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Enhancing Anaerobic Biodegradation of Phenanthrene in Polluted Soil by Bioaugmentation and Biostimulation: Focus on the Distribution of Phenanthrene and Microbial Community Analysis

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Abstract: The remediation of polycyclic aromatic hydrocarbon (PAH)-contaminated soils has received much attention in recent years, and most of the contaminated sites are in anaerobic environments, such as deep soils and flooded soils. We simulated the natural flooded soil environment, selected phenanthrene (PHE) as a model PAH contaminant, and designed batch experiments run for 63 days to comprehensively investigate the effects of the combined addition of anaerobic sludge and granular biochar on microbial community and function and the anaerobic biodegradation of PHE. Firstly, the residue, distribution, and removal of PHE in the flooded soil environment were quantified for each group. Secondly, the effects of bioaugmentation of soil indigenous microorganisms by the addition of anaerobic activated sludge and biostimulation of biochar on the removal of PHE from the soil were analyzed against each other. Lastly, the changes in the structure of the microbial community under the effect of bioaugmentation and biostimulation were illustrated by sequencing analyses. The results of this study showed that the removal efficiency of PHE reached 72.0% after the addition of anaerobic activated sludge. The incorporation of anaerobic activated sludge and biochar resulted in a 25.3% increase in PHE removal compared to a single soil, suggesting that the combination of bioaugmentation and biostimulation can have a synergistic effect on the anaerobic biodegradation of PHE in contaminated soils. The results of sequencing analysis further indicated that the introduction of an exogenous microbial community changed the dominant genera associated with PHE degradation and introduced methanogenic archaea, which enriched the metabolic pathways of the carbon cycle in the system. On this basis, the addition of biochar resulted in higher anaerobic microbial community diversity, functional dominant species were enriched, and the direct interspecies electron transfer (DIET) process between electroactive bacteria (*Bacteroides*, *f_Geobacteraceae*) and Methanosaeta was facilitated, which accelerated the degradation of PHE by anaerobic microbial communities. The results of this study provide regulatory tools and basic data support for enhanced bioremediation of PAHs in flooded soils.

Keywords: PAH-contaminated soil; anaerobic biotransformation; bioaugmentation; biostimulation; phenanthrene degradation; microbial community

Citation: Xue, H.; Shi, Y.; Qiao, J.; Li, X.; Liu, R. Enhancing Anaerobic Biodegradation of Phenanthrene in Polluted Soil by Bioaugmentation and Biostimulation: Focus on the Distribution of Phenanthrene and Microbial Community Analysis. *Sustainability* **2024**, *16*, 366. https://doi.org/10.3390/su16010366

Academic Editor: Shibao Chen

Received: 30 November 2023
Revised: 26 December 2023
Accepted: 27 December 2023
Published: 30 December 2023

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are hydrocarbons with more than two benzene rings in their molecules and are a class of harmful organic pollutants widely distributed in nature [1]. PAHs can enter the ecosystem through combustion, volcanic eruptions, accidental spills, volatilization, exhaust emissions, etc., and can be classified as

either natural or anthropogenic sources [2]. Among them, accidental spills and emissions from petroleum extraction, smelting, transport, and the production and use of petroleum products are important sources of PAHs in the environment [3,4]. Globally, more than 1 billion petroleum pollutants are reported to be produced annually [5]. These PAHs generated by natural and anthropogenic activities enter the atmosphere, water bodies, soil, and other circles. Due to their unique hydrophobicity, persistence, bioaccumulation, and other characteristics, the PAHs from the atmosphere, as well as the water body, eventually converge into the sediment and soil through migration and transformation [6–8]. The soils are loaded with about 90% of the PAHs in the environment [9], so the current situation of PAH soil contamination cannot be ignored [10]. According to the survey, the concentration of PAHs in surface soils in China ranged from 0 to 261 mg/kg, with an average value of 0.63 mg/kg [11]. In some heavily polluted areas, the concentration of PAHs was higher. Fu et al. collected 138 soil samples from the junction of an oil field and a suburban area in the Yellow River Delta. After analyzing the samples, it was found that the total concentration of 16 kinds of PAHs in the samples ranged from 278.7 to 733.5 mg/kg (mean value 382.5 $\pm$ 128.4 mg/kg). Moreover, the concentration of 16 PAHs increased with the duration of exploitation of the nearby oil wells [12,13]. The accumulation of PAHs in soil can be further transmitted through the food chain and ultimately pose carcinogenic, teratogenic, and mutagenic risks to human beings [14,15]. The problem of PAHs contaminated soil is receiving more and more attention, and there is an urgent need for an efficient remediation strategy.

Most of the actual contaminated sites, such as flooded soils and deep soils, are anoxic or anaerobic environments, and the microbial communities are mainly dominated by facultative and anaerobic microorganisms. Therefore, how to improve the efficiency of anaerobic biodegradation of PAHs has gradually become a focal issue in the soil remediation process. Currently, bioaugmentation and biostimulation are the two main ways to enhance the remediation effect of PAH-contaminated soil. Bioaugmentation refers to the addition of pre-selected or domesticated cultured microorganisms to a microbial community to enhance the ability of that microbial community to degrade pollutants [16]. The introduction of functional exogenous microorganisms into the contaminated environment will enhance the rate of pollutant degradation. This technique has been developed for the remediation of PAH-contaminated soils [5]. Li et al. used functional microbial colonies cultured from oil-contaminated soils to enhance the biodegradation of 16 PAHs (initial concentration: 15.72 mg/kg) in soils, and the treatment efficiency was increased from about 30% to more than 50% [17]. Biostimulation is a method to promote the degradation of PAHs by indigenous microorganisms through the addition of nutrients, surfactants, functional materials, and the provision of electron acceptors [18]. Among them, the addition of functional materials applied in the remediation of contaminated soil is an emerging category of pathways. Biochar, as a common carbon material, is a product obtained by pyrolysis of organic matter at high temperatures under anoxic or adiabatic conditions [19,20]. Biochar has been widely used for the removal of pollutants such as heavy metals, dyes, nitrobenzene, and polycyclic aromatic hydrocarbons due to its porous nature, high specific surface area, abundance of organic functional groups, and environmental friendliness [21,22]. Biochar has also shown good performance in a number of areas, such as fertility improvement, nutrient retention, enhancement in microbial activity, and pollutant immobilization. It has been shown that biochar can inhibit the uptake of toxic metals and increase the activity of antioxidant enzymes to effectively improve soil quality and plant physiological properties [23]. Biochar is also able to promote the growth of native microorganisms by neutralizing pH and providing nutrient support [24]. Although many previous investigations have been reported on the remediation of PAH-contaminated soils using bioaugmentation or biostimulation, relatively few studies have been conducted on the application of a combination of the two methods to PAH-contaminated soils. It was hypothesized that anaerobic activated sludge and biochar would be synergistic for the anaerobic biodegradation of PAHs in contaminated soils.

Thus, this study selected PHE as a PAH model pollutant and designed a long-term (63-day) batch experiment to simulate a flooded soil environment. To investigate whether the anaerobic microbial community and granular biochar domesticated by PHE can promote the anaerobic biodegradation of PHE in soil, whether biochar can strengthen the anaerobic microbial community to repair PHE-contaminated soil, and whether the combination of biostimulation and bioaugmentation could have a synergistic effect on the anaerobic biological removal of PHE, which would provide technical and theoretical support for the microbial regulation and remediation of actual PAH-contaminated soil.

2. Materials and Methods

2.1. Feedstock, Inoculum, and Operating Procedure

The soil used in this study was collected from paddy fields in the Yellow River Delta region (N: 37.8°; E: 118.8°). The physical and chemical properties of the soil used for the experiment are presented in Table S2. The inoculated anaerobic sludge (TS = 11.03 ± 0.30%; VS = 5.41 ± 0.13%) was obtained from a UASB reactor that was continuously operated (no PHE was added to the influent water, and the PHE in the sludge was tested to be sufficiently degraded after 6 months). The granular biochar for the experiments was sieved by a sieve with a particle size range of 2–5 mm. The biochar was the commercial biochar, whose characteristics are presented in Table S1 and Figure S1. PHE (97%) was procured from Sigma-Aldrich (Shanghai, China). Prior to the experiment, the soil was air-dried in an indoor environment, then ground and passed through a 0.5 mm diameter sieve. The soil was uniformly contaminated with a concentrated solution of PHE dissolved in acetone for 4 h at room temperature until the acetone evaporated completely, and then sealed in glass vials and aged for 7 days. Sterilized soil and sludge were obtained by means of autoclaving (121 °C, 0.103 MPa). The contamination of sterilized soil was carried out in sterile containers and treated as above. The content of PHE in the aged and sterile soil was 46.74 ± 1.40 mg/kg soil and 48.79 ± 3.46 mg/kg soil, respectively. The nutrient salt buffers (g/L in distilled water) used in the experiments mainly included $MgCl_2$-$6H_2O$ (1.0), KH_2PO_4 (2.0), K_2HPO_4-$3H_2O$ (2.0), $CaCl_2$ (1.0), KCl (5.0), and $NaHCO_3$ was used to adjust initial pH to 7.5 ± 0.05 [25].

2.2. Experimental Design

A serum bottle with a volume of 120 mL and a working volume set at 90 mL was used for this experiment. The experiment was carried out at 35 ± 1 °C and 120 rpm. Sterilized soil, sterilized sludge, soil, or anaerobic activated sludge were dropped in different vials. sterilized soil or sludge was used in order to form the control group. The microbial community in the sterilized soil or sludge was inactivated; thus, it could be used to investigate the PHE removal in the soil without microbes. The specific experimental protocol is shown in Table 1. The mass of soil added in the batch experiment was 10.0 g, the weight of inoculated anaerobic activated sludge was 3.0 g, and the concentration of biochar injected was 10 g/L. Soil, inoculated sludge, 80 mL of nutrient salt buffer, and granular biochar were added to the serum vials according to the experimental design, and then the vials were purged with pure nitrogen for 10 min to remove the oxygen and ensure anaerobic conditions and sealed with rubber gaskets and aluminum caps [25]. The vials were placed in a set water bath for 5 min to equilibrate the air pressure. Liquid samples and soil samples were removed from the vials at 7 d, 21 d, 35 d, 49 d, and 63 d of the experimental run for analysis, and PHE adsorbed on the biochar and microbial communities were analyzed at the end of the batch test. In this experiment, S1, S2, S3, and S4 were control groups, and S5–S10 were experimental groups.

2.3. Analytical Methods

2.3.1. Chemical Analysis

Total solids (TS) and volatile solids (VS) of anaerobic sludge were measured according to the standard methods [26]. Chemical oxygen demand (COD) was quantified by applying

ultraviolet–visible spectrophotometry (Shimadzu, Japan, UV-2600) and using potassium dichromate as an oxidant to quantify soluble COD (SCOD), the aqueous samples were filtered by 0.45 μm hydrophilic polyethersulfone filters [27]. High-performance liquid chromatography (HPLC) (Shimadzu, Japan, LC-2030) equipped with a UV detector was used to test the concentration of VFAs, mainly including formate, acetate, propionate, and butyrate [28].

Table 1. The experimental design of batch test.

Classification	Groups	Sterilization Soil (g)	Sterilized Sludge (g)	Soil (g)	Nutritive Salt Buffer (mL)	Anaerobic Sludge (g)	Biochar (g)
Control group	S1	10.0	/	/	80.0	/	/
	S2	10.0	3.0	/	80.0	/	/
	S3	10.0	/	/	80.0	/	1.0
	S4	10.0	3.0	/	80.0	/	1.0
Experimental group	S5	/	/	10.0	80.0	/	/
	S6	/	3.0	10.0	80.0	/	/
	S7	/	3.0	10.0	80.0	/	1.0
	S8	/	/	10.0	80.0	/	1.0
	S9	/	/	10.0	80.0	3.0	/
	S10	/	/	10.0	80.0	3.0	1.0

Note: Sterilization soil and soil in the table were PHE-contaminated and aged.

2.3.2. PHE Detection

Extraction and determination of PAHs in soil, liquid, and granular biochar in vials were carried out with reference to the literature [29–31]. For soil and biochar samples, approximately 0.1 g of PHE in freeze-dried soil or biochar was extracted using accelerated solvent extraction (Thermo, America, ASE 350) with hexane as the solvent. The ASE conditions were 10 mL extraction tank (soil or biochar samples, 5 mL of hexane), temperature 100 °C, and pressure 11.03 MPa. Each sample was extracted three times. Afterward, the samples were evaporated to near dryness under a stream of nitrogen. Then, the extracts were diluted to 2 mL of hexane. The mixture was filtered through a nylon 66 filter for subsequent analysis. For water samples, 2 mL of sample and 6 mL of hexane were added to a glass separatory funnel, shaken vigorously for 20 min, and then allowed to stand for 15 min. Each sample was extracted three times. The post-extraction treatment was similar to that for soil samples. The content of PHE was monitored by high-performance liquid chromatography (HPLC, Shimadzu, Japan, LC-2030) equipped with a UV detector and a reversed-phase C18 column (Shimadzu, 5 μm, 4.6 × 250 mm). The mobile phases were acetonitrile (70%) and Milli-Q water (30%) at a flow rate of 1.0 mL/min, and the column temperature was 40 °C. The detection wavelength of PHE was 253 nm. The recoveries of PHE in soil samples, biochar samples, and water samples by these methods were 95.5 ± 2.5%, 91.8 ± 3.1%, and 94.4 ± 1.7%, respectively.

2.3.3. Sequencing

At the end of the experiment, S5, S8, S9, and S10, as well as the original soil and inoculated sludge samples, were collected and then stored at −20 °C. The DNA was extracted by washing the samples three times with PBS buffer. All samples were washed three times with PBS buffer pH 7.2–7.4 before DNA extraction. Whole genomic DNA from samples was extracted using the Magen Hipure Soil DNA Kit (Magen, Guangzhou, China) according to the manufacturer's protocols, and the DNA concentration was determined by the Qubit® dsDNA HS Assay Kit (Thermo, America). DNA libraries were prepared by GENEWIZ® multiplexed and loaded onto an Illumina MiSeq instrument according to the manufacturer's instructions (Illumina, San Diego, CA, USA) [29]. We used Tax4Fun2 (v1.1.6) [32] software to make functional predictions of communities from sequencing results and combined them with the Kyoto Encyclopedia of Genes and Genomes (KEGG)

database (https://www.genome.jp/kegg/pathway.html accessed on 29 November 2023) to reveal potential metabolic responses at the microbial community level.

2.3.4. Data Analysis

Concentrations of PHE on soil, liquid, and biochar are expressed as mean ± standard deviation. Correlations of performance indicators were analyzed using the R program (version 4.2.1) with 95% confidence intervals. *p*-values less than 0.05 indicate statistically significant results (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The graphs of chemical indicators, as well as microbial communities, were plotted using Origin 2018.

3. Results and Discussion

3.1. Changes in Dissolved Organic Matter in Flooded Soil

In this experiment, soil and inoculated sludge provided the main carbon source, while microorganisms preferred to use the organic carbon sources in the liquid environment [33]. So, it was necessary to analyze the changes in the concentration of soluble organics in the aqueous environment, and the results of the experiment are shown in Figure 1. At day 7, the SCOD concentration in S6 and S7 of supplemented sterilized sludge was higher than those of the control (S5) and experimental (S9, S10) groups, suggesting that anaerobic sludge can provide a source of dissolved organic carbon and that the bio-microbial community in the anaerobic activated sludge is able to accelerate the depletion of the bioavailable carbon source. The same phenomenon was reported in the study of Shi et al. [34]. This was mainly due to the fact that a large number of organic components (lipids, proteins, carbohydrates, etc.) were released from sludge through the process of hydrolysis, and the high-temperature and high-pressure pre-treatment can destroy the structure of organic matter and improve its bioavailability. It is evident that the SCOD concentration was the lowest in all groups at day 35, which may be due to the faster microbial metabolism that promotes the consumption of organic matter at this stage [35]. The addition of biochar stimulated microbial community metabolism, resulting in faster release of organic matter from the soil and faster consumption of organic matter by microorganisms (days 7–35) in group S8 than in group S5. This observation was also confirmed by group S7 compared to S6. In addition, the SCOD concentration in S10 was higher than that in S9 on day 7 and lower on day 35, indicating that biochar enhanced the metabolic activity of the microbial community and enhanced the hydrolytic release of organic matter from the solid-phase environment as well as the bioavailability of dissolved organic matter. After the 35th day, the concentration of SCOD increased significantly in all groups, probably due to the reduced metabolic activity of some of the functional microorganisms and the slower rate of utilization of organic matter.

VFAs are metabolites produced by the hydrolytic fermentation process of anaerobic microorganisms and carbon sources necessary for some microbial metabolism [36]. The experimental results showed that formate and acetate were the main VFA components in this experimental system in the time series, while propionate and butyrate had lower concentrations (Figure 1b). Formate concentration was higher in S5 and S8, whereas acetate concentration was higher in the other groups supplemented with anaerobic sludge. A high acetate concentration in bioaugmentation experiments can be indicative of microbial activity in metabolizing PAHs, as reported in a previous study [37]. It is commonly assumed that higher concentrations of acetate favored PHE degradation by anaerobic microorganisms via the co-metabolic pathway. And this assumption was confirmed by Wang et al., who showed that sodium acetate as a co-metabolized carbon source enhanced the anaerobic biodegradation of PAHs in sediments [38]. The concentration of acetate was lower in S7 and S10 supplemented with both biochar and anaerobic sludge compared to S6 and S9 supplemented with only anaerobic sludge. This suggests that biochar stimulated the utilization of acetate by the anaerobic microbial community, which may enhance the biodegradation efficiency of PHE. From the change in tVFA concentration in each system, the tVFA concentration decreased after the addition of biochar compared with the

experimental groups without biochar (group S8 compared to S5, group S7 compared to S6, group S10 compared to S9). It indicates that biochar promoted the metabolic activity of the anaerobic microbial community, accelerated the consumption of VFAs, and improved the efficiency of anaerobic digestion [39]. At the same time, the experimental group to which anaerobic activated sludge was added had a lower concentration of tVFA by 35 days. The utilization of VFAs, as well as COD by anaerobic microorganisms, showed a consistent trendiness in terms of rate, corroborating the changes in metabolic activity of some functional microorganisms.

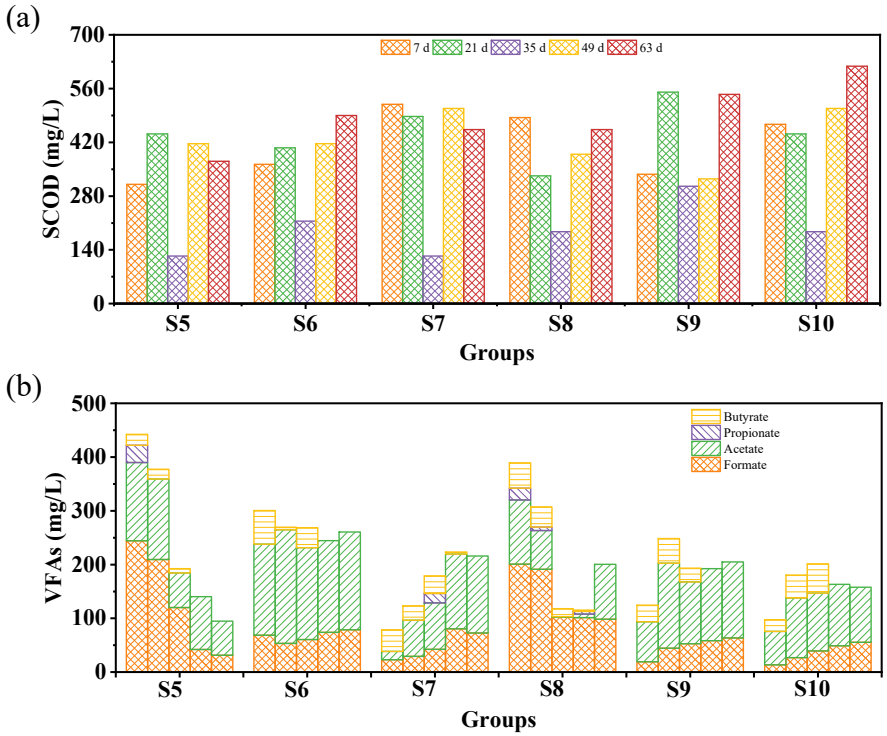

Figure 1. The variations in soluble organics, including SCOD (**a**) and VFAs (**b**), in experimental groups.

The anaerobic digestion process can be divided into four stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Among them, the acetogenesis stage is shown in Figure 2; the acetogenesis pathways mainly include homoacetogenesis (HOM), syntrophic butyrate oxidation (SBO), and syntrophic propionate oxidation (SPO) [40]. From Figure 2a, it can be seen that both bioaugmentation and biostimulation strategies favor HOM. The relative abundance of enzymes of the HOM in S9 with the addition of anaerobic activated sludge was 1.78 times higher than that in S5. The addition of biochar also stimulated the HOM with a 19.51% increase in the relative abundance of functional enzymes. Figure 2b shows the relative abundance of enzymes common to these three pathways, and their abundance variations follow a similar pattern to that of HOM. In the VFAs of each experimental system, the concentration of propionate was low, and the abundance of enzymes related to SPO did not differ much. In SBO, on the other hand, the addition of biochar did not cause changes in the abundance of functional enzymes, but rather, the introduction of anaerobic activated sludge weakened the syntrophic butyrate oxidation pathway.

3.2. The Removal, Residues, and Distribution of PHE in Flooded Soil

The removal, residues, and distribution of PHE in every group are shown in Figure 3. After 63 days of experiment, the concentration of PHE in the soil of S1 changed very little, and the removal rate was only 7.11%; the results of this experiment reflect the persistence as well as the difficult degradation of PAHs. In S3 and S4, where biochar was added, the concentration of PHE in the soil decreased significantly after the 7th day, and the concentration of PHE in the soil of S1 at the end of the experiment was 3.7 times higher than

that in S3, and the adsorption of biochar played a great influence on the distribution of PHE. In the control group, adsorption of biochar resulted in a decrease in PHE concentration in S3 and S4 soils from 48.79 ± 3.46 mg/kg soil (starting concentration) to 12.36 ± 2.58 mg/kg soil and 12.36 ± 2.58 mg/kg soil (after 63 days), respectively, whereas the final PHE concentrations in the S3 and S4 biochar were 0.32 ± 0.02 mg/g BC and 0.30 ± 0.02 mg/g BC, respectively. S1, S2, S3, and S4 were controls that were sterilized, and the removal of PHE was less than 10%, suggesting that human factors such as sampling operations had a low impact on the experiment.

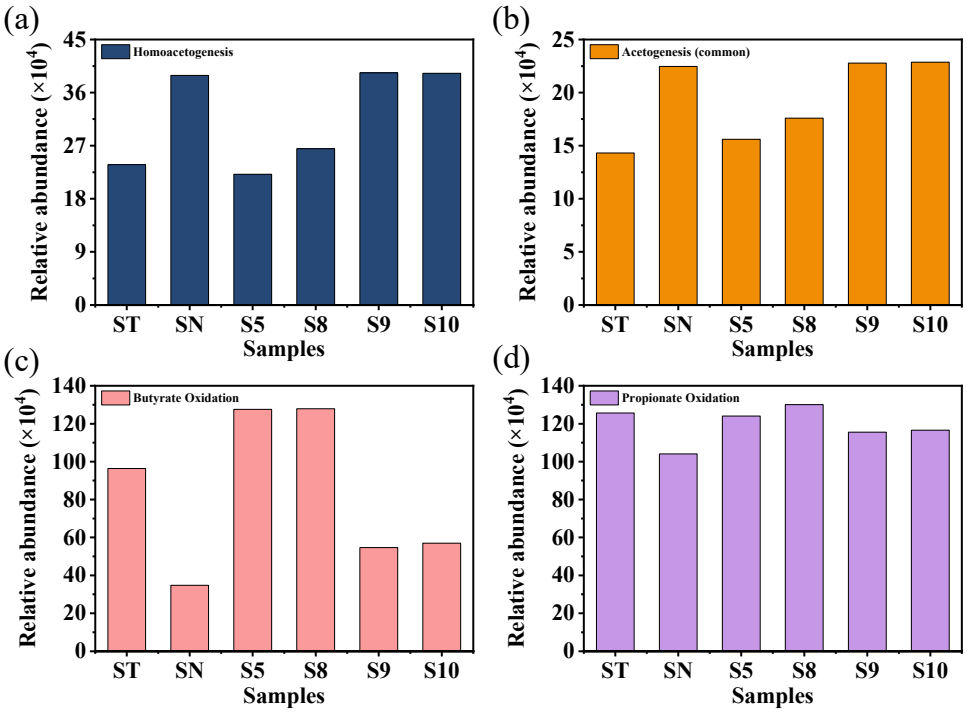

Figure 2. The relative abundances of modules involved in acetogenesis in anaerobic digestion (AD) system based on KEGG database. (Homoacetogenesis (HOM) (**a**), the relative abundance of enzymes common to these three pathways (**b**), synthonous butyric acid oxidation (SBO) (**c**), synthonous propionic acid oxidation (SPO) (**d**)) (Note: ST: original soil samples; SN: inoculated sludge samples; S5, S8, S9, and S10 are microbial samples taken from the corresponding groups at the end of the experiment).

In S5 without any enhancement measures, the final PHE concentrations in soil and water were 20.40 ± 1.54 mg/kg soil and 0.035 ± 0.006 mg/L, and the removal efficiency of PHE by indigenous microorganisms in soil was 55.7%. Sterilized sludge can be used as an additional carbon source to stimulate the metabolism of the microbial community, and the removal efficiency of PHE was elevated to 62.1% in S6. Bianco et al. also reported that anaerobic digestion products as an organic amendment could effectively stimulate the anaerobic biodegradation of PAHs in marine sediments in a methanogenic environment [41]. Granular biochar was added in S8. As shown in Figure S1, the C content of the biochar used in the experiment reached 84.86%. Granular biochar presents a remarkable porous structure, which facilitates microbial attachment. Organic functional groups on the surface of carbon materials can act as an ability to transfer electrons. FTIR analysis showed (Figure S1b) that phenol-OH (3415 cm^{-1}) as well as ketone group C=O (1590 cm^{-1}) on the surface of biochar can give redox activity to the biochar by means of electron conversion [42]. In addition, lower O:C (0.14) and H:C (0.014) ratios can indicate that biochar is highly aromatic and chemically stable [43]. The removal efficiency of PHE in S8 supplemented with biochar only (57.3%) was slightly higher than that of S5, suggesting that the addition of a single biochar did not have a significant enhancing effect on the biodegradation of PHE in soil. Previous

studies have shown [44] that the adsorption and immobilization of biochar reduces the bioavailability of PAHs and is detrimental to the removal of PAHs from soil. It has also been noted [45,46] that low molecular weight organic acids can promote the desorption of PAHs from biochar and that biochar addition can enrich PAHs-degrading bacteria as well as enhance the cooperation between functional flora, which is beneficial for the anaerobic biodegradation of PAHs. Therefore, the removal efficiency of PHE increased to 66.3% after biochar and sterilized sludge addition in S7. The addition of anaerobic sludge not only provided an exogenous carbon source but also introduced exogenous anaerobic microbial communities, and the removal efficiency of PHE in S9 increased significantly to more than 70%. Similar to the findings of Sigmund et al., the addition of compost products introduced exogenous microbial strains to the indigenous microbial community of the soil and accelerated the degradation of PAHs in the soil [47]. The highest PHE removal efficiency (81.0%) was achieved in S10, indicating that biochar enhanced the metabolism of PHE by the anaerobic microbial community consisting of anaerobic activated sludge and soil. The results in Figure 3d also showed that the final PHE concentration in the biochar of S10 (0.021 ± 0.005 mg/g BC) was significantly lower than that of S8 (0.067 ± 0.005 mg/g BC) and S7 (0.033 ± 0.009 mg/g BC).

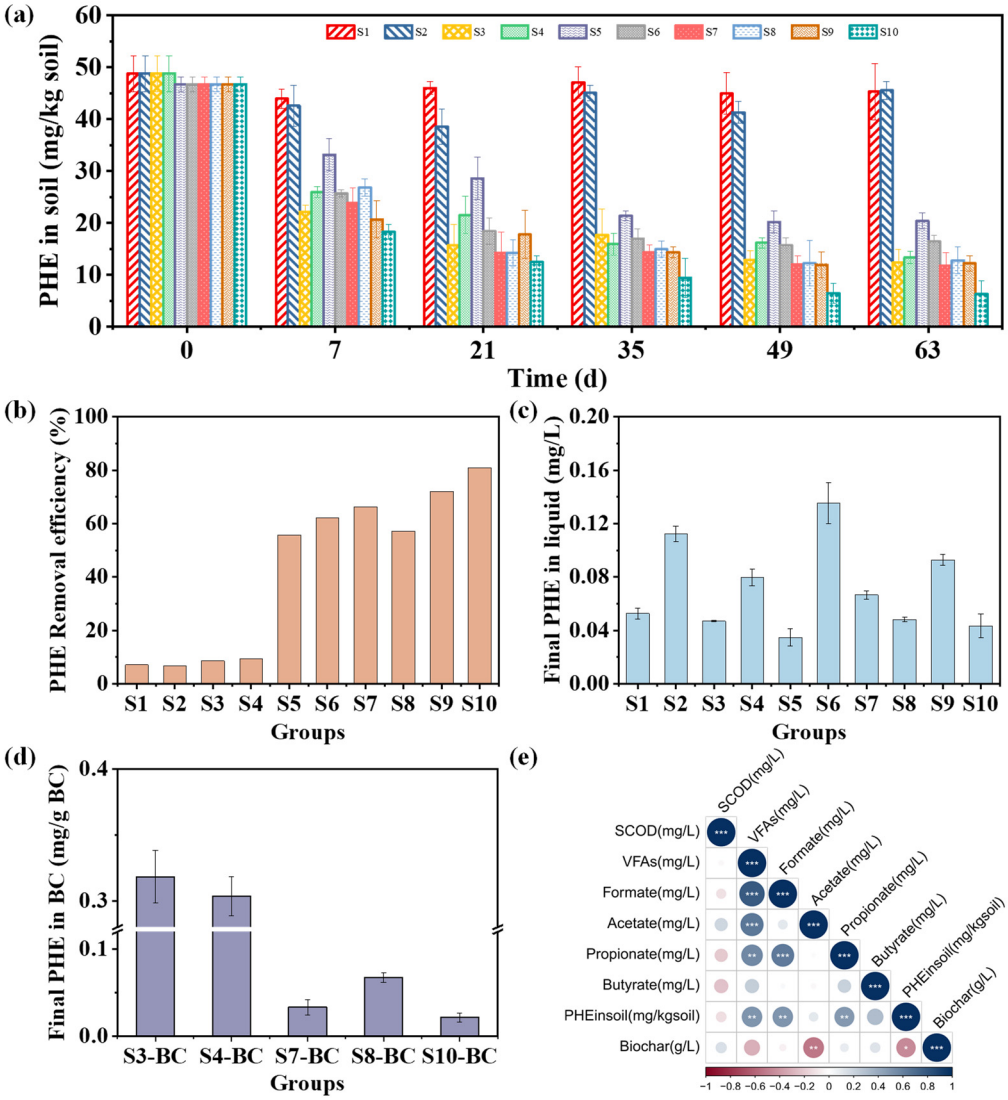

Figure 3. The changes in PHE concentration in soil (**a**) and the removal of PHE (**b**), the PHE concentrations in liquids and biochar (**c**,**d**), and the heatmap of correlations in performance indicators (**e**).

As shown in Figure 3c, comparing S1 with S2, S3 with S4, and S5 with S6, respectively, it can be seen that the addition of sterilized sludge promotes the distribution of PHE in the water while decreasing the amount of residue in the soil [48], and we speculated that the large amount of organic matter (such as polysaccharide) contained in sterilized sludge contributed to the solubility of PHE in water. In Figure 3d, the amount of PHE adsorbed on the biochar of S3 (only sterilized soil in the system) was the highest among all the groups to which biochar was added. The presence of native microorganisms in the soil reduced the amount of FHE adsorbed on the biochar in S7 compared to S4, and the anaerobic functional microorganisms enriched on the biochar utilized the PHE more completely. The negative correlation between biochar and the concentration of PHE in soil can be seen in Figure 3e, which highlights the enhanced effect of biochar on the removal of pollutants in soil [49]. Meanwhile, the concentration of PHE in soil showed a correlation with VFAs, especially formate and propionate in VFAs, which showed a significant positive correlation. We speculate that formate and propionate favor the distribution of PHE in the system to the soil, which echoes the previously mentioned low molecular weight organic acids that promote the desorption of PAHs from biochar.

3.3. Microbial Community Analysis

3.3.1. Evaluation of Microbial Diversity

The microbial community α-diversity indexes (Ace, Chao1, Shannon, Simpson) were calculated based on the 16S rRNA high-throughput sequencing; the results are presented in Table 2. Ace and Chao1 were used to estimate the index of the number of OUTs in the microbial community, and the larger value of the two indicated the total number of strains in the community. Shannon and Simpson were used to estimate the diversity of microorganisms in the samples, and the values of both can reflect the diversity of the microbial community. As shown in Table 2, the number of strains in the inoculated sludge (SN) was more than in the soil (ST), but the diversity of the microbial community was lower. At the end of the experiment, compared with ST, the values of Ace and Chao1 in the soil of S5 were elevated, while the values of Shannon and Simpson were almost unchanged, indicating that the indigenous microorganisms in the soil could adapt to the experimental environment for amplification and reproduction. The addition of biochar in S8 resulted in a significant decrease in all four indicators of α-diversity, suggesting that the biochar in this experiment has biostimulatory properties and may support the growth of some dominant functional bacterial species in the soil [21]. In S9, inoculated sludge and soil together form an anaerobic microbial community. All four indicators of α-diversity were significantly higher in S9 with the addition of anaerobic sludge compared to SN. This indicates that the introduction of anaerobic sludge both increased the diversity of the community and promoted the growth of microbial strains. There was no increase in community diversity in S9 compared to ST and S5, but the number of microbial strains increased significantly, suggesting that the growth of certain functional strains was favored. After the introduction of biochar, the diversity of the microbial community in S10 was slightly elevated compared with S9, while the values of Ace and Chao1 were reduced, probably because some dominant microbial strains were enriched.

Table 2. The changes in α-diversity of microbial community.

Sample	Ace	Chao1	Shannon	Simpson	Goods Coverage
ST	322.071	330.882	5.867	0.961	0.999
SN	391.730	387.786	5.657	0.911	0.999
S5	335.706	352.750	5.865	0.967	0.999
S8	325.510	330.000	4.725	0.849	0.999
S9	476.796	492.500	5.812	0.946	0.999
S10	476.128	472.692	5.893	0.949	0.999

Note: ST: original soil samples; SN: inoculated sludge samples; S5, S8, S9, and S10 are microbial samples taken from the corresponding groups at the end of the experiment.

3.3.2. The Variations in Microbial Community Structure

Figure 4 shows the structure of microbial communities at the phylum level (a–c) and at the genus level (d–e). The dominant phyla (top 3) were different in ST and SN samples: In ST, the dominant phyla were Firmicutes (53.4%), Proteobacteria (16.8%), and Acidobacteriota (14.5%). Some studies have reported the prevalence of Firmicutes, Proteobacteria, and Acidobacteriota in untreated soil samples [50]. In SN, the dominant phyla were Thermotogota (28.8%), Chloroflexi (19.1%), and Desulfobacterota (7.8%). The first dominant phylum in the S5 evolved to be Proteobacteria (45.0%), indicating that Proteobacteria played a dominant role in the anaerobic biodegradation of PHE in soil. Previous studies have demonstrated that strains of Proteobacteria are resistant to toxic PAHs, are enriched by PAHs in soil environments, and that the majority of PAH-degrading bacteria are attributed to Proteobacteria [51]. Biochar addition increased the relative abundance of Firmicutes (55.3%) and Proteobacteria (24.8%) in S8 soil compared to ST. Many articles have also reported that functional genes for PAH degradation are also present in strains of Firmicutes and that Firmicutes are better adapted to harsh environmental conditions, such as high temperatures, than Proteobacteria. And that PAH-degrading bacteria are mainly composed of Firmicutes and Proteobacteria under anoxic conditions [52,53]. This can also indicate that biochar stimulated the growth of dominant functional bacteria species with PAHs degradation function. In S9, the relative abundances of the top 3 dominant phyla were similar: Chloroflexi (18.9%), Thermotogota (18.3%), and Proteobacteria (16.84%). The introduction of an exogenous anaerobic microbial community changed the microbial community structure of PHE-contaminated soil, and Chloroflexi and Thermotogota had the function of hydrolytic fermentation, which promoted the carbon cycle metabolism among microbial communities. Lin et al. also found [31] that Chloroflexi was enriched in anaerobic digestion reactors with additional electrodes for the treatment of PHE-containing contaminated sludge. The addition of biochar in S10 did not result in very significant changes in microbial community structure (compared to S9), and of interest was the increase in the relative abundance of Bacteroidota and Euryarchaeota, which may have facilitated the process of DIET between microbial communities [35].

The changes in relative abundance of the major genera (abundance > 1%) were further analyzed at the genus level. As can be seen from Figure 4d–e, the major genera in SN were completely different from those in ST. SN was introduced as an exogenous microbial community into the PHE-contaminated soil environment, and the community structure in S9 was very different from that in S5, and the dominant genera were almost all derived from SN, which indicated that the anaerobic microbial community in SN could be adapted to the simulated inundated soil environment. Among them, the dominant genera (top 5), including Mesotoga, f_Rhodocyclaceae, SBR1031, Zixibacteria, and f_Anaerolineaceae, favored the process of hydrolytic fermentation of organic carbon sources with PHE biodegradation. In addition, methanogens (Methanolinea, Methanosaeta) were introduced by SN in S9 with a relative abundance of bacteria greater than 1%, expanding the metabolic pathway of organic carbon sources. CO_2, as an electron acceptor in methanogenic environments, can promote PHE degradation [41]. The addition of biochar did not alter the species of the major genera in the anaerobic microbial community composed of sludge and soil, but the abundance of the bacterial genera *Bacteroides*, *f_Geobacteraceae*, and the methanogenic genera Methanolinea and Methanosaeta were elevated compared to S9. Analysis of the results of this study revealed that Bacteroides and Methanolinea enrichment favors PHE biodegradation. Moreover, Bacteroides and f_Geobacteraceae can release intracellular electrons to form reciprocal metabolism with Methanosaeta to enhance the methanogenesis process through the DIET pathway. Therefore, biochar in S10 may enhance the degradation of PHE by anaerobic microbial communities by promoting the DIET process.

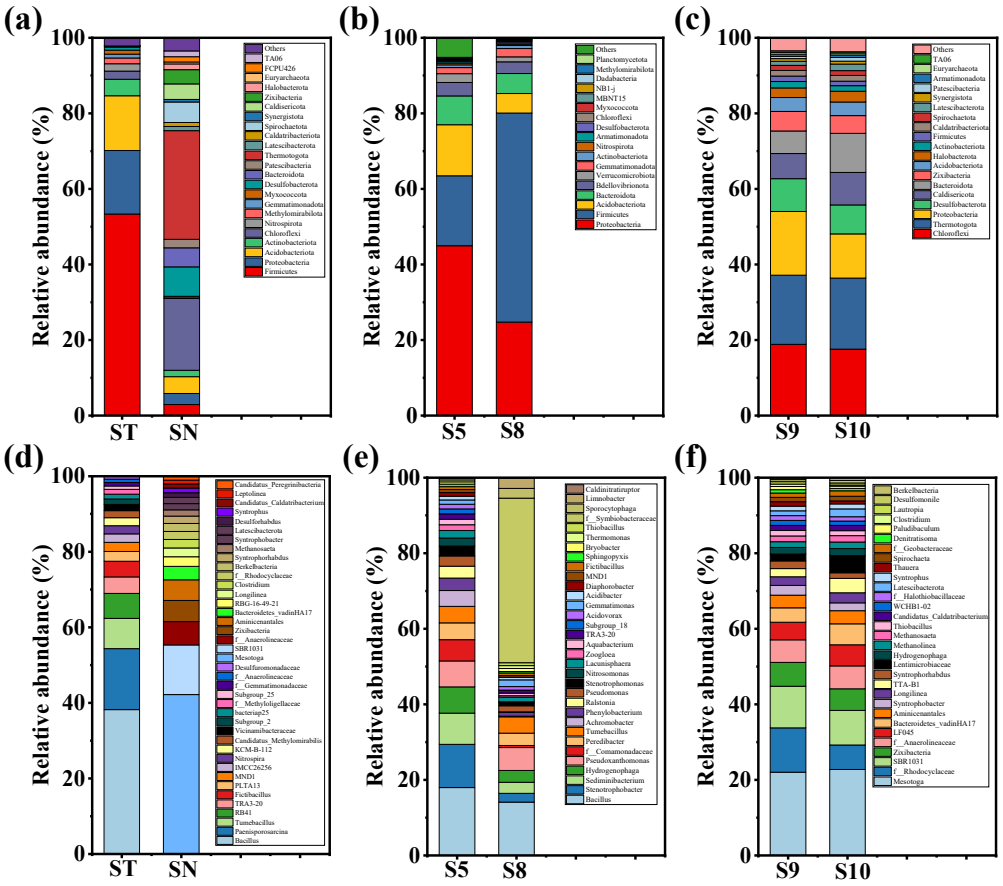

Figure 4. The effects of biochar and anaerobic sludge on microbial community in batch experiments. (Microbial community structure at the phylum level of ST and SN (**a**), microbial community structure at the phylum level of S5 and S8 (**b**), microbial community structure at the phylum level of S9 and S10 (**c**). Microbial community structure at the genus level of ST and SN (**d**), microbial community structure at the genus level of S5 and S8 (**e**), microbial community structure at the genus level of S9 and S10 (**f**)).

3.3.3. Predicted Potential Functions of Microbial Community

In order to further investigate the effects of adding biochar and anaerobic activated sludge on soil microbial metabolic pathways, the potential functions of microbial communities were predicted and analyzed in conjunction with the KEGG database, and the results are shown in Figure 5. Six functional subgroups were included at level 1, which in order of abundance were metabolism (69.19–73.55%), environmental information processing (10.93–14.40%), cellular processes (6.71–8.28%), genetic information processing (3.18–5.09%), human diseases (2.33–3.51%), and organismal systems (0.69–1.56%). At level 1, metabolism was the highest abundance in all samples, and at level 2, it can be seen that metabolism was refined into 11 pathways, with carbohydrate metabolism, amino acid metabolism, and energy metabolism being the main three pathways. In Figure 5b, it can be seen that the addition or absence of biochar had little effect on the metabolism pathway; rather, the addition of anaerobic sludge played an important role. The carbohydrate metabolism abundance in the experimental group with the addition of anaerobic sludge was relatively high, increasing from 8.60% in S5 to 9.82% in S9 and from 8.71% in S8 to 9.82% in S10. Meanwhile, the addition of anaerobic sludge also increased the abundance of energy metabolism, suggesting that the introduction of the anaerobic sludge microbial community stimulated the metabolism of the carbon source in the system and also facilitated the energy conversion and transfer capacity [54]. However, the abundance of genes in the functions of amino acid metabolism, lipid metabolism, and xenobiotics biodegradation and metabolism showed a trend of decreasing in the experimental group with biostimulation. The cellular

processes pathway showed that cellular community function was enhanced, but cell motility function was weakened after the addition of anaerobic sludge. At level 3, it can be seen that anaerobic sludge addition mainly enhances glycolysis/gluconeogenesis in carbohydrate metabolism, amino sugar and nucleotide sugar metabolism, starch, and sucrose. The anaerobic sludge itself can also provide an organic carbon source with a high SCOD detected (Figure 1a). Moreover, it could also stimulate the expression of corresponding genes and enhance the metabolism of the microbial community, making the PHE-contaminated system more stable and further promoting the degradation and utilization of pollutants (Figure 5c).

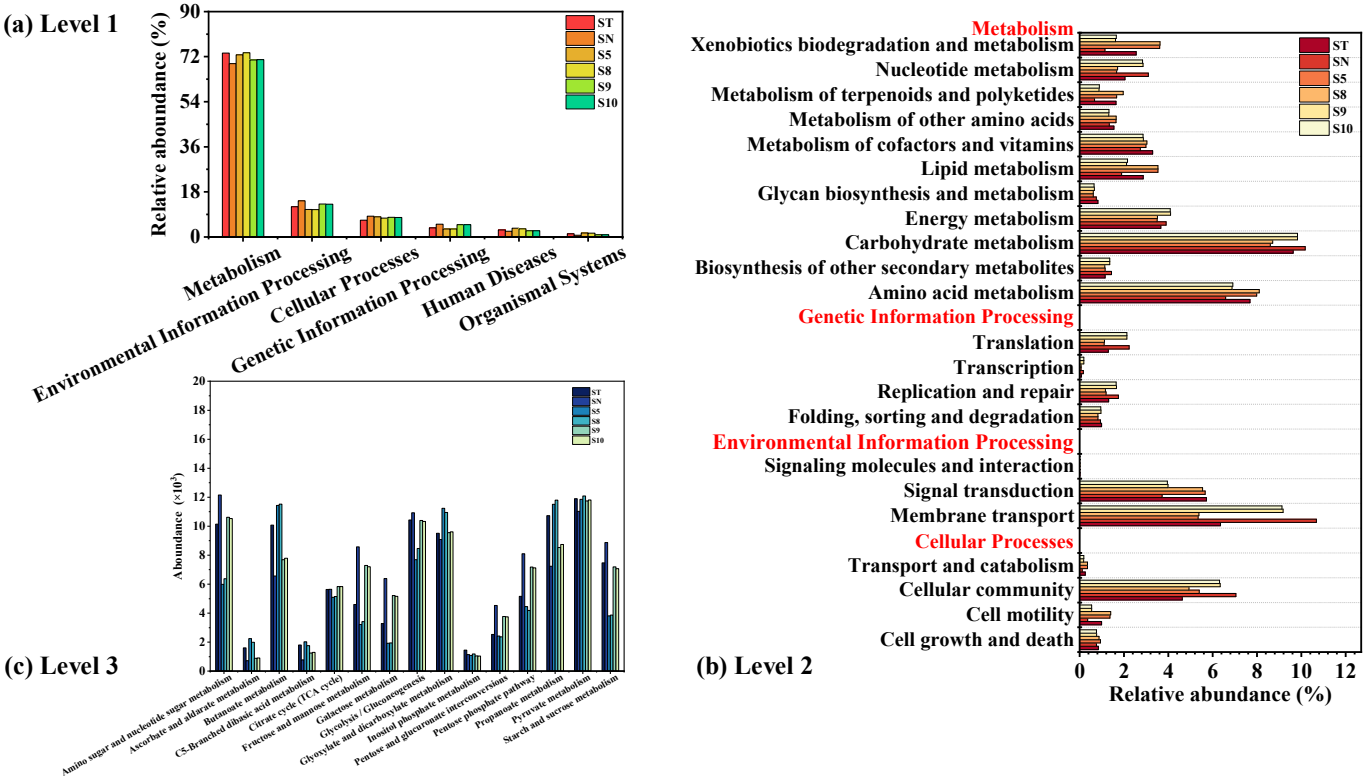

Figure 5. Changes in potential functions of microbial community during AD: the RA of metabolic pathways on KEGG categories at level 1 (**a**); the RA of metabolic pathways on KEGG categories at level 2 (**b**); the RA of KO metabolic pathways in carbohydrate metabolism on KEGG categories at level 3 (**c**).

3.4. The Removal, Residues, and Distribution of PHE in Flooded Soil

Bioremediation of PAHs in contaminated soils still faces numerous bottlenecks, and the remediation efficiency is affected by a variety of factors, so there is an urgent need to explore methods to enhance the biodegradation of PAHs. For example, the ability to degrade PAHs is strengthened by adding functional microorganisms enriched by domesticated bacterial colonies, or the biodegradation of PAHs is stimulated by adding functional materials (carbon materials). This study combined bioaugmentation and biostimulation to explore the distribution of PHE accumulation and the regulation of biodegradation in anaerobic environments. The addition of enriched and domesticated anaerobic sludge enhanced the removal of PHE in soil from 55.7% to 72.0%. The removal of PHE was not significantly enhanced when biochar was singly dosed. However, when anaerobic sludge and biochar were jointly dosed, the highest degradation efficiency of PHE in soil was 81.0%. Through a systematic experimental design, the study of key factors that can promote the anaerobic biodegradation of PHE is of theoretical and practical significance for the remediation of PHE pollution in polluted sites.

4. Conclusions

In this study, batch experiments were used to investigate the effect of combined bioaugmentation and biostimulation on anaerobic biodegradation of PHE in contaminated soil. Enriched and domesticated anaerobic sludge was used as a means of bioaugmentation for inoculation into PHE-contaminated flooded soil, and the removal efficiency of PHE from the soil was increased by 16.3%. Although the addition of a single biochar did not have a significant promoting effect on the removal of PHE, when the biochar and anaerobic sludge were jointly added, the biochar played an enhancing effect on the anaerobic microbial community co-constituted by the sludge and the soil, and the PHE removal effect reached 81.0%. In addition, the dominant microbial communities associated with PHE degradation evolved into Chloroflexi and Proteobacteria after the addition of anaerobic sludge, changing the microbial community structure. The addition of biochar not only increased the diversity of anaerobic microorganisms but also promoted the DIET process between electroactive bacteria (*Bacteroides*, *f_Geobacteraceae*) and Methanosaeta, which accelerated the degradation of PHE by the microbial community. This study provides ideas and references for enhancing the bioremediation of PAH-contaminated soils. Further work should focus on direct evidence of functional changes in microbial communities after biostimulation and bioaugmentation, as well as synergistic cooperation between different functional microorganisms.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/su16010366/s1, Table S1: Physicochemical characteristics of biochar. Table S2: Physicochemical characteristics of soil used in this work. Figure S1: Physiochemical properties of biochar: scanning electron microscope (SEM) images (a), the organic functional groups on the surface of biochar (b), elemental analysis (c).

Author Contributions: Conceptualization, H.X. and Y.S.; methodology, H.X.; software, R.L.; validation, R.L., J.Q., and X.L.; formal analysis, H.X.; investigation, Y.S.; resources, H.X.; data curation, Y.S.; writing—original draft preparation, H.X.; writing—review and editing, H.X.; visualization, J.Q. and X.L.; supervision, Y.S.; project administration, R.L.; funding acquisition, R.L. All authors have read and agreed to the published version of the manuscript.

Funding: The authors' research was funded by the National High Technology Research and Development Program of China (2022YFC3700187), the National Natural Science Foundation of China (U1806216 and 51608304), and the Young Scholars Program of Shandong University (2018WLJH53).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

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

References

1. Kariyawasam, T.; Doran, G.; Howitt, J.; Prenzler, P. Polycyclic aromatic hydrocarbon contamination in soils and sediments: Sustainable approaches for extraction and remediation. *Chemosphere* **2022**, *291*, 132981. [CrossRef] [PubMed]
2. Ismail, N.; Kasmuri, N.; Hamzah, N. Microbial Bioremediation Techniques for Polycyclic Aromatic Hydrocarbon (PAHs)-a Review. *Water Air Soil Pollut.* **2022**, *233*, 124–151. [CrossRef]
3. Zhu, Y.; Xu, Y.; Xu, J.; Meidl, P.; He, Y. Contrasting response strategies of microbial functional traits to polycyclic aromatic hydrocarbons contamination under aerobic and anaerobic conditions. *J. Hazard. Mater.* **2023**, *454*, 131548. [CrossRef] [PubMed]
4. Wang, Y.; Zhang, Q.; Zhang, Y.; Zhao, H.; Tan, F.; Wu, X.; Chen, J. Source apportionment of polycyclic aromatic hydrocarbons (PAHs) in the air of Dalian, China: Correlations with six criteria air pollutants and meteorological conditions. *Chemosphere* **2019**, *216*, 516–523. [CrossRef] [PubMed]
5. Hu, G.; Li, J.; Zeng, G. Recent development in the treatment of oily sludge from petroleum industry: A review. *J. Hazard. Mater.* **2013**, *261*, 470–490. [CrossRef] [PubMed]
6. Zhang, J.; Liu, F.; Huang, H.; Wang, R.; Xu, B. Occurrence, risk and influencing factors of polycyclic aromatic hydrocarbons in surface soils from a large-scale coal mine, Huainan, China. *Ecotoxicol. Environ. Saf.* **2020**, *192*, 110269. [CrossRef]
7. Kuśmierz, M.; Oleszczuk, P.; Kraska, P.; Pałys, E.; Andruszczak, S. Persistence of polycyclic aromatic hydrocarbons (PAHs) in biochar-amended soil. *Chemosphere* **2016**, *146*, 272–279. [CrossRef]

8. Yang, L.; Han, D.; Jin, D.; Zhang, J.; Shan, Y.; Wan, M.; Hu, Y.; Jiao, W. Soil physiochemical properties and bacterial community changes under long-term polycyclic aromatic hydrocarbon stress in situ steel plant soils. *Chemosphere* **2023**, *334*, 138926. [CrossRef]
9. Zhao, L.; Lyu, C.; Li, Y. Analysis of factors influencing plant–microbe combined remediation of soil contaminated by polycyclic aromatic hydrocarbons. *Sustainability* **2021**, *13*, 10695. [CrossRef]
10. Barth, J.; Steidle, D.; Kuntz, D.; Gocht, T.; Mouvet, C.; Tümpling, W.; Lobe, I.; Langenhoff, A.; Albrechtsen, H.; Janniche, G.; et al. Deposition, persistence and turnover of pollutants: First results from the EU project AquaTerra for selected river basins and aquifers. *Sci. Total Environ.* **2007**, *376*, 40–50. [CrossRef]
11. He, M.; Shangguan, Y.; Zhou, Z.; Guo, S.; Yu, H.; Chen, K.; Zeng, X.; Qin, Y. Status assessment and probabilistic health risk modeling of polycyclic aromatic hydrocarbons (PAHs) in surface soil across China. *Front. Environ. Sci.* **2023**, *11*, 107–116. [CrossRef]
12. Fu, X.; Li, T.; Ji, L.; Wang, L.; Zheng, L.; Wang, J.; Zhang, Q. Occurrence, sources and health risk of polycyclic aromatic hydrocarbons in soils around oil wells in the border regions between oil fields and suburbs. *Ecotoxicol. Environ. Saf.* **2018**, *157*, 276–284. [CrossRef] [PubMed]
13. Zhang, J.; Yang, J.; Wang, R.; Hou, H.; Du, X.; Fan, S.; Liu, J.; Dai, J. Effects of pollution sources and soil properties on distribution of polycyclic aromatic hydrocarbons and risk assessment. *Sci. Total Environ.* **2013**, *463–464*, 1–10. [CrossRef] [PubMed]
14. Mallah, M.; Changxing, L.; Mallah, M.; Noreen, S.; Liu, Y.; Saeed, M.; Xi, H.; Ahmed, B.; Feng, F.; Mirjat, A.; et al. Polycyclic aromatic hydrocarbon and its effects on human health: An overeview. *Chemosphere* **2022**, *296*, 133948. [CrossRef]
15. Imam, A.; Suman, S.; Kanaujia, P.; Ray, A. Biological machinery for polycyclic aromatic hydrocarbons degradation: A review. *Bioresour. Technol.* **2022**, *343*, 126121. [CrossRef]
16. Zhou, N.; Guo, H.; Liu, Q.; Zhang, Z.; Sun, J.; Wang, H. Bioaugmentation of polycyclic aromatic hydrocarbon (PAH)-contaminated soil with the nitrate-reducing bacterium PheN7 under anaerobic condition. *J. Hazard. Mater.* **2022**, *439*, 129643. [CrossRef]
17. Li, X.; Lin, X.; Li, P.; Liu, W.; Wang, L.; Ma, F.; Chukwuka, K. Biodegradation of the low concentration of polycyclic aromatic hydrocarbons in soil by microbial consortium during incubation. *J. Hazard. Mater.* **2009**, *172*, 601–605. [CrossRef]
18. Kong, L.; Gao, Y.; Zhou, Q.; Zhao, X.; Sun, Z. Biochar accelerates PAHs biodegradation in petroleum-polluted soil by biostimulation strategy. *J. Hazard. Mater.* **2018**, *343*, 276–284. [CrossRef]
19. Cha, J.; Park, S.; Jung, S.; Ryu, C.; Jeon, J.; Shin, M.; Park, Y. Production and utilization of biochar: A review. *J. Ind. Eng. Chem.* **2016**, *40*, 1–15. [CrossRef]
20. El-Naggar, A.; El-Naggar, A.; Shaheen, S.; Sarkar, B.; Chang, S.; Tsang, D.; Rinklebe, J.; Ok, Y. Biochar composition-dependent impacts on soil nutrient release, carbon mineralization, and potential environmental risk: A review. *J. Environ. Manag.* **2019**, *241*, 458–467. [CrossRef]
21. Hao, Z.; Wang, Q.; Yan, Z.; Jiang, H. Novel magnetic loofah sponge biochar enhancing microbial responses for the remediation of polycyclic aromatic hydrocarbons-contaminated sediment. *J. Hazard. Mater.* **2021**, *401*, 123859. [CrossRef] [PubMed]
22. Gao, F.; Ahmad, S.; Tang, J.; Zhang, C.; Li, S.; Yu, C.; Liu, Q.; Sun, H. Enhanced nitrobenzene removal in soil by biochar supported sulfidated nano zerovalent iron: Solubilization effect and mechanism. *Sci. Total Environ.* **2022**, *826*, 153960. [CrossRef] [PubMed]
23. Aborisade, M.; Geng, H.; Oba, B.; Kumar, A.; Ndudi, E.; Battamo, E.; Liu, J.; Chen, D.; Okimiji, O.; Ojekunle, O.; et al. Remediation of soil polluted with Pb and Cd and alleviation of oxidative stress in *Brassica rapa* plant using nanoscale zerovalent iron supported with coconut-husk biochar. *J. Plant Physiol.* **2023**, *287*, 154023. [CrossRef] [PubMed]
24. Narayanan, M.; Ma, Y. Influences of biochar on bioremediation/phytoremediation potential of metal-contaminated soils. *Front. Microbiol.* **2022**, *13*, 929730. [CrossRef] [PubMed]
25. Yao, Y.; Wei, Y.; Li, J.; Han, R.; Jing, C.; Liu, R.; Niu, Q. Microbial electron flow promotes naphthalene degradation in anaerobic digestion in the presence of nitrate electron acceptor: Focus on electron flow regulation and microbial interaction succession. *J. Hazard. Mater.* **2023**, *459*, 132293. [CrossRef]
26. Chinese State Environmental Protection Administration. *The Water and Wastewater Monitoring Analysis Method Editorial Board. Water and Wastewater Monitoring Analysis Method*; China Environmental Science Press: Beijing, China, 2002.
27. Hallaji, S.; Kuroshkarim, M.; Moussavi, S. Enhancing methane production using anaerobic co-digestion of waste activated sludge with combined fruit waste and cheese whey. *BMC Biotechnol.* **2019**, *19*, 19. [CrossRef]
28. Khitous, M.; Saber, M.; Tirichine, N.; Aiouaz, F. Enhancing mesophilic anaerobic co-digestion of slaughterhouse waste with household waste under a pilot-scale reactor. *J. Mater. Cycles Waste Manag.* **2022**, *24*, 1786–1796. [CrossRef]
29. Shi, Y.; Xue, H.; Li, J.; Yao, Y.; Liu, R.; Niu, Q. Response of methanogenic system to long-term polycyclic aromatic hydrocarbon exposure: Adsorption and biodegradation, performance variation, and microbial function assessment. *J. Environ. Manag.* **2023**, *329*, 117010. [CrossRef]
30. Aemig, Q.; Chéron, C.; Delgenès, N.; Jimenez, J.; Houot, S.; Steyer, J.; Patureau, D. Distribution of Polycyclic Aromatic Hydrocarbons (PAHs) in sludge organic matter pools as a driving force of their fate during anaerobic digestion. *Waste Manag.* **2016**, *48*, 389–396. [CrossRef]
31. Lin, C.; Wu, P.; Liu, Y.; Wong, J.; Yong, X.; Wu, X.; Xie, X.; Jia, H.; Zhou, J. Enhanced biogas production and biodegradation of phenanthrene in wastewater sludge treated anaerobic digestion reactors fitted with a bioelectrode system. *Chem. Eng. J.* **2019**, *365*, 1–9. [CrossRef]
32. Wemheuer, F.; Taylor, J.; Daniel, R.; Johnston, E.; Meinicke, P.; Thomas, T.; Wemheuer, B. Tax4Fun2: Prediction of habitat-specific functional profiles and functional redundancy based on 16S rRNA gene sequences. *Environ. Microbiome* **2020**, *15*, 11. [CrossRef] [PubMed]

33. Wang, X.; Jin, P.; Zhang, A.; Gao, J.; Zhang, B.; Hou, Y. Effect of mechanical elutriation on carbon source recovery from primary sludge in a novel activated primary tank. *Chemosphere* **2020**, *240*, 124820. [CrossRef] [PubMed]
34. Shi, Z.; Usman, M.; He, J.; Chen, H.; Zhang, S.; Luo, G. Combined microbial transcript and metabolic analysis reveals the different roles of hydrochar and biochar in promoting anaerobic digestion of waste activated sludge. *Water Res.* **2021**, *205*, 117679. [CrossRef] [PubMed]
35. Shi, Y.; Liu, M.; Li, J.; Yao, Y.; Tang, J.; Niu, Q. The dosage-effect of biochar on anaerobic digestion under the suppression of oily sludge: Performance variation, microbial community succession and potential detoxification mechanisms. *J. Hazard. Mater.* **2022**, *421*, 126819. [CrossRef] [PubMed]
36. Chen, Y.; Ping, Q.; Li, D.; Dai, X.; Li, Y. Comprehensive insights into the impact of pretreatment on anaerobic digestion of waste active sludge from perspectives of organic matter composition, thermodynamics, and multi-omics. *Water Res.* **2022**, *226*, 119240. [CrossRef] [PubMed]
37. Ferraro, A.; Massini, G.; Miritana, V.; Panico, A.; Pontoni, L.; Race, M.; Rosa, S.; Signorini, A.; Fabbricino, M.; Pirozzi, F. Bioaugmentation strategy to enhance polycyclic aromatic hydrocarbons anaerobic biodegradation in contaminated soils. *Chemosphere* **2021**, *275*, 130091. [CrossRef] [PubMed]
38. Wang, F.; Dong, W.; Wang, H.; Zhao, Y.; Zhao, Z.; Huang, J.; Zhou, T.; Wu, Z.; Li, W. Enhanced bioremediation of sediment contaminated with polycyclic aromatic hydrocarbons by combined stimulation with sodium acetate/phthalic acid. *Chemosphere* **2022**, *291*, 132770. [CrossRef]
39. Johnravindar, D.; Wong, J.; Chakraborty, D.; Bodedla, G.; Kaur, G. Food waste and sewage sludge co-digestion amended with different biochars: VFA kinetics, methane yield and digestate quality assessment. *J. Environ. Manag.* **2021**, *290*, 112457. [CrossRef]
40. Schmidt, O.; Hink, L.; Horn, M.; Drake, H. Peat: Home to novel syntrophic species that feed acetate-and hydrogen-scavenging methanogens. *ISME J.* **2016**, *10*, 1954–1966. [CrossRef]
41. Bianco, F.; Race, M.; Papirio, S.; Esposito, G. Removal of polycyclic aromatic hydrocarbons during anaerobic biostimulation of marine sediments. *Sci. Total Environ.* **2020**, *709*, 136141. [CrossRef]
42. Li, Q.; Gao, X.; Liu, Y.; Wang, G.; Li, Y.; Sano, D.; Wang, X.; Chen, R. Biochar and GAC intensify anaerobic phenol degradation via distinctive adsorption and conductive properties. *J. Hazard. Mater.* **2020**, *405*, 124183. [CrossRef] [PubMed]
43. Zhou, H.; Brown, R.; Wen, Z. Biochar as an Additive in Anaerobic Digestion of Municipal Sludge: Biochar Properties and Their Effects on the Digestion Performance. *ACS Sustain. Chem. Eng.* **2020**, *8*, 6391–6401. [CrossRef]
44. Cao, Y.; Yang, B.; Song, Z.; Wang, H.; He, F.; Han, X. Wheat straw biochar amendments on the removal of polycyclic aromatic hydrocarbons (PAHs) in contaminated soil. *Ecotoxicol. Environ. Saf.* **2016**, *130*, 248–255. [CrossRef] [PubMed]
45. Li, X.; Song, Y.; Bian, Y.; Gu, C.; Yang, X.; Wang, F.; Jiang, X. Insights into the mechanisms underlying efficient Rhizodegradation of PAHs in biochar-amended soil: From microbial communities to soil metabolomics. *Environ. Int.* **2020**, *144*, 105995. [CrossRef] [PubMed]
46. Ni, N.; Wang, F.; Song, Y.; Bian, Y.; Shi, R.; Yang, X.; Gu, C.; Jiang, X. Mechanisms of biochar reducing the bioaccumulation of PAHs in rice from soil: Degradation stimulation vs immobilization. *Chemosphere* **2018**, *196*, 288–296. [CrossRef] [PubMed]
47. Sigmund, G.; Poyntner, C.; Pinar, G.; Kah, M.; Hofmann, T. Influence of compost and biochar on microbial communities and the sorption/degradation of PAHs and NSO-substituted PAHs in contaminated soils. *J. Hazard. Mater.* **2018**, *345*, 107–113. [CrossRef] [PubMed]
48. Fernández-Luqueño, F.; Marsch, R.; Espinosa-Victoria, D.; Thalasso, F.; Hidalgo-Lara, M.; Munive, A.; Luna-Guido, M.; Dendooven, L. Remediation of PAHs in a saline–alkaline soil amended with wastewater sludge and the effect on dynamics of C and N. *Sci. Total Environ.* **2008**, *402*, 18–28. [CrossRef]
49. Stefaniuk, M.; Tsang, D.; Ok, Y.; Oleszczuk, P. A field study of bioavailable polycyclic aromatic hydrocarbons (PAHs) in sewage sludge and biochar amended soils. *J. Hazard. Mater.* **2018**, *349*, 27–34. [CrossRef]
50. Aborisade, M.; Oba, B.; Kumar, A.; Liu, J.; Chen, D.; Okimiji, O.; Zhao, L. Remediation of metal toxicity and alleviation of toxic metals-induced oxidative stress in *Brassica chinensis* L. using biochar-iron nanocomposites. *Plant Soil.* **2023**, *493*, 629–645. [CrossRef]
51. Chen, B.; He, R.; Yuan, K.; Chen, E.; Lin, L.; Chen, X.; Sha, S.; Zhong, J.; Lin, L.; Yang, L.; et al. Polycyclic aromatic hydrocarbons (PAHs) enriching antibiotic resistance genes (ARGs) in the soils. *Environ. Pollut.* **2017**, *220*, 1005–1013. [CrossRef]
52. Lee, D.; Lee, H.; Lee, A.; Kwon, B.; Khim, J.; Yim, U.; Kim, B.; Kim, J. Microbial community composition and PAHs removal potential of indigenous bacteria in oil contaminated sediment of Taean coast, Korea. *Environ. Pollut.* **2018**, *234*, 503–512. [CrossRef] [PubMed]
53. Sun, Z.; Wang, L.; Yang, S.; Xun, Y.; Zhang, T.; Wei, W. Thermally enhanced anoxic biodegradation of polycyclic aromatic hydrocarbons (PAHs) in a highly contaminated aged soil. *J. Environ. Chem. Eng.* **2022**, *10*, 107236. [CrossRef]
54. Li, J.; Yao, Y.; Shi, Y.; Tang, J.; Gadow, S.; Liu, R.; Niu, Q. [Bmim] $FeCl_4$ mediated inhibition and toxicity during anaerobic digestion: Dose-response kinetics, biochar-dependent detoxification and microbial resistance. *Water Res.* **2022**, *210*, 117969. [CrossRef] [PubMed]