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Enhanced Anaerobic Biodegradation of PAHs by Rhamnolipid and Earthworm Casts in Contaminated Soil

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Abstract: Rhamnolipids and earthworm casts, as efficient and environmentally friendly biostimulants, influence the biodegradation of organic pollutants. However, it remains unclear how rhamnolipids and earthworm casts affect the anaerobic biodegradation of polycyclic aromatic hydrocarbons (PAHs). This work aimed to investigate the efficacy and mechanism of biostimulants on the anaerobic biodegradation of PAHs through PAH degradation, functional gene abundance, and bacterial community structure. The results revealed that both stimulants promoted the anaerobic degradation of typical PAHs, such as phenanthrene, pyrene, and benzo(a)pyrene. Rhamnolipids and earthworm casts promoted the degradation of phenanthrene and pyrene more significantly, with the degradation rate increasing by 13.75% and 16.92%, respectively, and the degradation rate of benzo(a)pyrene increased by 10.26% and 11.7%, respectively. The addition of rhamnolipids and earthworm casts significantly stimulated the abundance of functional genes (*UbiD*, *UbiE*) in bacterial communities, and this study indicated a strong association between the abundance of functional genes and PAH degradation efficiency. Furthermore, biostimulants altered the microbial community structure and affected microbial diversity and function. Earthworm casts significantly promoted the *Azospirillum* (0.02–20.17%) and *Acinetobacter* (0.01–15.70%) genera, which played an important role in the degradation process of PAHs. Therefore, these findings suggested that the enhancement of anaerobic biodegradation of PAHs by rhamnolipids and earthworm casts is probably due to an increase in abundance of both PAH-degraders and their degrading genes (*UbiD*, *UbiE*). This study could provide valuable insights for advancing the sustainable remediation of PAH-contaminated soils.

Keywords: polycyclic aromatic hydrocarbons (PAHs); earthworm casts; rhamnolipids; functional genes; microbial communities

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are persistent environmental organic pollutants (POPs) formed by the combination of multiple benzene rings and are widely present in the environment [1]. PAHs are primarily produced from natural processes and anthropogenic activities, including the incomplete combustion of some organic materials such as coal, oil, gasoline, and wood [2]. Due to their structural stability, hydrophobicity, and low water solubility, PAHs are difficult to remove from the environment and accumulate long-term residues in soil [3]. It has been reported that 90% of the world's PAHs are stored in soil [4,5]. Furthermore, PAHs exhibit genotoxicity, carcinogenicity, and mutagenicity, posing serious hazards to human health and ecosystems [6]. Current methods for removing PAHs from soil include physical, chemical, and bioremediation. Although

physical–chemical methods have significant treatment effects, they also have drawbacks such as secondary contamination and high cost [7,8]. In contrast, bioremediation can be used as an exogenous bioremediation method that is environmentally friendly, economical, and sustainable, addressing the limitations of most physical–chemical methods and receiving increasing attention and promising development prospects [7,9].

In recent decades, the degradation of PAHs in aerobic environments has been extensively studied, with the associated degradation mechanisms elucidated [10,11]. However, PAHs are also prevalent in anoxic or anaerobic environments (e.g., subsurface soils, groundwater, river sediments, and marine sediments), where PAH contamination is typically more severe than in aerobic environments. Recently, the anaerobic degradation of PAHs has received increasing attention, and several studies have investigated anaerobic microbial degradation pathways and mechanisms, which are still not comprehensive enough [12,13]. Anaerobic biodegradation of PAHs is particularly challenging because molecular oxygen, which acts as a terminal electron acceptor in the first step of aerobic degradation, cannot participate in the anaerobic degradation process [14]. Several studies have demonstrated that the strategy of adding biostimulants can significantly enhance the potential for microbial degradation of PAHs by increasing soil microbial enzyme activity and optimizing PAHs bioavailability compared to natural attenuation [15,16]. However, the anaerobic biodegradation of certain high molecular weight PAHs is constrained by their low water solubility, which limits microbial accessibility [17]. The application of biosurfactants has emerged as an effective strategy to overcome this limitation. Rhamnolipids, produced by various microorganisms, possess a characteristic molecular structure comprising one or two rhamnose moieties and one or two 3-hydroxy fatty acid chains containing 8–14 carbon atoms [18]. Owing to their favorable physicochemical and biological properties, as well as environmental and economic benefits, rhamnolipids have been widely investigated and applied as biostimulants in soil bioremediation [19]. By promoting the transfer of PAHs from the nonaqueous to the aqueous phase, rhamnolipids enhance solubility and bioavailability, thereby facilitating biodegradation [20,21]. Previous studies have demonstrated that rhamnolipid addition can markedly increase PAH degradation rates by reducing surface and interfacial tension, which enhances microbial utilization of PAHs even at low concentrations [22]. Furthermore, the anaerobic biodegradation of PAHs can be promoted by increasing soil organic matter, and earthworm casts, as an excellent soil amendment, are considered an economical, eco-friendly, and resource-sustainable method for removing organic contaminants from soil [23,24]. Research has also shown that introducing supplemental carbon substrates or nutrients can improve soil microbial diversity and activity, facilitating the co-metabolic degradation of PAHs and thereby enhancing anaerobic PAH degradation rates [25]. Nevertheless, the mechanisms underlying the effects of rhamnolipids and earthworm casts on PAH biodegradation in anaerobic environments are still unclear.

Microorganisms are crucial for PAHs biodegradation. This process is primarily mediated by multiple genes and gene clusters within the bacterial community [26–28]. Therefore, within bacterial communities, the expression and abundance of functional genes are critical to the PAH biodegradation process, and changes in gene abundance can serve as key indicators of the bacterial community's PAH-degrading capacity [29,30]. Microbial community structure and diversity are also key factors that regulate the dissipation of organic pollutants [31]. Based on the preceding background information, we hypothesized that rhamnolipids and vermicompost effectively stimulate the expression of functional genes, enhance microbial metabolic activity, and facilitate the anaerobic degradation of PAHs. Furthermore, an association was speculated between gene abundance and the degradation rate.

Nowadays, few studies have comprehensively explored the effect of biosurfactants and organic amendments on anaerobic degradation of PAH-contaminated soil, and the diversity, function, community structure, and functional gene abundance of indigenous microorganisms and their potential response mechanisms during the degradation process remain inadequately understood. Therefore, this study aimed to investigate the mechanism and pattern of the effects of rhamnolipids and earthworm casts on the anaerobic biodegradation process of PAHs. In this experiment, three typical PAHs, low-molecular-weight phenanthrene, high-molecular-weight pyrene, and benzo(a)pyrene, were chosen as target pollutants. Quantitative Real-Time PCR (qPCR) Analysis was used to reveal the characteristics of degradation genes (*UbiD*, *UbiE*) in response to biostimulants. The effects on microbial diversity and bacterial community structure in the PAH-contaminated soil were analyzed using high-throughput sequencing. This study would provide a theoretical basis for further elucidating the mechanism of anaerobic degradation of PAH-contaminated soil promoted by rhamnolipids and earthworm casts, as well as information for the future development of sustainable bioremediation applications.

2. Materials and Methods

2.1. Soil

This study was conducted in Beijing, China, and the soil used was collected from the topsoil layer (0–20 cm) on the Beijing University of Architecture campus. All soil samples were air-dried and sieved through a 100-mesh sieve. A portion of the soil was used to measure basic physicochemical properties, and the remainder was used for the microcosm PAHs degradation experiments. All analyses were in triplicate. Table 1 (mean ± SD, n = 3) provides detailed information on the physicochemical properties of the soil. The soil samples were spiked with PHE (Macklin, Shanghai, China), PYR (Macklin, Shanghai, China), and BAP (Macklin, Shanghai, China) dissolved in acetonitrile solution, air-dried in a fume hood, and then mixed thoroughly to achieve initial concentrations of 35, 14, and 10 mg/kg [16], respectively.

Table 1. Physicochemical characteristics of the soil (mean ± SD, n = 3).

Parameters	Value	Methods	Instrument
Moisture content (%)	6.35 ± 0.84	Oven-drying method [32]	Analytical balance
pH	6.84 ± 0.17	Potentiometric method [32]	pH meter
Organic matter ($g \cdot kg^{-1}$)	14.40 ± 1.52	Loss on Ignition [33]	Muffle furnace
Total N ($g \cdot kg^{-1}$)	0.77 ± 0.02	Kjeldahl digestion [34]	Elemental analyzer
Total P ($g \cdot kg^{-1}$)	0.95 ± 0.06	Molybdenum blue Colorimetric method [35]	Spectrophotometer
Bulk density ($g \cdot cm^{-3}$)	1.02 ± 0.11	pycnometer method [36]	Densimeter
Soil particle density ($g \cdot cm^{-3}$)	2.71 ± 0.04		Pycnometer
Salinity (%)	0.16 ± 0.05	Saturated paste extract EC [37]	EC meter

Data are means ± standard deviation from three replicates (n = 3).

2.2. Experimental Design

Experiments were conducted in 250 mL glass vials with 60 g of soil added. Three treatment groups were designed: a blank control (O), rhamnolipid (R), and earthworm casts (E). Group O served as a control without added stimulants for natural attenuation, while group R received 1 g/L rhamnolipid (Macklin, Shanghai, China) [38], and group E contained 50 mg of earthworm casts per gram [23]. Earthworm manure was purchased from Xuzhou Earthworm Farm (Xuzhou, China). Sterile distilled water was added to maintain a water content of 60%, as previous studies indicated higher microbial activity at this ratio [39]. Each bottle was inflated with argon gas for 30 min to ensure an anaerobic

environment. During the experiment, the sample bottles were transferred to an anaerobic incubator (Shanghai CIMO Medical Instrument Co., Ltd., Shanghai, China) for sampling. After the sampling was completed, the sample bottles were sealed and transferred to a constant temperature incubator at 30 °C for the experiment. Three replicates were used for each treatment. On days 7, 20, 32, 45, and 72 after incubation, triplicate samples were collected for chemical and biological analyses. Samples were stored at −80 °C for subsequent analysis.

2.3. PAHs Extraction and Analysis in Soil

Soil samples were pre-treated using a freeze dryer and subsequently freeze-dried for 10 h to eliminate moisture. Soil samples were freeze-dried, and PAH residues were extracted using a mixture of hexane/dichloromethane (Macklin, Shanghai, China). After two or three repetitions of ultrasonication and centrifugation (TG16G, 4000 rpm for 10 min), rotary evaporation was performed at 70 °C, followed by the addition of acetonitrile to dissolve and filter through a filter membrane with a pore size of 0.22 μm [40,41]. An ultra-performance liquid chromatography (ACQUITY UPLC, Milford, MA, USA) system was used to analyze the PAH concentrations. Three replicates were used for each treatment.

2.4. DNA Extraction from Soil and Quantitative Real-Time PCR

The genes related to the anaerobic degradation of PAHs (*UbiD* and *UbiE*) were quantified using Quantitative Real-Time PCR (qPCR). Three replicates were used for each treatment. Total genomic DNA was extracted using the E.Z.N.A™ MagBind Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA), according to the manufacturer's instructions. The DNA was used for qPCR experiments; genomic DNA was first extracted from soil samples; primers were synthesized on the sequences of the *UbiD* and *UbiE* genes [30], and the sequence information is shown in Table 2. qPCR amplification of the *UbiD* and *UbiE* genes was performed. The specificity of the primer pairs was verified by analysis of the melting curves, while amplification efficiency was assessed through the construction and analysis of standard curves. All standard curves exhibited R^2 values of 0.99, and the amplification efficiencies ranged from 91% to 97%. The DNA amplification protocol involved an initial pre-denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. After the final cycle, a final extension step was carried out at 72 °C for 10 min. Once the reaction was complete, the amplification products were analyzed by 1% agarose gel electrophoresis, and the target DNA fragment was extracted from the gel. All procedures were conducted in accordance with the guidelines outlined in the Combi Century Agarose Gel DNA Recovery Kit manual.

Table 2. Primer sequences utilized in this study.

Primer	Sequences
UbiD F:	GAGCATCCTGCGACAGTTCA
UbiD R:	GTAGTCGAGGGCGTGTTC
UbiE F:	CCGGCCTACGACTGGTATTC
UbiE R:	GAAGTACTGCACCCGTTCCA
16 s F:	CCTACGGGNGGCWGCAG
16 s R:	GACTACHVGGGTATCTAATCC

2.5. Microbial Community Analysis

Soil samples were analyzed at Sangon Biological Engineering (Shanghai) Co., Ltd. (Shanghai, China). The microbial community was analyzed in three independent biological replicates per treatment group (n = 3). Soil bacterial communities were characterized using Illumina high-throughput sequencing of the 16S rRNA gene (V3–V4 region). The highly

variable region V3–V4 of the bacterial 16S rRNA gene was targeted. The 16S rRNA V3–V4 amplicon was amplified using 2×Hieff® Robust PCR Master Mix (Yeasen, Shanghai, China). The 16S rRNA gene was amplified using the universal specific primers listed in Table 2. This study evaluated the sequencing depth (Clean reads 75,103–96,881 per sample) and coverage (>0.99), confirming that the data quality met the requirements for microbial diversity analysis. Valid tags were clustered into operational taxonomic units (OTUs) with ≥97% similarity by using Usearch software (version 11.0.667). The most abundant tag sequence was chosen as the representative sequence for each cluster. Representative sequences of bacterial OTUs were classified taxonomically by blasting against the Silva Database. Alpha diversity indices, reflecting the richness and evenness of bacterial communities within individual samples, were calculated based on the rarefied OTU table (rarefied to 75,000 sequences per sample to account for uneven sequencing depth). The following indices were computed using the bioinformatics pipeline provided by Sangon Biotech: observed OTUs (richness), Chao1 index (estimated richness), Shannon index (diversity incorporating richness and evenness), and Simpson index (diversity weighted towards dominant species). Principal Coordinates Analysis (PCoA) is an ordination method that visualizes similarities and dissimilarities among samples based on a distance matrix. Through eigen decomposition, it determines key dimensions (principal coordinates) that effectively rotate the dataset without altering the inherent relationships among samples. Consequently, the resulting plot clusters samples with similar community structures in close proximity while distinctly separating those exhibiting greater differences.

2.6. Prediction of Microbial Community Function

Microbial functional profiling was performed using the FAPROTAX 1.2.4 database (n = 3). Microbial taxa were mapped to predefined ecological functional groups based on OTU/ASV abundance tables derived from 16S rRNA gene amplicon data and their taxonomic annotations (confidence threshold ≥ 0.7).

2.7. Statistical Analysis

All data were dealt with in Excel 2021 (Microsoft, Redmond, WA, USA). Data in the figures and tables represent the average of three replicates. Pearson's correlation analysis and analysis of variance (ANOVA) were conducted using the SPSS statistical package 29.0 for Windows (SPSS, Chicago, IL, USA). Figures were produced by Origin 2021 (OriginLab, Northampton, MA, USA).

3. Results

3.1. Biostimulants Enhanced the Biodegradation of PAHs in Soil

As shown in Figure 1, the addition of rhamnolipid and earthworm casts increased PAH degradation compared to the control group O. The maximum degradation of PHE reached 81.6% in the rhamnolipid-treated group R, and 79.26% in the earthworm casts-treated group E, increasing degradation rates by 13.75% and 11.47%, respectively ($p < 0.05$). Maximum PYR and BAP degradation rates were 62.17% and 50.2% in the earthworm casts-treated group E, 16.92% and 11.7% higher than controls ($p < 0.05$), while in the rhamnolipid-treated group R, rates were 58.78% and 48.76%. Among the three typical PAHs, PHE showed the highest degradation efficiency, followed by PYR and BAP. Meanwhile, the highest degradation efficiency of PAHs was generally observed in the three different treatment groups from day 7 to day 20, with the degradation rate gradually slowing down after three weeks. This is probably due to the reduction in available carbon sources in the soil, which, in turn, caused competitive effects among microorganisms.

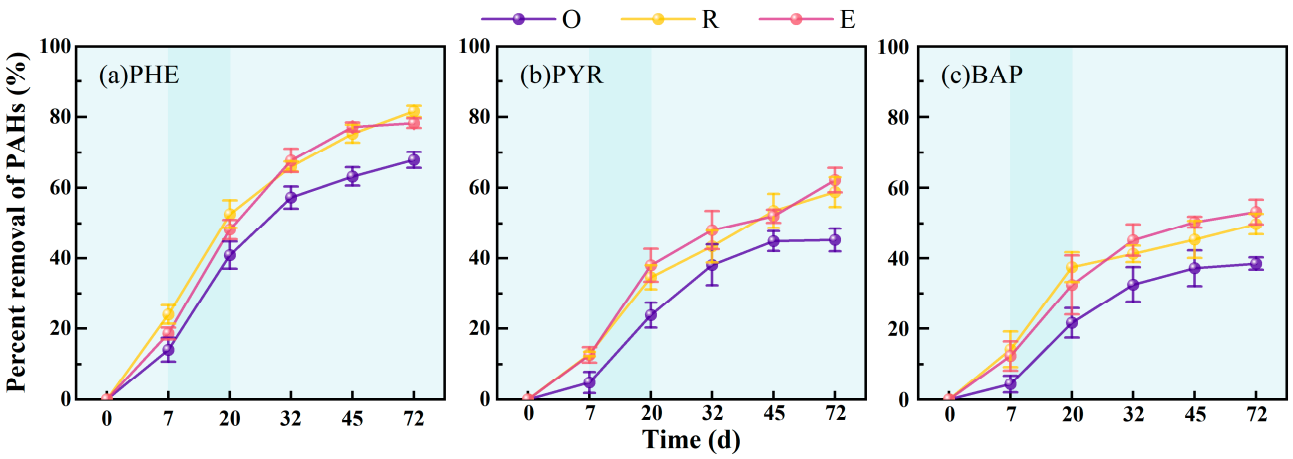


Figure 1. Percentage removal of (**a**) Phenanthrene (PHE), (**b**) Pyrene (PYR), and (**c**) Benzo(a)pyrene (BAP) at different treatment groups during the 72-day incubation. Error bars represent standard deviations of triplicate samples (n = 3).

3.2. Abundance of PAHs Anaerobic Degradation Genes

Quantitative analysis of *UbiD* and *UbiE* gene abundance was performed using qPCR, as shown in Figure 2. The abundance of *UbiD* and *UbiE* genes generally increased during the pre-culture period in both rhamnolipid and earthworm cast-treated groups, then stabilized and slowly declined. The increase in *UbiD* gene abundance was more significant ($p < 0.05$) in group E than in group R and the control group, whereas there was no significant difference between *UbiD* gene abundance in group R and the control group.

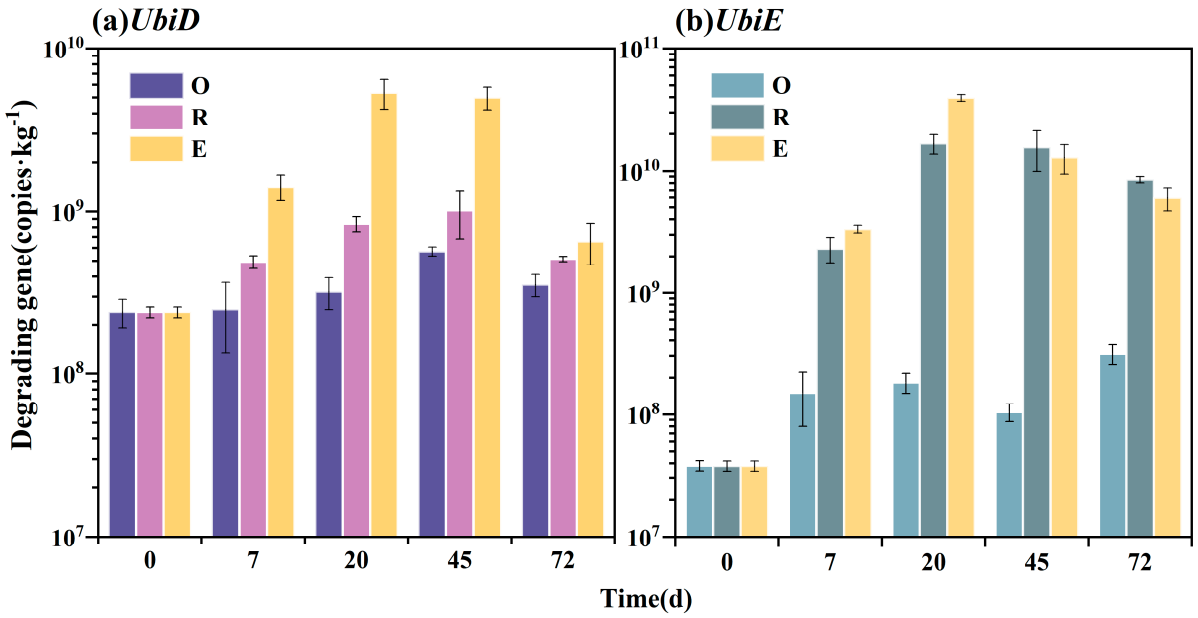


Figure 2. Abundance of *UbiD* gene (**a**) and abundance of *UbiE* gene (**b**) during the PAH degradation. The treatments include O (Controls), R (Rhamnolipids), and E (Earthworm casts). Data are means ± standard deviation from three replicates (n = 3). Different letters indicate significant differences ($p < 0.05$).

From 0 to 20 days, rhamnolipid and earthworm casts significantly promoted the enrichment of the *UbiE* gene ($p < 0.01$), reaching maximum copy numbers of 1.67×10^{10} copies·kg^{-1} and 3.95×10^{10} copies·kg^{-1}, respectively, compared to controls. In contrast, *UbiE* gene abundance did not differ significantly between the rhamnolipid group R and the

earthworm cast group E. The abundance of *UbiD* and *UbiE* genes tended to stabilize after 20 days.

3.3. Changes in Microbial Alpha Diversity Analysis

Alpha diversity is commonly utilized to evaluate the changes in bacterial diversity and abundance during degradation. Several commonly used α-diversity indices were selected to analyze the α-diversity of soil samples at 0 d, 45 d (M), and 72 d (F), as shown in Table 3 (mean ± SD, n = 3). The maximum OTUs, Shannon, Chao, and Ace values were obtained for the bacterial community in soil samples on day 0. The Chao and Ace indices reflect the abundance of the bacterial community, while the Shannon index is a composite index characterizing the abundance and evenness of the species distribution, with higher values indicating higher diversity [42]. The community richness and diversity of group R (R–M) and group E (E–M) were higher than that of the control group (O–M) on day 45, with group E showing higher values than group R, but both lower than on day 0. This indicates that bacterial diversity and richness changed dynamically during the incubation process. On day 72, there was no significant change in community richness and diversity in group R–F and group E–F, but community diversity and richness increased in the control group O–F. In addition, the structure and diversity of microbial communities changed with the dissipation of PAHs.

Table 3. Alpha diversity comparison in soil samples (mean ± SD, n = 3).

	OTUs	Shannon	Chao1	Ace	Simpson	Coverage
O	2748 ± 124	6.63 ± 0.11	2779.04 ± 201	2844.84 ± 58	0.0035	0.9965
O–M	1364 ± 102	1.85 ± 0.08	1259.23 ± 126	1314.74 ± 151	0.2900	0.9944
O–F	1738 ± 84	4.65 ± 0.05	1722.67 ± 96	1784.16 ± 89	0.0357	0.9956
R–M	1478 ± 132 *	4.51 ± 0.19 **	1898.66 ± 189 **	1972.18 ± 174 **	0.0398	0.9959
R–F	1700 ± 76	4.72 ± 0.09	1903.10 ± 69	1969.96 ± 153 *	0.0503	0.9953
E–M	2013 ± 141 **	5.00 ± 0.23 **	2203.21 ± 176 **	2326.94 ± 211 **	0.0289	0.9937
E–F	1999 ± 136 *	5.48 + 0.19 **	2168.63 ± 164 *	2239.59 ± 195 **	0.0132	0.9955

The treatments include O (Controls), R (Rhamnolipids), and E (Earthworm casts). M and F represent days 45 and 72, respectively. Data are means ± standard deviation from three replicates (n = 3). * Indicates significant difference at $p < 0.05$. ** Indicates significant difference at $p < 0.01$.

3.4. Changes in Microbial Community Structure

Figure 3 illustrates changes in soil bacterial communities at different taxonomic levels in three treatment groups on days 0, 45, and 72. Twelve dominant phyla were identified in all soil samples (Figure 3, and the abundance of dominant phyla varied with time. The bacterial phyla with the highest abundance on day 0 were *Pseudomonadota* (32.15%), *Actinomycetota* (19.19%), and *Acidobacteriota* (16.39%). The phyla that changed mainly in controls were *Pseudomonadota* (32.15%–38.77%–68.73%), *Bacillota* (0.55%–20.95%–16.80%), *Bacteroidota* (6.29%–20.18%–3.46%); in R group, the changed phyla were *Pseudomonadota* (32.15%–43.78%–28.35%), *Bacillota* (0.53%–27.52%–21.71%), and *Bacteroidota* (6.29%–4.62%–18.30%), and in group E, the changed phyla were *Pseudomonadota* (32.15%–37.77%–92.27%) and *Bacillota* (0.55%–25.49%–5.48%). PAH dissipation was generally strongly positively correlated with *Bacillota*. Furthermore, while it correlated with *Pseudomonadota* in groups O and E, in group R, it correlated with *Bacteroidota*.

The relative abundances of bacteria in soil at the genus level are shown in Figure 4. The dominant genera in control group O were *Acinetobacter* (0.03–11.55%), *Azospirillum* (7.67–9.67%), and *Massilia* (6.76–18.01%). In group E, the dominant genera were *Azospirillum* (5.52–20.17%), *Acinetobacter* (0.15–15.70%), and *Massilia* (2.82–9.10%).

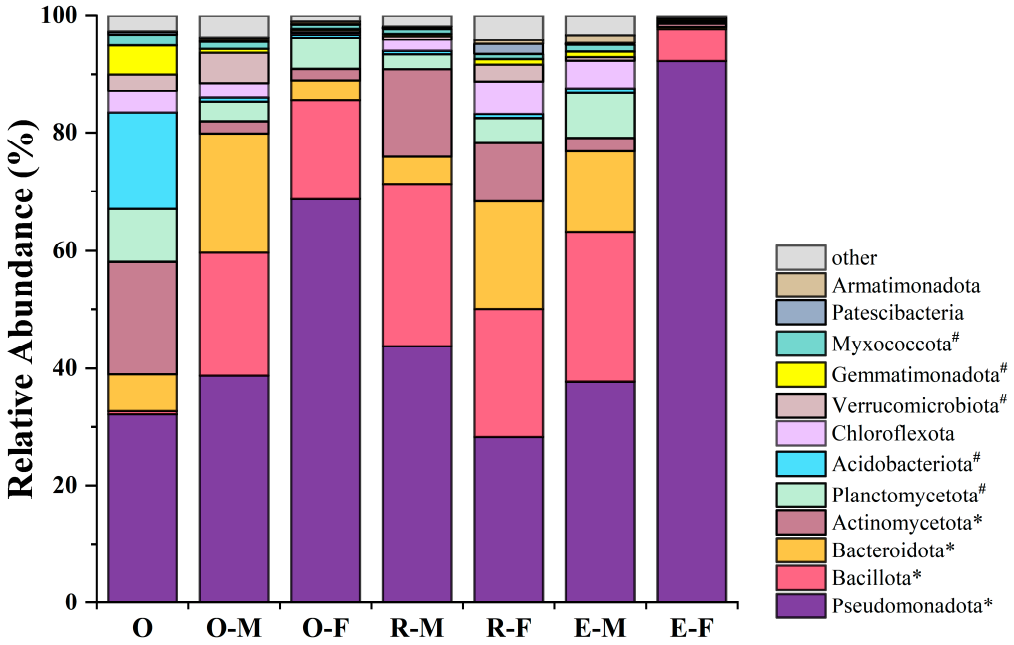


Figure 3. Relative abundance of dominant bacteria at phylum level at 45 and 72 days (n = 3). The x-axis is the sample name. O (Controls), R (Rhamnolipids), E (Earthworm casts). The y-axis is the relative abundance proportion. Different colors represent each species at that taxonomic level, with the block width indicating the relative abundance proportion of different species. * Symbolizes strains primarily involved in the degradation of PAHs. # Indicates that the phylum is dominated by aerobic bacteria.

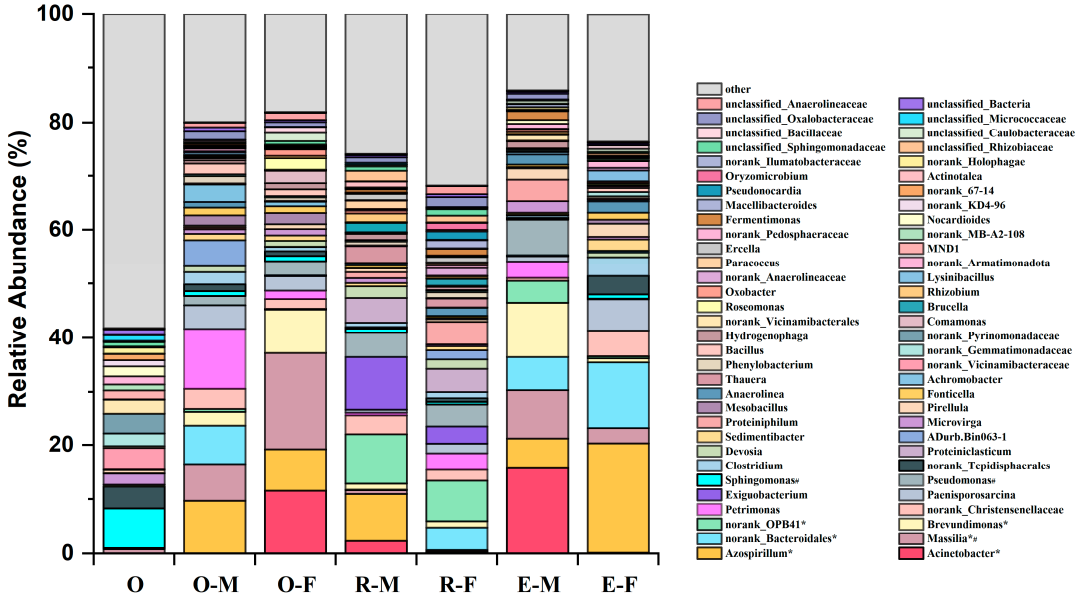


Figure 4. Relative abundance of dominant bacteria at genus level at 45 and 72 days (n = 3). The x-axis is the sample name; O (Controls); R (Rhamnolipids); E (Earthworm casts); and the y-axis is the relative abundance proportion. Different colors represent each species at that taxonomic level, with the block width indicating the relative abundance proportion of different species. * Symbolizes strains primarily involved in the degradation of PAHs. # Indicates that the phylum is dominated by aerobic bacteria.

3.5. Metabolic Function of Soil Bacteria

The function of the bacterial community in soil changed in the three treatment groups, as shown in Figure 5. The FAPROTAX function predictions focused on sulfur, nitrogen,

hydrogen, and carbon cycling. Bacterial community metabolic functions changed in all treatment groups due to different biostimulants. The chemoenergetic heterotrophic function increased in all treatment groups, and the functions of carbon cycling, methylation, and nitrate utilization related to PAH degradation also altered over time. The hydrocarbon degradation function was generally found to be positively correlated ($r > 0.7$) with functional gene abundance (*UbiD*, *UbiE*). Furthermore, nitrate respiration and nitrite respiration functions indicated an association with both *UbiD* and *UbiE* genes in group R ($r > 0.94$), while a stronger association with *UbiE* gene abundance was also expressed in group E.

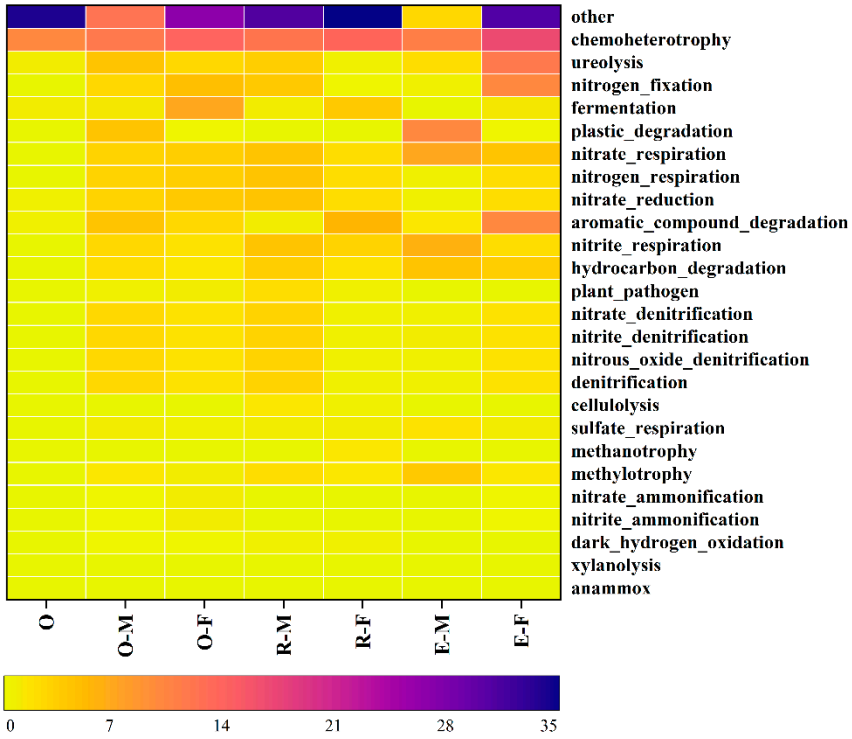


Figure 5. FAPROTAX functional abundance heatmap, plotted with a functional abundance matrix where each column represents a sample; rows represent functions (n = 3). The treatments include O (Controls), R (Rhamnolipids), and E (Earthworm casts). M and F represent days 45 and 72, respectively.

4. Discussion

In this study, we investigated the anaerobic biodegradation of PHE(LMW-PAHs), PYR, and BAP (HMW-PAHs). Results showed that the degradation efficiencies of PHE, PYR, and BAP were in descending order, consistent with previous research [12,17]. The difference in degradation rates among the three PAHs may be related to their molecular structure, as water solubility decreases with increasing molecular weight, which leads to lower bioavailability and makes microbial degradation more difficult [1,43].

The anaerobic biodegradation of PAHs was promoted by both rhamnolipids and earthworm casts. Rhamnolipids enhanced the degradation of both LMW-PAHs (13.75–16.28%) and HMW-PAHs (10.26–13.57%). As surfactants, rhamnolipids increase PAH solubility in water through micellar solubilization, thereby improving PAH bioavailability and enhancing microbial utilization [44]. Compared to LMW-PAHs, HMW-PAHs exhibit greater resistance to microbial degradation due to their structural stability and lower water solubility. Rhamnolipids mitigate this limitation by facilitating microbial utilization of HMW-PAHs, consequently increasing their degradation rates [45]. Earthworm casts also effectively improved PAH degradation rates in the present study. As a bioremediation strategy for composting, earthworm casts enriched with organic matter and nutrients can

serve as a carbon and energy source, providing auxiliary growth substrates for microorganisms. This facilitates PAH degradation via co-metabolic mechanisms [23,46]. Earthworm casts also stimulated the degradation of the HMW-PAHs pyrene (PYR) and benz(a)pyrene (BAP). Furthermore, previous studies have indicated that the presence of LMW-PAHs can enhance the biodegradation of HMW-PAHs [47,48]. Moreover, the degradation of PAHs was generally slower in the first week, probably because microorganisms preferentially select more readily available rhamnolipids and earthworm casts as carbon sources to sustain vital metabolic activities [44,45,49]. The PAH degradation rate gradually decreased after 32 days since the added biostimulants also promoted the growth of other non-PAH-degrading microorganisms that competed with potential PAH-degrading bacteria, thus restricting the rate of PAH degradation in the soil; this phenomenon was also observed in previous studies, thus providing further evidence for our conclusions [50–52]. In addition, previous studies have indicated that reduced availability of carbon sources, the accumulation of toxic intermediate metabolites during PAH degradation, and elevated terminal electron acceptor activity (e.g., nitrate) in anaerobic environments likely inhibit microbial degradation processes, thereby decelerating the overall degradation rate [53].

Alpha diversity analysis revealed lower richness and diversity of microbial communities in all treatment groups in earlier stages, compared to the initial values, and increased or stabilized microbial diversity and richness in later stages (Table 3). This may be ascribed to the negative impact of PAH contamination on the microbial community [54]. Toxicity disrupted microbial physiological structure and caused mortality of some bacterial organisms, reducing the richness and diversity of the community structure. Previous studies have also confirmed these findings [55–57]. Stimulation with rhamnolipid and earthworm casts increased microbial abundance and diversity compared to controls, with the highest values in the earthworm casts treatment. These phenomena might be due to the microbial utilization of the biosurfactant and the earthworm casts as an auxiliary substrate or nutrient source, which stimulated the growth and activity of microorganisms [58–60]. Earthworm casts provide diverse living space for microorganisms through their porous structure and also have pH buffering capacity, which benefits the metabolic activities of most microorganisms. The rich nutrient matrix provides diverse carbon and nitrogen sources that promote the growth and collaboration of different functional microflora [23], thus creating a multi-level ecological niche for PAH anaerobic degrading microorganisms. Importantly, this enhanced microbial community diversity directly contributes to the key mechanisms underpinning efficient PAH degradation. Microbial community diversity plays a crucial role in PAH degradation [20]. Higher diversity promotes synergistic interactions among microorganisms, which is particularly critical for degrading recalcitrant high-molecular-weight (HMW) PAHs like benzo(a)pyrene. Furthermore, increased diversity enhances functional redundancy, bolstering community resilience to environmental perturbations and overall stability. This enhanced robustness facilitates effective PAH degradation across diverse environmental conditions [31]. However, available carbon sources and nutrients were gradually decomposed in the later stages, and competition and starvation among the flora led to the inactivation or death of some microorganisms, which, in turn, showed lower diversity and abundance. Furthermore, biosurfactants exhibit a high tolerance to fluctuations in environmental conditions, such as pH, temperature, and ionic strength [61]. It is plausible that the presence of biosurfactant initially provided a degree of buffering or stabilization against such fluctuations in the soil environment, potentially mitigating environmental pressure on the microbial community. However, this effect diminished as available carbon sources, including the biosurfactants, gradually decomposed during later stages.

The present study revealed significant changes in microbial community structure. Principal coordinate analysis (PCoA) revealed a significant separation between biostimulation treatments and the control group (Figure 6), indicating that rhamnolipid and earthworm cast amendments substantially altered microbial community structure, which is consistent with preceding α-diversity analyses. Notably, specific microbial taxa were significantly enriched during PAH degradation, among which *Pseudomonadota*, *Bacillota*, and *Bacteroidota* are recognized as highly efficient PAH-degrading bacteria [62]. This suggests that the succession of community composition is not random but closely linked to the PAH degradation process, and community reconfiguration represents a critical ecological response that could drive degradation efficiency. Furthermore, the elevated abundance of these key taxa and the positive correlation with PAH removal rates point directly to their central role in PAH dissipation. The phyla *Pseudomonadota*, *Bacillota*, and *Bacteroidota* contributed predominantly to PAHs degradation, while rhamnolipid stimulation increased *Bacteroidota* abundance, and earthworm casts significantly stimulated *Pseudomonadota*, suggesting that rhamnolipid and earthworm cast addition promoted biodegradation of PAHs through enrichment of potential PAH-degrading bacteria. Additionally, there was a significant increase in the abundance of the phylum *Bacillota* in all treatment groups, indicating an important role in anaerobic PAH degradation. The dominant PHE-degrading genus *Massilia* was detected in Groups O and E (Figure 4), previously identified as a bioindicator for soil PAH contamination [63,64]. Earthworm casts significantly increased *Azospirillum* abundance in soil and have the potential to degrade PYR [65]. Rhamnolipid addition favored *Pseudomonas* and *Proteiniclasticum* growth in group R. Previous studies found that *Pseudomonas* increased with increasing rhamnolipid concentrations, suggesting that it contributed to rhamnolipid-potentiated PAHs degradation, providing further evidence for our conclusions [44]. Furthermore, in a previous study of anaerobic waste-activated sludge degradation by biosurfactants, *Proteiniclasticum* was found to be responsible for secondary metabolite degradation, indicating that rhamnolipids can be metabolized as carbon sources [66].

In addition to changes in soil bacterial communities, assessment of bacterial metabolic functions is necessary for the PAH degradation potential of bacteria [67]. In this study, bacterial functions varied among different treatments. Chemoheterotrophy reflects the ability of the bacterial community to utilize carbon sources, while hydrocarbon degradation and aromatic compound degradation reflect the ability to degrade PAHs. Compared with the control group, chemoheterotrophy, nitrate respiration, hydrocarbon degradation, aromatic compound degradation, and methylotrophy functions were significantly improved in the earthworm casts-treated group, where nitrate respiration was associated with anaerobic degradation of PAHs [44]. And nitrate respiration function and *UbiE* gene abundance indicated a strong association. This suggested that earthworm casts enhanced the anaerobic degradation capacity of soil bacteria for PAHs by stimulating gene expression, providing further support for our conclusions [12]. The addition of rhamnolipids enhanced both cellulose hydrolysis and aromatic compound degradation, as well as hydrocarbon degradation and nitrate respiration. A previous study found that rhamnolipids increased the activity of hydrolytic enzymes [44], indicating that rhamnolipids improved PAH degradation by the bacterial community through enhanced cellulose hydrolysis. Nitrate denitrification, nitrate respiration, and nitrite respiration functions of the bacterial communities in groups R and E intensified with time and peaked during the middle of this experiment. Moreover, fermentation was significantly enhanced in controls compared to the other groups, and previous studies have revealed that the bacterial community can increase the PAH degradation rate through fermentative metabolism under reducing conditions when PAH degradation lacks a co-substrate [68].

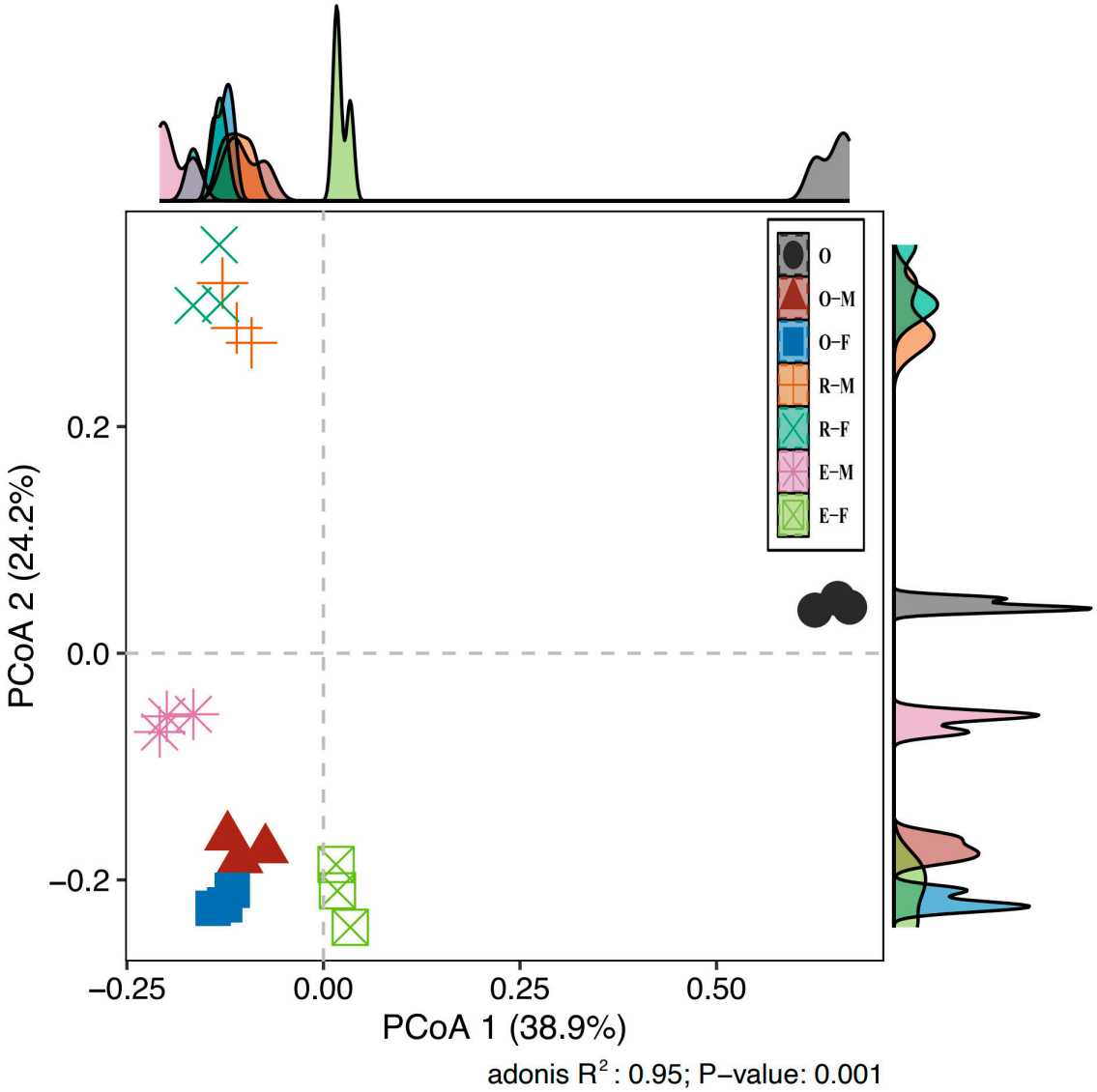


Figure 6. PCoA analysis of bacterial communities in different treatment groups ($n = 3$). The treatments include O (Controls), R (Rhamnolipids), and E (Earthworm casts). M and F represent days 45 and 72, respectively.

The anaerobic biodegradation of PAHs depends on various functional genes, which serve as valuable biomarkers for assessing the potential of bacterial communities to degrade PAHs [29]. Carboxylation and methylation reactions have been shown to be the most critical initiation reactions for anaerobic degradation and are regulated by *UbiD* and *UbiE* genes, respectively. The expression and abundance of these genes are crucial for PAH biodegradation [13,27,28]. Our study detected the expression of both *UbiE* and *UbiD* genes (Figure 2). Compared to controls, both gene abundances increased with rhamnolipid and earthworm casts, and the stimulatory effect of earthworm casts was more significant ($p < 0.05$), probably due to the enrichment of organic matter, nitrogen, and phosphorus in earthworm casts that increased microbial activity and facilitated gene expression [25,69]. Previous studies have found that biostimulants enhance degradation gene abundance [31]. Similarly, Yuan et al. observed that nutrient nitrogen increased *UbiD* and *UbiE* abundance and promoted anaerobic PAH degradation [30]. However, the stimulatory effect of rhamnolipids on the *UbiD* gene was not significant, suggesting that the enhancement of anaerobic PAH degradation by rhamnolipids may not rely on the carboxylation reaction mediated by *UbiD*; rather, it may be attributed to the promotion of the methylation reaction regulated by

UbiE. In this study, the PAHs degradation rate positively correlated with *UbiE* abundance (r = 0.85–0.88; r = 0.75–0.83) in the rhamnolipid and earthworm casts treatment groups. A slowly declining gene trend was observed in the later stages, probably related to reduced available carbon sources and nutrients in the soil that limited PAH-degrading flora growth and gene expression [70].

Rhamnolipids and earthworm casts are both biostimulants with high potential for the degradation of organic pollutants and have promising applications. Compared to natural attenuation, rhamnolipids and earthworm casts promote anaerobic biodegradation. Furthermore, as environmentally friendly stimulants, they are ecologically harmless, improve the soil environment, and benefit soil health [71]. In addition to biostimulation remediation, bioaugmentation remediation can also effectively improve PAH remediation but is constrained by factors such as the low bioavailability of PAHs, sensitivity to environmental conditions, and competition from indigenous microorganisms. Bioaugmentation combined with biostimulation has been found to achieve better PAH degradation results [72]. The combined application of bioaugmentation and biostimulation overcomes key limitations in PAHs biodegradation—particularly for HMW congeners—by synergistically integrating functional microbial consortia with optimized microenvironmental conditions. This strategy achieves high efficiency (>90% degradation) while maintaining ecological sustainability. It also demonstrates superior performance in complex scenarios, including co-contaminated (e.g., heavy-metal PAHs) and alkaline soils. Therefore, further investigation of the synergistic effect of earthworm casts or rhamnolipids with biofortification on anaerobic PAH degradation is warranted. Moreover, applying a multi-omics strategy (e.g., genomics and metabolomics) to explore the bioremediation process of PAH-contaminated soil with a deeper understanding is meaningful.

5. Conclusions

This study investigated the anaerobic biodegradation of PAHs and evaluated the promotion of this process by rhamnolipids and earthworm casts. Multiple factors were assessed, including PAH removal efficiency, the expression and abundance of functional genes, and microbial community response characteristics. The results demonstrated that the addition of rhamnolipids and earthworm casts significantly increased the abundance of the *UbiD* and *UbiE* functional genes, which may facilitate the anaerobic degradation of PAHs. Furthermore, both biostimulants promoted the growth and abundance of PAH-degrading microbial communities while enhancing their functional capacity. This study confirms the feasibility of biostimulation as an environmentally sustainable strategy to enhance the anaerobic degradation of PAHs. This remediation strategy advances Sustainable Development Goals (SDGs) 6, 12, and 15 by reducing PAH groundwater leaching (SDG 6: Clean Water and Sanitation), valorizing waste-derived amendments (SDG 12: Responsible Consumption and Production), and restoring the ecosystem and improve soil biodiversity (SDG 15: Life on Land). The findings offer novel insights for developing cost-effective, scalable bioremediation approaches that align with circular economy principles and sustainable land management goals.

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