ryegrass cultivation. Following the 12-week experimental period, systematic sample collection was performed, with plant biomass and rhizosphere soils being processed and preserved under distinct protocols. Soil samples underwent fractionation through wet sieving methodology into four aggregate size classes: (i) whole soil (unfractionated), (ii) macro-aggregates (1–2 mm), (iii) meso-aggregates (0.25–1 mm), and (iv) micro-aggregates (<0.25 mm). For subsequent analyses, aliquots were either air-dried at 25 ± 2 °C for determination of soil physicochemical parameters or immediately flash-frozen in liquid nitrogen and stored at –80 °C for molecular characterization (including microbial community profiling and functional gene analysis).

2.3. Soil and Plant Properties Analysis

Following sample collection, comprehensive analyses were conducted to quantify: (1) petroleum hydrocarbon content, (2) total zinc concentration, (3) DTPA-extractable (bioavailable) zinc, and (4) zinc speciation fractions in soil samples, along with (5) total zinc accumulation in plant tissues. Detailed analytical protocols are provided in Supplementary Materials Method X3.

2.4. Soil Enzyme Activity Assay

Dehydrogenase activity (DHA) in soil samples was determined spectrophotometrically through enzymatic reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to triphenyl formazan (TPF), following optimized protocols described in previous studies [6]. Catalase (CAT) activity in soil samples was determined through UV spectrophotometric monitoring

of hydrogen peroxide (H_2O_2) decomposition at 240 nm, following established enzymatic protocols. Soil urease activity was quantified through the phenol-hypochlorite reaction method, wherein enzyme-catalyzed urea hydrolysis releases ammonium nitrogen ($\text{NH}_3\text{-N}$) that forms indophenol blue with alkaline phenol and sodium hypochlorite. The colored product was measured spectrophotometrically at 630 nm, with activity expressed as milligrams of $\text{NH}_3\text{-N}$ generated per gram of dry soil per 24 h incubation. For alkaline protease assessment, the modified Anson method was employed, monitoring casein hydrolysis through tyrosine liberation. The reaction mixture containing 2% (w/v) casein in Tris-HCl buffer was incubated with soil at 37 °C for 1 h, followed by trichloroacetic acid precipitation. The supernatant reacted with Folin–Ciocalteu reagent to form phosphomolybdic tungstate complexes, measured at 660 nm.

2.5. Soil Microbial Community Analysis

2.5.1. Soil Phospholipid Fatty Acids Determination

The soil PLFA (phospholipid fatty acid) profile was characterized through a sequential extraction and fractionation protocol [23]. Briefly, total lipids were extracted from freeze-dried soil samples using a chloroform-methanol-phosphate buffer system, followed by solid-phase fractionation to isolate phospholipids. The derived fatty acid methyl esters (FAMES) were analyzed by gas chromatography-mass spectrometry (GC-MS; 7890B/5977A, Agilent Technologies, Santa Clara, CA, USA). Complete analytical procedures and biomarker assignments are documented in Supplementary Materials Method X4 and Table S3.

2.5.2. DNA Extraction and PCR Amplification

Genomic DNA was extracted from soil samples following the cetyltrimethylammonium bromide (CTAB) protocol. To assess the quality and quantity of the extracted DNA, electrophoresis was performed on a 1% agarose gel. The samples were then diluted with sterilized water to a concentration of $1 \mu\text{g mL}^{-1}$. The universal bacterial primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') were used to amplify the V3 + V4 hypervariable region of the 16S rRNA gene. These primers specifically target the conserved flanking regions of the V3 + V4 region, allowing for the selective amplification of a 460-base pair amplicon, which is an optimal size for Illumina sequencing. The PCR program was as follows: Template: Diluted genomic DNA; Primers: Barcode-labeled primers designed for the V3 + V4 region of the 16S rRNA gene; Enzyme and buffer: New England Biolabs' Phusion® High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs, Ipswich, MA, USA). The Phusion® High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs, Ipswich, MA, USA) contains high-quality and highly processive enzymes that ensure efficient and accurate PCR amplification. PCR reaction mixture (30 μL) contain 15 μL Phusion Master Mix (2 $\times$), 1 μL Forward Primer (0.2 $\mu\text{M } \mu\text{L}^{-1}$), 1 μL Reverse Primer (0.2 $\mu\text{M } \mu\text{L}^{-1}$), 10 μL DNA template (1 ng μL^{-1}), and 2 μL H_2O , followed by pre-denaturation at 98 °C for 1 min; 30 cycles of (98 °C, 10 s; 50 °C, 30 s; 72 °C, 30 s); 72 °C, 5 min. Electrophoresis on a 2% agarose gel was used to detect PCR products.

2.5.3. Illumina Novaseq Sequencing

The PCR products were combined in equal amounts based on their respective concentrations. After being mixed thoroughly, the purification of the products was carried out using 2% agarose gel electrophoresis in a 1 $\times$ TAE buffer solution. Subsequently, the bands of interest were excised from the gel matrix and subsequently retrieved via the Qiagen gel recovery kit (Qiagen, Hilden, Germany). The library was constructed using the Illumina TruSeq® DNA PCR-Free Sample Preparation Kit, manufactured by Illumina (San Diego,

CA, USA). The built library was quantified using Qubit and then checked for quality. The qualified library was then sequenced on a NovaSeq 6000 PE250 sequencer (Illumina, San Diego, CA, USA).

2.5.4. Bioinformatic Analysis

The analysis was conducted in accordance with the lesson entitled “Atacama soil microbiome tutorial” as outlined in the QIIME2 manual [24]. The raw sequence fastq files were imported into QIIME2 using the QIIME tools import plugin, and then quality-controlled, trimmed, denoised, merged, and decontaminated using the dada2 plugin. The generation of the final feature sequence table was completed [25]. The ASVs’ representative sequences were aligned with the pre-trained 13_8 version 99% similarity GREENGENES database. The database was reduced to the V3-V4 area using the 341F/806R primer pairs. This alignment was performed using the QIIME2 feature-classifier plugin, and the taxa classification table was obtained. Subsequently, the QIIME2 feature-table plugin was employed to exclude any mitochondrial and chloroplast sequences that may have introduced contamination.

2.6. Statistical Analysis

Following the evaluation of normality and homogeneity of the dataset, a one-way analysis of variance (ANOVA) was utilized to examine the overall variability of soil parameters across various groups. Subsequently, to assess differences in soil parameters among groups, statistical comparisons were conducted using Fisher’s least significant difference (LSD) test. Microbial community composition was characterized through phospholipid fatty acid (PLFA) profiling, followed by principal component analysis (PCA) implemented in R (v. 4.2.1) with the ‘vegan’ package. Additionally, microbial co-occurrence networks were generated based on pairwise Spearman correlations between taxa, with network visualization performed in Cytoscape (v. 3.9.1). Topological properties, including node-level Zi-Pi metrics and network robustness, were computed and graphically represented using the ‘igraph’ package in R (v. 3.9.1).

3. Results and Discussion

3.1. Physicochemical Characterizations and Adsorption Capacity of FeBC

The scanning electron microscopy (SEM) analysis demonstrated distinct morphological differences between ferrate-modified biochar (FeBC) and pristine corn cob biochar (BC), with FeBC exhibiting a characteristic smooth lamellar structure (Figure 1a–d), consistent with previous modification study. This structural transformation suggests potential improvements in biochar-soil-plant interactions due to increased surface contact area [26].

Energy dispersive X-ray spectroscopy (EDS) elemental mapping confirmed successful iron deposition on the biochar surface alongside carbon, nitrogen, and oxygen (Figure 1e–h), while X-ray photoelectron spectroscopy (XPS) quantification revealed an iron loading of approximately 2.64% on FeBC surfaces (Figure 1i–k). Surface charge analysis through zeta potential measurements indicated a more positive surface charge for FeBC, which may enhance its affinity for negatively charged contaminants like petroleum hydrocarbons. Brunauer–Emmett–Teller (BET) surface area measurements provided direct evidence of the potassium ferrate modification’s effectiveness, showing significantly improved adsorption capacity that was subsequently confirmed through batch adsorption experiments demonstrating FeBC’s superior performance in capturing both organic dyes and zinc ions compared to unmodified biochar, leading to its selection as an amendment for enhanced phytoremediation of contaminated soils.

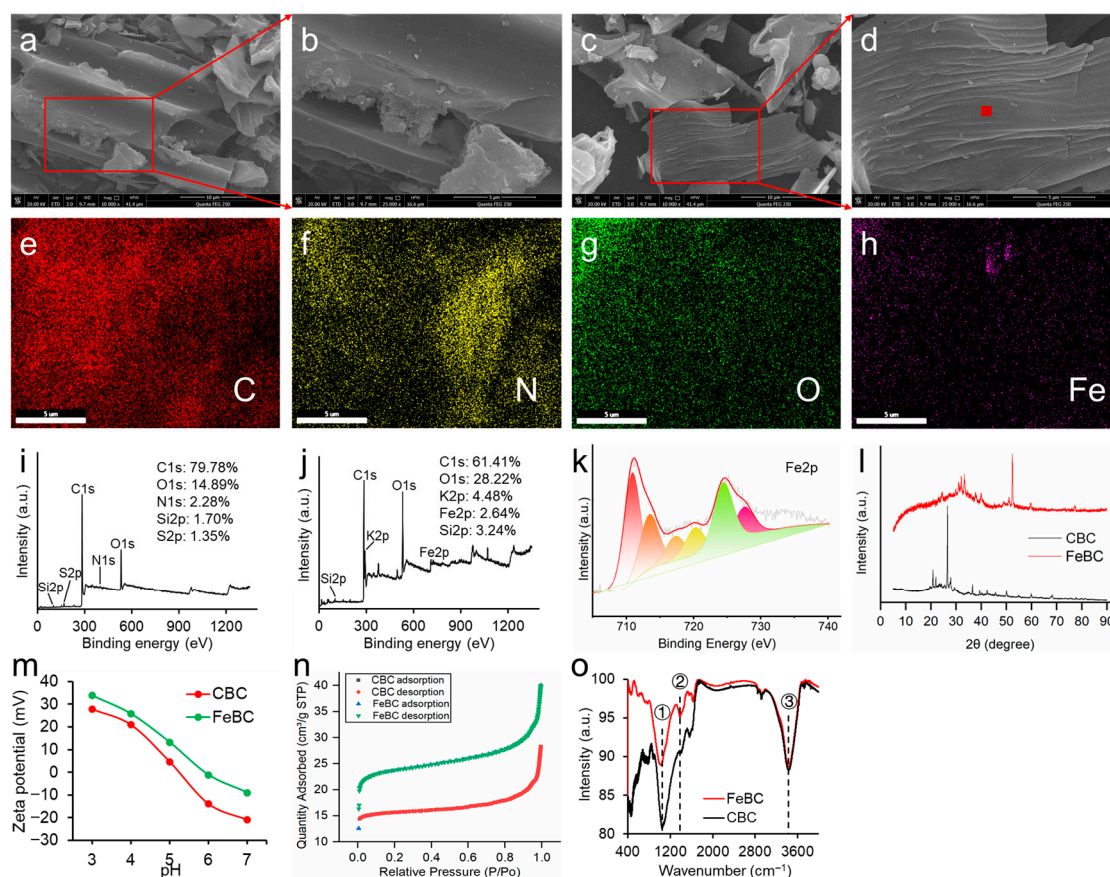


Figure 1. Comparative characterization of unmodified biochar (CBC) and ferrate-treated biochar (FeBC). (a–d) Scanning electron microscopy images showing CBC (a,b) and FeBC (c,d) morphologies, the red box is an EDS point.; (e–h) Elemental distribution maps of FeBC; (i,j) X-ray photoelectron spectroscopy survey scans comparing CBC (i) and FeBC (j); (k) High-resolution XPS analysis of Fe 2p orbitals, and different colors represent different XPS peaks.; (l) X-ray diffraction patterns; (m) Surface charge measurements via zeta potential; (n) Porosity analysis through pore size distribution; (o) Chemical functional group identification by FT-IR spectroscopy, different numbers correspond to different IR peak positions.

3.2. Soil and Plant Properties

Our analytical results demonstrated significant treatment-dependent variations in both concentration and speciation of soil pollutants across experimental groups (Figure 2a–d), with the combined biochar-ryegrass treatment showing particularly pronounced reductions in total petroleum hydrocarbon (TPH) concentrations that were consistently observed across all three aggregate fractions. Notably, macro-aggregates retained substantially higher residual petroleum hydrocarbon levels compared to meso- and micro-aggregates, likely attributable to their more complex pore architectures that physically sequester contaminants and limit their accessibility for degradation processes [27,28], a finding that highlights the critical need for future remediation research to develop targeted strategies for overcoming this macro-aggregate-associated contaminant persistence.

The experimental data revealed a statistically significant reduction in bioavailable zinc concentrations in soils subjected to the integrated phytoremediation-biochar treatment (PBC) compared to control samples (Figure 2e–h). This consistent pattern was observed across all aggregate size fractions, though micro-aggregates exhibited comparatively elevated levels of exchangeable zinc. The differential zinc distribution among aggregate classes may be explained by distinct physical protection mechanisms: macro-aggregates likely immobilize metallic contaminants through physical encapsulation within their complex pore

networks, thereby reducing metal lability and phytoavailability, whereas micro-aggregates, characterized by greater specific surface area and reactive sites, tend to preferentially accumulate but maintain higher mobility of heavy metal species [29,30]. These findings underscore the importance of aggregate-scale heterogeneity in determining metal bioavailability during remediation processes.

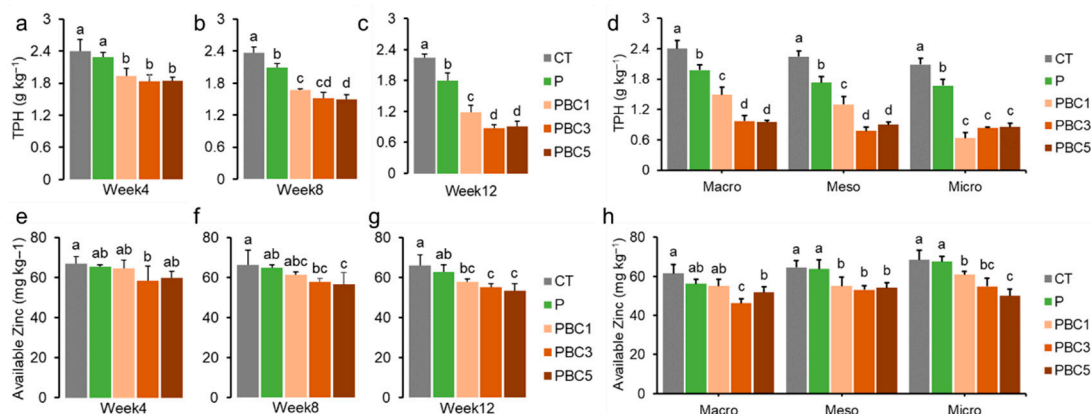


Figure 2. Remediation effects on contaminated soils: (a–c) Residual total petroleum hydrocarbon (TPH) concentrations across treatment groups; (d) TPH distribution patterns among soil aggregates. (e–g) Bioavailable zinc levels under various treatments; (h) Zinc fractionation in soil aggregates. Control (CT), phytoremediation alone (P), and three phytoremediation-biochar combinations with varying biochar application rates (1% PBC1, 3% PBC3, 5% PBC5). Intergroup differences were tested with statistical significance set at $p < 0.05$.

Analysis of plant tissue metal accumulation (Figure S3) demonstrated that ferrate-modified biochar (FeBC) amendment substantially reduced zinc translocation to ryegrass leaves compared to phytoremediation alone, with the PBC3 treatment group exhibiting the lowest foliar Zn concentrations. This observation substantiates FeBC's capacity to immobilize heavy metals and prevent their phytoextraction from contaminated soils. Complementary sequential extraction data (Figure S4) revealed a progressive transformation of Zn speciation, characterized by decreasing exchangeable fractions (C1) and increasing residual (C3) and organic-bound (C4) fractions following FeBC application, further confirming its metal stabilization efficacy. These results align with established findings regarding biochar's dual functionality in simultaneously enhancing hydrocarbon degradation and metal immobilization [31,32], highlighting FeBC's potential as a multifunctional soil amendment for co-contaminated site remediation.

Three-dimensional fluorescence spectroscopic characterization of dissolved organic matter (DOM) in ryegrass rhizosphere soils revealed distinct compositional variations among treatment groups (Figure 3), with fluorescence intensity exhibiting an increasing gradient from macro- to micro-aggregates. This aggregate-size dependent DOM distribution pattern corresponds with previous findings by Xu et al. [33] regarding the inverse relationship between aggregate size and both DOM concentration and structural complexity, which may be mechanistically explained by the elevated total organic carbon content and greater abundance of micropores in smaller aggregates that facilitate the retention of labile carbon fractions [34]. Notably, the PBC3 treatment induced significant modifications in DOM characteristics, increasing the fluorescence index while decreasing the humification index (Supplementary Table S4). Furthermore, while the P treatment elevated total dissolved carbon (TDC), dissolved inorganic carbon (DIC), and dissolved organic carbon (DOC) levels in rhizosphere soils, subsequent FeBC addition produced the opposite effect, markedly reducing these carbon pool constituents (Figure S5), suggesting that ferrate-

modified biochar may alter carbon cycling dynamics through mechanisms warranting further investigation.

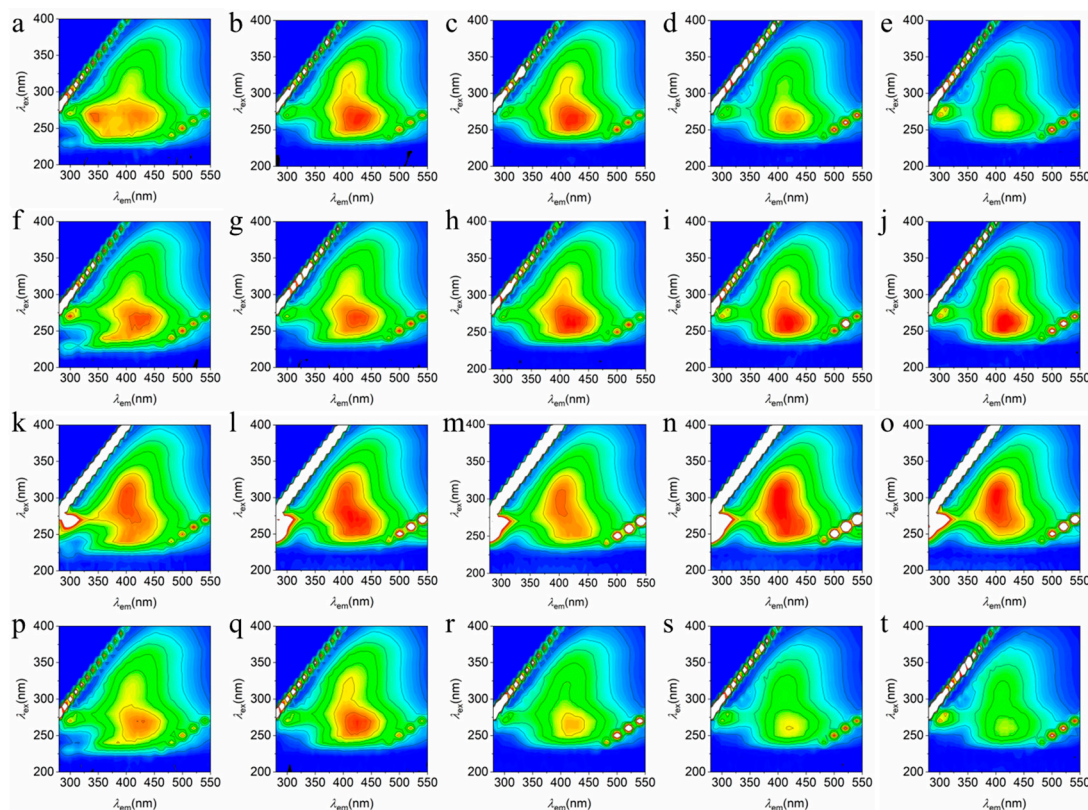


Figure 3. Three-dimensional excitation-emission matrix (3D-EEM) spectra of dissolved organic matter from: (a,f,k,p) untreated control (CT); (b,g,l,q) phytoremediation treatment (P); (c,h,m,r) 1% ferrate-treated biochar and phytoremediation treatment (PBC1); (d,i,n,s) 3% ferrate-treated biochar and phytoremediation treatment (PBC3); and (e,j,o,t) 5% ferrate-treated biochar and phytoremediation treatment (PBC5) treated soils. From top to bottom, spectra represent total dissolved organic matter, macro-aggregate, meso-aggregate, and micro-aggregate fractions, respectively.

Our microscopic characterization using transmission electron microscopy (TEM) coupled with energy-dispersive X-ray spectroscopy (EDS) provided direct evidence of iron plaque formation in ryegrass root tissues following FeBC application (Supplementary Figure S6). These observations corroborate previous reports of similar iron oxide depositions in rice root systems amended with nano zero-valent iron [35,36], suggesting a common plant response mechanism to iron-based amendments. The formation of these stable iron oxide coatings on root surfaces is believed to serve as a protective barrier, potentially enhancing plant tolerance to various environmental stressors through multiple mechanisms including heavy metal immobilization and oxidative stress mitigation.

3.3. Soil Enzyme Activities

Our enzymatic analyses revealed treatment-dependent variations in soil catalase (CAT, EC 1.11.1.6) activity profiles across experimental conditions (Figure 4). The Control and P treatments exhibited elevated CAT levels in bulk soil, with a progressive decline observed with increasing FeBC application rates (Figure 4a). This aggregate-size dependent pattern was particularly pronounced in macro-aggregates, which consistently maintained higher CAT activity compared to smaller aggregates. As a critical oxidoreductase with an exceptional turnover rate of approximately 6×10^6 reactions per second [37,38], catalase plays a pivotal role in cellular defense mechanisms by detoxifying hydrogen peroxide (H_2O_2)

generated during aerobic respiration [39]. While catalase and dehydrogenase activities are well-established biomarkers for evaluating hydrocarbon degradation efficiency during bioremediation [40,41], our observed CAT suppression with FeBC amendment contrasts with previous reports of biochar-induced CAT enhancement [42,43]. This discrepancy may be attributed to system-specific factors including soil characteristics, contaminant profiles, and potential feedback inhibition resulting from successful contaminant removal, highlighting the context-dependent nature of enzymatic responses during remediation processes.

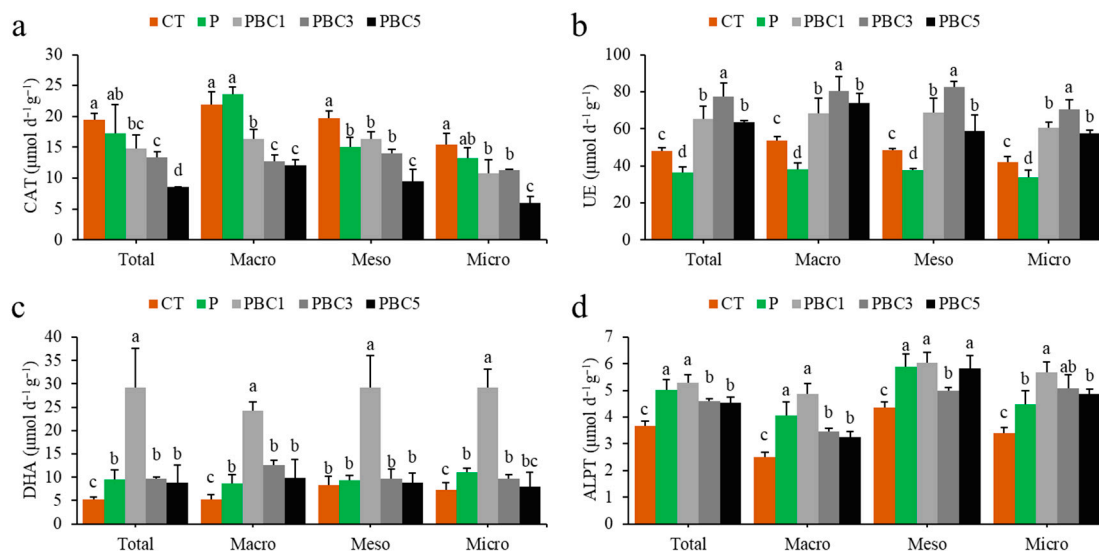


Figure 4. Enzymatic activities in treated soils: (a–d) Bulk and aggregated soil catalase, urease, dehydrogenase, and alkaline phosphatase levels across treatments. Control (CT), phytoremediation alone (P), and three phytoremediation-biochar combinations with varying biochar application rates (1% PBC1, 3% PBC3, 5% PBC5).

Enzymatic analysis demonstrated significantly enhanced urease activity in combined biochar-phytoremediation treatments compared to both control and phytoremediation-only groups (Figure 4b), with this pattern being consistently observed across all aggregate size fractions. As a key hydrolase (EC 3.5.1.5) governing urea hydrolysis in terrestrial ecosystems, urease assumes particular importance in petroleum-contaminated soils where nitrogen availability frequently limits hydrocarbon degradation [6]. The observed biochar-mediated stimulation of urease activity suggests improved nitrogen cycling capacity in these co-contaminated systems, which may explain the concomitant enhancement of petroleum hydrocarbon removal efficiency. This synergistic effect between biochar amendment and phytoremediation highlights the potential for integrated approaches to overcome nutrient limitations that often constrain bioremediation processes in hydrocarbon-impacted soils.

The enzymatic profile analysis revealed distinct treatment effects on dehydrogenase (DHA) and alkaline phosphatase activities (Figure 4c,d). Maximum DHA activity was recorded in the PBC1 treatment, exceeding Control levels by approximately 2.5-fold, consistent with its established role as a sensitive indicator of microbial oxidative metabolism during organic pollutant degradation [44]. This observation aligns with our previous findings demonstrating biochar-induced stimulation of DHA activity correlating with enhanced petroleum hydrocarbon removal rates [6]. Similarly, alkaline phosphatase activity showed significant elevation in biochar-amended treatments (PBC1, PBC3, PBC5) compared to the Control, suggesting improved phosphorus cycling capacity in the remediated soils. These enzymatic responses collectively indicate that biochar amendment

enhances multiple microbial metabolic pathways critical for contaminant transformation in co-contaminated systems.

3.4. Soil Phospholipid Fatty Acids

Phospholipid fatty acid (PLFA) profiling revealed significant treatment- and aggregate-dependent variations in soil microbial biomass (Figure 5). In bulk soil analyses, both phytoremediation (P) and combined phytoremediation-biochar (PBC) treatments enhanced total PLFA content, with particularly pronounced increases in biomarkers for Gram-positive bacteria (G+), Gram-negative bacteria (G−), and total bacterial populations compared to control (CT) samples. Biochar, a carbon-rich material produced through biomass pyrolysis under oxygen-limited conditions, possesses high specific surface area and porosity, providing favorable microhabitats for microbial colonization. Aggregate-specific analyses demonstrated fraction-dependent responses: macro-aggregates showed reduced PLFA content in P treatments but partial recovery with PBC amendment, whereas meso-aggregates mirrored the bulk soil pattern with elevated microbial biomass in both P and PBC groups. Micro-aggregates exhibited a distinct response profile, with P treatment decreasing but PBC treatment significantly increasing PLFA concentrations relative to CT. These findings demonstrate that microbial community responses to remediation strategies are strongly mediated by soil aggregate hierarchy, with biochar amendment particularly enhancing microbial colonization in smaller aggregate fractions. Gram-negative bacteria (G−), classified as copiotrophic microorganisms, exhibit rapid growth rates and preferentially utilize readily decomposable carbon sources, typically dominating the initial stages of plant residue degradation. In contrast, Gram-positive bacteria (G+) are oligotrophic microorganisms characterized by slower growth but broader metabolic versatility, enabling them to exploit both labile and recalcitrant carbon substrates, thus becoming predominant during later degradation phases. Fungi generally demonstrate superior capability over bacteria in decomposing complex compounds such as cellulose and lignin.

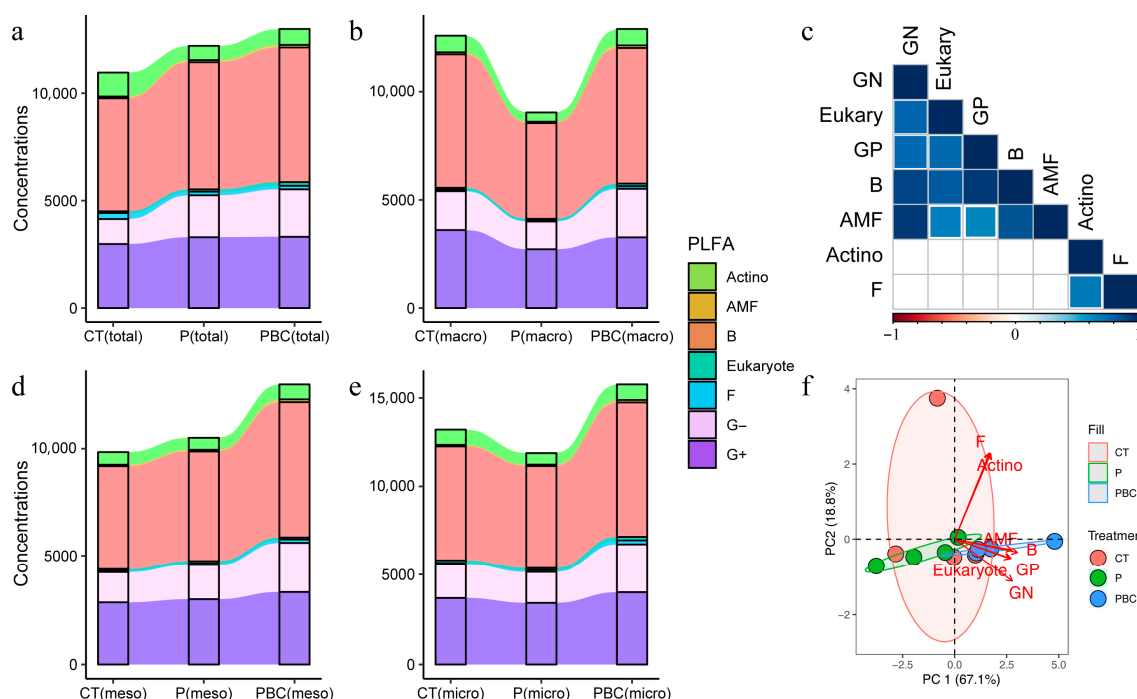


Figure 5. Phospholipid fatty acid (PLFA) profiles in treated soils: (a) Total microbial biomass; (b,d,e) PLFA distribution in macro-, meso-, and micro-aggregates, respectively. (c) Correlation matrix of microbial community markers; (f) Principal component analysis of PLFA composition. Control (CT), phytoremediation alone (P), and phytoremediation-biochar combinations (PBC).

Multivariate statistical analysis revealed distinct microbial community structures across treatment groups, with principal component analysis (PCA) clearly discriminating between control (CT), phytoremediation (P), and phytoremediation-biochar (PBC) treatments based on phospholipid fatty acid (PLFA) profiles. Correlation analyses identified significant positive associations ($p < 0.05$) between specific microbial groups: Gram-negative bacteria (G[−]) showed strong co-occurrence patterns with eukaryotic microorganisms, while Gram-positive bacteria (G⁺), total bacteria, and arbuscular mycorrhizal fungi (AMF) formed another closely linked functional group. These microbial assemblages, recognized as fundamental drivers of soil ecological processes [45,46], were particularly enhanced by FeBC amendment, which significantly increased biomass of key bacterial groups (G⁺, G[−], and total bacteria) in the ryegrass rhizosphere. The observed microbial network patterns suggest that FeBC-mediated phytoremediation promotes the development of synergistic relationships among diverse soil microbiota, potentially creating more robust microbial consortia for contaminant degradation.

3.5. Soil Microbial Communities

High-throughput 16S rDNA sequencing analysis revealed substantial FeBC-induced modifications in bacterial community structure within ryegrass rhizosphere soils across aggregate fractions (Figure 6). The observed reduction in Chao1 richness indices in both P and PBC treatments relative to CT controls (Figure 6a,b) likely reflects rhizosphere selection pressures, where root exudates promote the proliferation of specialized microbial taxa while reducing overall diversity. Moreover, FeBC exhibits inhibitory effects on certain microbial taxa, which could concurrently contribute to the observed reduction in rhizospheric microbial diversity and the selective enrichment of specific microbial groups. This ecological filtering effect was further evidenced by principal coordinates analysis (PCoA), which demonstrated clear treatment-driven differentiation of microbial assemblages (Figure 7a–c). Principal components 1 to 4 (PC1–PC4) explained 12%, 9.2%, 5%, and 4.5% of microbial community variance, respectively. Despite these modest explanatory powers, they still revealed significant differentiation among sample groups. Such community restructuring suggests that FeBC amendment mediates niche partitioning in the rhizosphere microenvironment, potentially favoring microbial taxa with enhanced capacities for both metal resistance and hydrocarbon degradation in these co-contaminated soils.

Soil microorganisms constitute a vital living component within the soil micro-ecological environment, participating directly or indirectly in all biochemical processes occurring in soils. As a key element of the soil ecosystem, they play a pivotal role in regulating soil physicochemical reactions. The intensity of microbial activity serves as a sensitive indicator of soil environmental changes, making microbial abundance and community structure critical parameters for assessing soil quality [6]. Taxonomic profiling identified Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Gemmatimonadetes as the predominant bacterial phyla across all treatments, with notable representation of *Luteimonas*, *Nocardioides*, *Longimicrobium*, *Sphingomonas*, *Marmoricola*, *Chryseolinea*, and *Lysobacter* at the genus level (Figure 6c,d). Comparative analysis revealed treatment-specific shifts in microbial populations, with P and PBC amendments significantly decreasing Actinobacteria (by 15–22%) and *Lysobacter* abundance while increasing Bacteroidetes (by 18–25%), *Chryseolinea*, and *Longimicrobium* populations relative to CT. These compositional changes contrast with previous reports [6,47,48], potentially reflecting system-specific responses mediated by interactions between soil physico-chemical properties (e.g., pH, organic matter content) and ryegrass-specific rhizosphere effects. Such context-dependent microbial community dynamics underscore the importance of site-specific factors in shaping bioremediation outcomes.

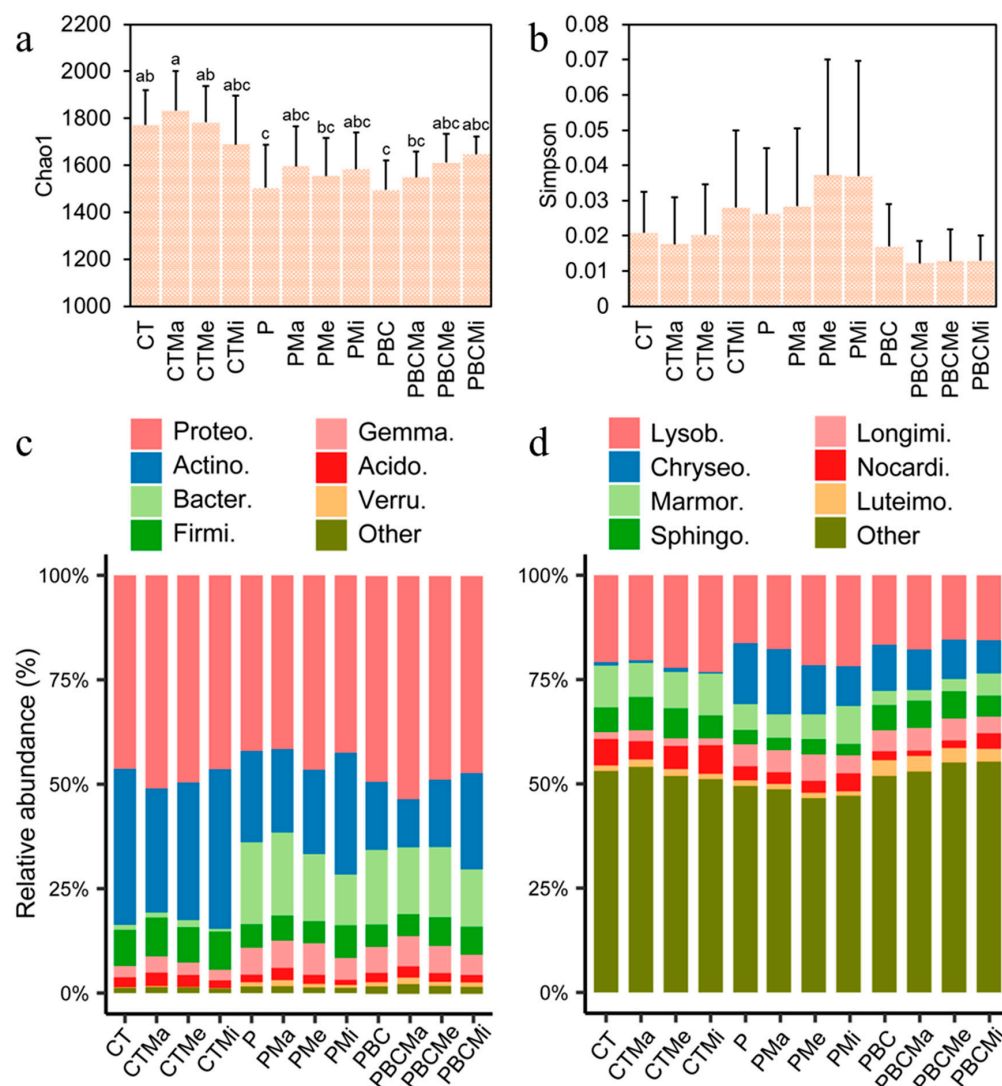


Figure 6. Microbial diversity analysis of treated soils: (a,b) α -diversity indices (Chao1 richness and Simpson diversity); (c,d) Taxonomic composition showing relative abundance at phylum (c) and genus (d) levels across treatments. Control (CT), phytoremediation alone (P), and phytoremediation-biochar combinations (PBC).

3.6. Soil Microbial Co-Occurrence Networks

Molecular ecological networks provide a visual representation of microbial correlations. The advent of ecological network analysis has offered researchers a powerful tool to investigate potential microbial interactions, including mutualism, symbiosis, competition, and antagonism. In these networks, positive and negative correlations are commonly employed to infer different interaction types. However, it should be noted that detected correlations do not necessarily demonstrate direct microbial interactions, as they may reflect indirect relationships or environmental influences [49]. Network analysis of microbial interactions, based on Spearman's rank correlations of taxonomic abundances, revealed distinct topological patterns among treatment groups (Figure 8a–c; Supplementary Table S5). The constructed networks exhibited significant structural variations, with P and PBC treatments showing increased node complexity (28–35% more nodes) but reduced connectivity (40–45% fewer links) compared to CT. Notably, the proportion of positive correlations (indicating potential mutualistic interactions) followed a treatment gradient, peaking in P networks ($72 \pm 5\%$) and reaching minimum values in CT ($48 \pm 3\%$). This shift from competitive to cooperative microbial relationships suggests that phytoremediation alleviates pollutant-induced stress,

enabling more synergistic community assembly [50,51]. Furthermore, modularity analysis demonstrated that PBC networks achieved the highest compartmentalization (modularity index = 0.65 ± 0.03), significantly exceeding both P (0.52 ± 0.04) and CT (0.41 ± 0.05) groups. This enhanced modular organization, a recognized stability indicator in ecological networks [49,52], implies that biochar amendment promotes the development of functionally resilient microbial consortia through niche differentiation and stress buffering.

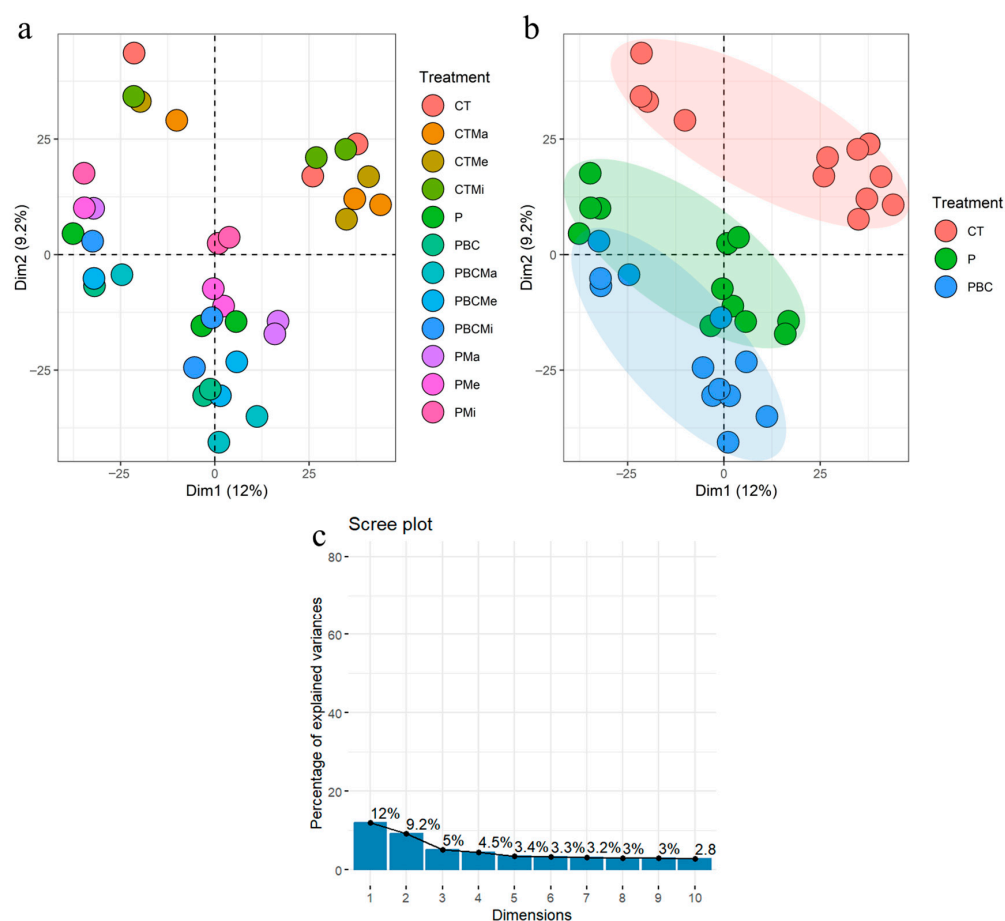


Figure 7. Microbial diversity analysis of treated soils: (a,b) β -diversity patterns; (c) Variance explanation ratios of the top 10 PCoA dimensions. Control (CT), phytoremediation alone (P), and phytoremediation-biochar combinations (PBC).

Zi-Pi topological analysis of microbial networks revealed distinct patterns of keystone species distribution across treatments (Figure 8a–c). While CT networks exhibited the lowest keystone species richness, both P and PBC treatments maintained significantly higher numbers (Supplementary Figure S7a–c), with these critical taxa playing pivotal roles in maintaining ecological network resilience through trophic interactions and niche facilitation [53,54]. Paradoxically, robustness analysis demonstrated an inverse relationship between keystone abundance and network stability, with CT networks showing the highest resistance to random node removal compared to PBC systems (Supplementary Figure S7d). Betweenness centrality metrics identified *Bradyrhizobium* and *Lysobacter* as predominant keystone taxa in PBC networks (Supplementary Table S6), both recognized as plant growth-promoting rhizobacteria (PGPR) capable of nitrogen fixation and pathogen suppression [55,56]. This selective enrichment of PGPR suggests FeBC amendment creates favorable rhizospheric conditions for beneficial plant-microbe interactions, potentially explaining the observed phytoremediation enhancement despite lower topological robustness.

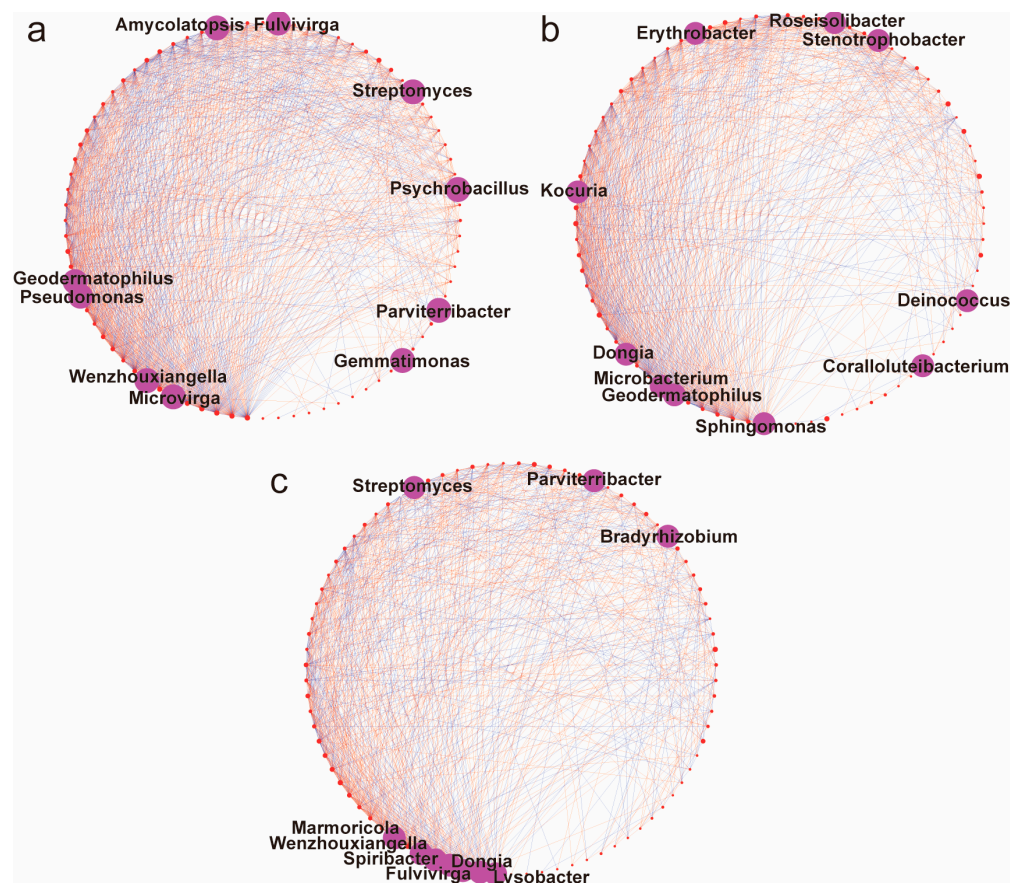


Figure 8. Network analysis of microbial communities: Microbial co-occurrence networks constructed using Spearman correlation analysis: (a) Control (CT), (b) phytoremediation-amended (P), and (c) biochar-assisted phytoremediation (PBC) groups. Nodes represent individual taxa, with red and blue edges indicating positive and negative correlations, respectively.

4. Conclusions

Ferrate treatment markedly alters the surface properties and elemental composition of biochar. When combined with ryegrass, Fe-modified biochar (FeBC) demonstrates enhanced effectiveness in degrading petroleum hydrocarbons while simultaneously mitigating heavy metal uptake in plant leaves and improving their immobilization in soil. The introduction of FeBC also modifies the dissolved organic matter (DOM) profile and total organic carbon (TOC) content in the rhizosphere soil of ryegrass, further stimulating iron plaque formation in root tissues. Additionally, FeBC and ryegrass synergistically boost the activity of key enzymes involved in pollutant degradation as well as nitrogen and phosphorus cycling. The co-application of FeBC and ryegrass also elevates soil phospholipid fatty acids (PLFAs) levels, particularly favoring bacterial population. However, the introduction of either plants or biochar alone reduces soil bacterial diversity and shifts community structure. These insights not only advance the development of phytoremediation strategies for soils contaminated with petroleum hydrocarbons and heavy metals but also provide a deeper mechanistic understanding of remediation processes. Future research should prioritize field-scale applications of ferrate-modified biochar, particularly for in situ soil remediation. Concurrently, investigations into cost reduction in biochar modification processes and scalability of implementation are critically needed.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/pr13092827/s1>, Figure S1: Batch adsorption experiments, Figure S2: Flowchart of the pot experiment, Figure S3: Total Zn content in ryegrass leaves, Figure S4: Fraction distribution

of Zn in soil, Figure S5: Soil organic carbon fractions, Figure S6: TEM images of ryegrass root intercellular spaces, Figure S7: Network Zi-Pi scatter plots and robustness of co-occurrence networks, Table S1: Several characteristics of the pristine biochar, Table S2: The physicochemical properties of experimental soil, Table S3: The PLFA biomarkers, Table S4: The fluorescence index, biological index, and humification index for soil organic matter, Table S5: Topological properties of soil microbial networks, Table S6: Keystone taxa for each soil microbial network.

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