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Research Article

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Posted Date: November 19th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11602675/v1

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Additional Declarations: No competing interests reported.

Running title: Bacterial consortia enhance crude-oil removal

Defined bacterial consortia selectively enhance the degradation of recalcitrant crude-oil hydrocarbons in soil while reshaping soil microbial communities

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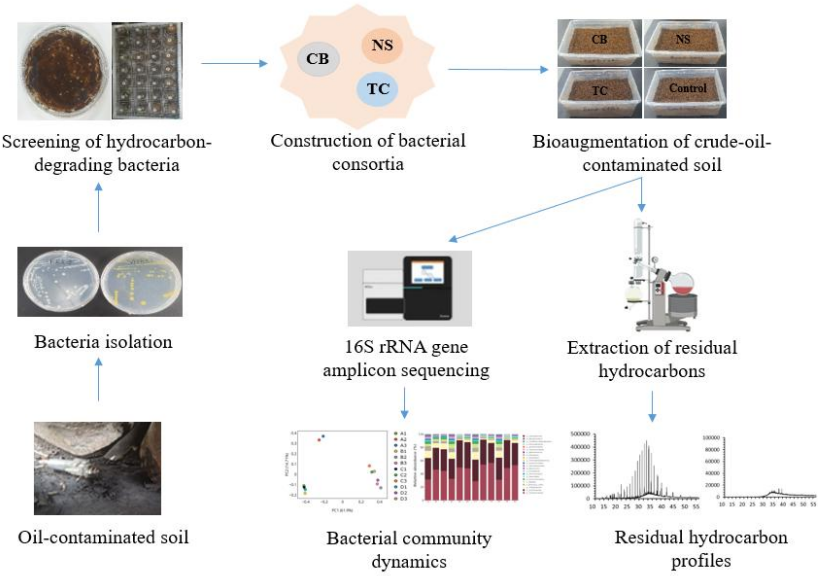
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Abstract

Bioaugmentation offers a promising strategy for remediating petroleum-contaminated soils, but its effectiveness is often constrained by the persistence of recalcitrant hydrocarbon fractions. Here, we isolated 370 bacterial strains from oil-contaminated soils in Korea, China, and Vietnam, selecting eight representative strains to assemble three defined consortia: CB (four high-efficiency degraders), NS (four novel species candidates), and TC (CB + NS). In soil microcosms contaminated with 2.5% (w/w) crude oil, bioaugmentation selectively enhanced the degradation of recalcitrant hydrocarbons. After 30 days, removal of long-chain n-alkanes (C_{25}–C_{33}) reached 88.58–98.30% across consortia, compared to 61.35–78.44% in unbioaugmented controls. Pristane, phytane, and cyclic compounds were also significantly more effectively degraded in bioaugmented soils. 16S rRNA gene amplicon sequencing revealed temporal and treatment-dependent restructuring of the soil bacterial community. Several hydrocarbon-associated genera were enriched, while inoculated genera (including *Gordonia*, *Mycolicibacterium*, *Stenotrophomonas*, and *Agrobacterium*) showed treatment-associated enrichment patterns. Notably, increasing consortium complexity from four to eight strains provided little additional benefit, indicating that greater inoculum diversity does not improve remediation performance. Overall, these findings demonstrate that defined bacterial consortia can selectively enhance recalcitrant hydrocarbon removal without requiring high consortium complexity.

Graphical abstract



Highlights

- Defined bacterial consortia enhanced the degradation of recalcitrant crude-oil hydrocarbons.
- Bioaugmentation increased cyclic-compound removal by 6.6–20.6-fold at day 30.
- Bioaugmentation reshaped soil bacterial communities and enriched hydrocarbon-associated taxa.
- Increasing consortium complexity from four to eight strains provided limited additional benefit.

Keywords: bioaugmentation; soil remediation; GC-MS fingerprinting; recalcitrant hydrocarbons; microbial consortium; 16S rRNA amplicon sequencing

1. Introduction

Oil-contaminated soils remain a major environmental hazard due to the persistence and complexity of petroleum mixtures, which contain hundreds of compounds spanning n-alkanes, branched alkanes, cyclic hydrocarbons, and aromatic fractions, as well as resins and asphaltenes (Dacco et al. 2020; Galitskaya et al. 2021). Accidental leaks can occur throughout petroleum exploration, refining, storage, and transport, and spilt oil can impair soil physical structure, fertility, and microbial function while increasing risks to groundwater, ecosystems, and human health (Hoang et al. 2021; Hu et al. 2013; Liu et al. 2022; Yazdanpanah et al. 2016). From a remediation perspective, the most challenging fractions are typically long-chain, branched, and cyclic hydrocarbons, which degrade more slowly and contribute to long-term persistence and risk (Dacco et al. 2020; Karishma et al. 2024).

Bioremediation is widely considered an attractive remediation option due to its low cost, minimal environmental impact, and ease of operation (Koshlaf and Ball 2017). Bioaugmentation has shown promise in enhancing the removal of petroleum hydrocarbons by introducing microorganisms with hydrocarbon-degrading capabilities into contaminated environments (Laothamteep et al. 2022; Lawniczak et al. 2020). The use of microbial consortia may further broaden the range of hydrocarbons targeted by bioaugmentation by combining organisms with complementary catabolic capacities (Karishma et al. 2024; Wang et al. 2019; Cerqueira et al. 2011; Zhang et al. 2021; Abbasian et al. 2016). However, outcomes remain inconsistent across studies, often due to limited survival or establishment of inoculated strains, competition with indigenous communities, and differences in soil physicochemical conditions (Laothamteep et al. 2022; Brzeszcz et al. 2020; Radwan et al. 2019; Ali et al. 2022). However, it remains unclear whether increasing consortium complexity by combining a larger number of hydrocarbon-degrading strains provides additional benefits for the removal of recalcitrant petroleum fractions under soil conditions. Thus, evaluating bioaugmentation requires not only measurement of overall petroleum removal but also assessment of which hydrocarbon fractions respond to inoculation and how the soil microbial community changes during remediation. Chemical fingerprinting can resolve treatment effects across individual compounds or hydrocarbon classes, whereas 16S rRNA gene amplicon sequencing can reveal community restructuring and treatment-associated enrichment of bacterial taxa during remediation. Combining these approaches can therefore provide a more integrated assessment of the chemical and ecological responses to bioaugmentation.

In this study, we integrated culture-based isolation and screening with soil microcosm

experiments to evaluate whether defined bacterial consortia can selectively enhance the degradation of recalcitrant crude-oil hydrocarbons. We isolated hydrocarbonoclastic bacteria from oil-impacted soils collected in Korea, China, and Vietnam; screened representative isolates for crude-oil depletion; and constructed three consortia comprising high-efficiency known degraders (CB), novel species candidates (NS), and their combination (TC). GC-MS was used to evaluate changes in specific hydrocarbon fractions over 0–60 days, focusing on n-alkanes, branched alkanes, and cyclic hydrocarbons, while 16S rRNA gene amplicon sequencing was used to characterize bacterial community restructuring and treatment-associated enrichment patterns during remediation**.** The aims were to (i) compare intrinsic attenuation with consortium-based bioaugmentation for different crude-oil hydrocarbon fractions, (ii) determine whether increasing consortium complexity from four to eight strains provides additional benefits for the degradation of recalcitrant fractions, and (iii) characterize microbial community restructuring and identify hydrocarbon-associated taxa enriched during bioaugmentation.

2. Materials and Methods

2.1. Collection of soil samples

Oil-contaminated soil samples were collected from agricultural machinery repair facilities in China. Chinese sampling sites included YingAn, China (42°16'05.6"N 130°04'52.4"E), Jilin, China (42°56'13.2"N 130°29'58.4"E) and Hunchunshi, China (42°47'54.5"N 130°08'29.9"E). Korean sampling sites included areas where construction machinery had been concentrated for extended periods, including Jinwi-myeon, Pyeongtaek, Gyeonggi-do, South Korea (37°06'40.8"N 127°05'18.0"E); Yonghyeon-dong, Nam-gu, Incheon, South Korea (37°27'08.6"N 130°08'29.9"E) and Cheoin-gu, Yongin-si, Gyeonggi-do, South Korea (37°13'58.6"N 127°15'11.8"E). Vietnamese soil samples were collected from motorbike repair shops, including Dak Bla, Kon Tum, Vietnam (14°20'43.7"N 108°04'48.9"E); Vinh Linh, Quang Tri, Vietnam (17°04'16.3"N 106°59'28.4"E); and Phu Vang, Thua Thien Hue, Vietnam (16°24'14.9"N 107°44'26.8"E). Samples were collected in 250 mL Schott bottles, kept on ice during transportation, and stored at 4°C until bacterial isolation.

2.2. Isolation and screening of hydrocarbon-degrading bacteria

For bacterial isolation, 3 g of oil-contaminated soil, after removal of rocks and debris, was placed in the bottom compartment of a six-well transwell plate (Corning Inc., Corning, NY, USA). The inserts were filled with 6 mL of 1% R2A broth (MBcell). A soil suspension was prepared by mixing 1 g of the soil sample with 9 mL of sterile 0.85% NaCl in a 15 mL conical tube and rotating for 30 min. Then, 100 µL of the prepared soil suspension was added to each insert. Plates

were incubated at 25°C and 100 rpm for 2 weeks. After incubation, 100 µL of serial dilutions (10^{-1}–10^{-7}) was spread onto 1% R2A agar and incubated at 28°C for up to 7 days. Colonies with distinct morphologies were repeatedly streaked onto R2A agar until pure cultures were obtained. Pure strains were stored at −80°C in 20% (v/v) glycerol.

The isolated strains were subsequently screened for their ability to utilize crude oil. MSM agar (Xia et al. 2017) plates were overlaid with 200 µL of crude oil (oil-MSM plates). Bacterial isolates were pre-cultured in R2A broth, and 10 µL of each culture was streaked onto oil–MSM plates. Plates were incubated at 28°C for 7 days, and bacterial growth on oil-MSM plates was used as a preliminary indicator of crude-oil utilization. The crude oil used in this study was collected from the MSP1/BK7 rig at the Vietsovpetro oil exploitation plant, Vung Tau, Vietnam and stored at room temperature until use.

2.3. Identification of bacterial isolates

16S rRNA genes were amplified using primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). PCR products were purified and sequenced by Macrogen Inc. (Seoul, Republic of Korea). The resulting 16S rRNA gene sequences were compared with reference sequences in the NCBI database and EzBioCloud to determine their closest taxonomic affiliations based on sequence similarity. Representative sequences were deposited in GenBank, and the corresponding accession numbers are provided in Table S2.

2.4. Crude oil degradation by individual strains (liquid assay)

Isolates showing robust growth on oil-MSM agar were evaluated for crude-oil degradation in liquid culture. Each strain was inoculated into 250 mL Erlenmeyer flasks containing 100 mL of mineral salts medium (MSM) (Xia et al. 2017) supplemented with 2 mL of crude oil (2%, v/v). Abiotic controls contained MSM and crude oil without bacterial inoculation. All treatments, including the abiotic controls, were performed in triplicate. Flasks were incubated at 30°C and 150 rpm for 5 weeks.

After incubation, residual oil was extracted with an equal volume of dichloromethane (DCM; culture = 1:1, v/v). The culture–DCM mixture was stirred for 30 min using a magnetic stirrer, after which the organic phase was separated using a separatory funnel. Extraction was repeated twice more at room temperature. The organic extracts were combined and transferred to a pre-weighed glass tube. The DCM was evaporated to dryness, and the mass of the recovered residual oil was determined gravimetrically.

Oil removal efficiency (ORE) was calculated as:

$ORE\ (\%) = [(W_1 - W_2) / W_1] \times 100$ (Das et al. 2023)

where W_1 is the mass of residual oil recovered from the abiotic control and W_2 is the mass of residual oil recovered from the inoculated culture**.**

2.5. Construction of the consortium, soil preparation and experimental design

Three consortia (CB, NS, and TC) were constructed based on the liquid-culture screening results. Consortium CB comprised four high-performing isolates affiliated with known species: *Mycolicibacterium fluoranthenivorans* CN3-95, *Stenotrophomonas maltophilia* CN5-326, *Pseudomonas aeruginosa* KR1-3, and *Agrobacterium pusense* VNQ86. Consortium NS comprised four novel species candidates representing different genera: *Gordonia* sp. VNQ95, *Sphingomonas* sp. VNH70, *Paenibacillus* sp. KR2-11, and *Acinetobacter* sp. VNH17. Consortium TC combined all eight strains from CB and NS (Table S1).

For inoculum preparation, each strain was cultured in R2A broth. During the logarithmic growth phase, the cultures were standardized to an OD_{600} of approximately 0.565. Equal volumes of the standardized cultures were then combined to prepare each consortium, with a final inoculum volume of 200 mL.

Soil microcosm experiments were performed using polypropylene (PP) boxes (30 cm × 20 cm × 10 cm; length × width × height) containing 4 kg of pristine garden soil collected from the botanical garden of the Department of Life Science, Kyonggi University, Republic of Korea. Soil physicochemical properties were determined as follows: texture by the hydrometer method, pH using a 1:2 soil-to-water suspension (USDA Handbook 18, 2017), organic carbon (OC) by the Walkley-Black method (Walkley and Black 1934), available phosphorus by the Bray-I method (Bray and Kurtz 1945), and total nitrogen by the Bremner method (Bremner 1960) (Table 1).

All soils were spiked with 100 g of light crude oil to obtain a final contamination level of 2.5% (w/w). Three bioaugmentation treatments were established by adding 200 mL of the CB, NS, or TC consortium inoculum, whereas the unbioaugmented control received 200 mL of sterile water. Microcosms were maintained under open environmental conditions for 60 days. To minimize excessive drying, 200 mL of sterile water was added twice per week, and the soils were thoroughly mixed after each water addition. Soil samples were collected on days 0, 30, and 60 for 16S rRNA gene amplicon sequencing and GC-MS analysis of residual hydrocarbons.

2.6. 16S rRNA gene amplicon sequencing and community analysis

Microbial community dynamics were assessed using 16S rRNA gene amplicon sequencing. A total of 12 soil samples, representing four treatments sampled on days 0, 30, and 60, were analyzed. Total DNA was extracted from the soil samples using the ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions.

DNA quality and concentration were assessed using 1% agarose gel electrophoresis, a Quantus™ Fluorometer (Promega, Madison, WI, USA), and a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

The V3–V4 region of the bacterial 16S rRNA gene was amplified using primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) and sequenced on an Illumina MiSeq platform (Macrogen Inc., Seoul, Republic of Korea). Adapter and primer sequences were removed using Cutadapt v3.2, and forward and reverse reads were truncated to 250 and 200 bp, respectively. Amplicon sequence variants (ASVs) were generated using DADA2 v1.18.0 through error correction, denoising, and paired-end read merging. Sequences with two or more expected errors were excluded. Chimeric sequences were removed using the consensus method implemented in the removeBimeraDenovo function of DADA2. ASVs shorter than 350 bp were removed using R v4.0.3. For community comparisons, ASV tables were normalized by subsampling each sample to the sequencing depth of the sample with the lowest read count using QIIME v1.9.0.

Taxonomic assignment was performed against the NCBI_16S reference database using a Bayesian classifier implemented in DADA2 v1.18.0 with a confidence threshold of 50. Relative abundances were summarized from the phylum to genus levels. Alpha diversity was evaluated using observed ASV richness, ACE, Chao1, Shannon, and Simpson diversity indices. Good's coverage and rarefaction curves were used to assess sequencing completeness and depth. Multiple sequence alignment of ASVs and phylogenetic tree construction were performed using MAFFT v7.475 and FastTreeMP v2.1.10, respectively. Beta diversity was assessed using Bray–Curtis dissimilarities, and community patterns were visualized using principal coordinate analysis (PCoA) and UPGMA clustering. The raw sequence data were deposited in the NCBI Sequence Read Archive under BioProject accession number PRJNA1237751.

2.7. Residual hydrocarbon extraction and GC-MS analysis

Residual hydrocarbon profiles were analyzed by gas chromatography–mass spectrometry (GC–MS). For each treatment and sampling time, three 10-g soil subsamples were collected from different locations within the microcosm and subjected to hydrocarbon extraction. Each soil subsample was extracted three times with 50 mL of dichloromethane (DCM) per extraction. The extracts were combined, concentrated using a rotary evaporator, and reconstituted in 2 mL of DCM. The resulting extracts were diluted 20-fold prior to GC–MS analysis.

GC–MS analysis was performed using an Agilent 6890N gas chromatograph coupled to a 5975B mass-selective detector (Agilent Technologies, USA) equipped with an HP-5MS column. One

microlitre of each extract was injected, with helium used as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. The oven temperature was initially maintained at 60°C for 5 min, increased to 280°C at 8°C min^{-1}, and then maintained at 280°C for 37 min. The GC–MS transfer line temperature was maintained at 280°C, and the solvent delay was 3 min. Hydrocarbon compounds were tentatively identified by comparison of their mass spectra with entries in the Wiley7n.L mass spectral library.

Biodegradation efficiency (%) for each hydrocarbon group (n-alkanes, substituted alkanes, and cyclic compounds) was calculated as:

Biodegradation efficiency (%) = [(RA - RB) / RA] × 100 (Ehiosun et al. 2023)

where RA is the concentration at day 0, and RB is the corresponding concentration at day 30 or day 60 in the control and bacteria-treated soils.

3. Results

3.1. Biodegradative potential of bacterial isolates

A total of 370 bacterial strains were isolated from oil-contaminated soils collected in Korea, China, and Vietnam. Among these, 142 isolates grew on MSM agar supplemented with crude oil as the sole carbon and energy source, indicating their potential to utilize crude-oil hydrocarbons under the screening conditions. Of these 142 isolates, 125 corresponded to known species, whereas 17 exhibited <98.63% 16S rRNA gene sequence similarity to their closest type strains and were therefore considered potential novel species candidates (Figure 1; Table S2).

Across all sites, *Pseudomonas*, *Mycolicibacterium*, and *Nocardioides* were the most frequently recovered genera; however, the composition of the culturable hydrocarbon-utilizing isolates differed among the three sampling regions. The Korean isolates were dominated by *Pseudomonas*, *Mycolicibacterium*, and *Nocardioides*, whereas the Vietnamese isolates were dominated by *Pseudomonas*, *Acinetobacter*, and *Gordonia*. In contrast, the Chinese isolates were predominantly represented by *Mycolicibacterium*, *Nocardioides*, *Paenibacillus*, and *Rhodococcus*. Notably, *Acinetobacter*, one of the predominant genera among the Vietnamese isolates, was not detected among the Korean or Chinese isolates. Similarly, *Rhodococcus*, one of the dominant genera among the Chinese isolates, was also present as a minor genus among the Korean isolates but was not detected among the Vietnamese isolates. *Nocardioides*, one of the dominant genera among the Korean and Chinese isolates, was absent among the Vietnamese isolates. In contrast, isolates belonging to *Pseudomonas*, *Mycolicibacterium*, *Gordonia*, *Microbacterium*, and *Sphingomonas* were recovered from all three regions. These genera include taxa previously reported to degrade petroleum hydrocarbons (Schippers et al. 2005; Wu et al. 2023; Kim et al. 2019; Zhou et al. 2016;

Brzeszcz et al. 2024).

To select strains for soil bioaugmentation, 31 isolates showing the fastest growth on oil-MSM plates were further evaluated for crude-oil removal in liquid MSM. Strains with ≥50% oil removal efficiency are summarized in Table S3. The top-performing isolates—*Pseudomonas aeruginosa* KR1-3, *Mycolicibacterium fluoranthenivorans* CN3-95, *Agrobacterium pusense* VNQ86, *Mycolicibacterium frederiksbergense* KR8-263, and *Stenotrophomonas maltophilia* CN5-326—removed 79.3 ± 7.49% to 86.42 ± 3.86% of crude oil after 5 weeks, whereas the remaining screened strains removed 41.06 ± 3.12% to 73.64 ± 6.19% over the same period. Putative novel taxa generally showed lower oil-removal efficiencies, although several still exhibited substantial activity, including VNQ95 (69.23 ± 3.60%), KR2-11 (56.32 ± 3.60%), VNH70 (52.60 ± 7.34%), and VNH17 (52.40 ± 8.36%).

Based on these results, two defined four-member consortia and one combined consortium were assembled for soil microcosm experiments (Table S1). For the CB consortium, high-performing isolates were selected while avoiding the inclusion of multiple strains from the same genus to maintain greater taxonomic diversity within the consortium. CB therefore comprised four selected high-performing known-species isolates (CN3-95, CN5-326, KR1-3, and VNQ86), whereas NS comprised four putative novel-species candidates (VNQ95, VNH70, KR2-11, and VNH17). TC combined all eight strains from CB and NS. This consortium design enabled comparison of the performance of the selected high-performing known-species isolates (CB) with that of the putative novel-species candidates (NS), while TC was used to assess whether increasing consortium complexity from four to eight strains provided additional benefits under soil conditions.

3.2. Bioaugmentation reshaped soil bacterial diversity and community structure over time

Illumina MiSeq sequencing of the V3–V4 region generated 979,492 raw reads across 12 soil microcosm samples (mean read length ~350 bp). After filtering, 752,482 clean reads were retained, and 403,599 non-chimeric reads were used for downstream analysis (mean 33,633 reads per sample; range 21,113–51,012; Table S4).

Good's coverage ranged from 99.57% to 99.98%, indicating high sampling completeness across all samples (Table 2). Rarefaction curves approached saturation across samples, further supporting adequate sequencing depth for community analysis (Figure 2).

Alpha diversity decreased after oil amendment and incubation in both unbioaugmented and bioaugmented soils. At day 0, the unbioaugmented control (A1) exhibited the highest richness and diversity (Chao1 and Shannon; Table 2). After 30 days, richness and diversity declined

markedly across all treatments. In the unbioaugmented control, Chao1 richness and Shannon diversity decreased by 47.95% and 20.95%, respectively, relative to day 0. In the bioaugmented treatments, Chao1 richness decreased by 61.19% in CB, 55.39% in NS, and 75.82% in TC, while Shannon diversity decreased by 33.60%, 27.16%, and 34.17%, respectively. These results indicate a pronounced reduction in bacterial richness and diversity during the first 30 days of incubation, with the magnitude of the change differing among treatments. By day 60, richness and diversity partially recovered in all bioaugmented treatments relative to day 30. Chao1 richness increased by 21.88%, 43.49%, and 50.00% in CB, NS, and TC, respectively, while Shannon diversity increased by 5.70%, 7.91%, and 8.13%, respectively. This partial recovery indicates continued restructuring of the bacterial communities during the later stage of incubation. TC showed the largest early decline in Chao1 richness; however, this pattern alone does not establish a direct relationship between inoculum complexity and the magnitude of diversity loss. The taxonomic distribution shown in Table 2 also varied over time, with changes in the numbers of detected phyla, classes, orders, families, and genera across the treatments and sampling times.

Beta-diversity analysis revealed distinct temporal and treatment-associated patterns in bacterial community structure. Bray–Curtis principal coordinate analysis (PCoA) accounted for 76.61% of the total variation on the first two principal coordinates (PC1 = 61.90% and PC2 = 14.71%; Figure 3). At day 0, samples from all treatments clustered together, indicating broadly similar initial bacterial community structures. After 30 and 60 days of incubation, the communities shifted markedly from their initial positions and diverged into two main groups corresponding to the unbioaugmented control and bioaugmented treatments. Within the bioaugmented treatments, NS separated from CB, whereas TC occupied an intermediate position between the two, consistent with its composition as a combination of the CB and NS consortia. Overall, these ordination patterns show pronounced temporal restructuring of the bacterial communities and differentiation between the unbioaugmented and bioaugmented microcosms during crude-oil remediation. At the phylum level, taxonomic shifts were dominated by *Actinomycetota* and *Pseudomonadota* across all microcosms (Figure 4). In the unbioaugmented control, oil exposure increased *Pseudomonadota* (from 31.56% to 46.47% at day 30) and *Thermodesulfobacteriota*, while reducing *Bacillota*, *Chloroflexota*, *Bacteroidota*, *Myxococcota*, *Gemmatimonadota*, and *Vulcanimicrobiota*. The relative abundances of *Acidobacteriota*, *Verrucomicrobiota*, and *Planctomycetota* decreased at day 30 and partially recovered by day 60. *Actinomycetota* remained relatively stable (~32%) in the control, whereas in bioaugmented soils *Actinomycetota* increased sharply at day 30 and then declined slightly by day 60. *Pseudomonadota* trajectories differed

among consortia: CB showed an increase at day 30 followed by a slight decline at day 60, whereas NS and TC increased continuously. Overall, these phylum-level profiles demonstrate distinct temporal changes in community composition among the unbioaugmented and bioaugmented microcosms.

At the genus level, day 0 communities were similar in the control and bioaugmented treatments, with *Nocardia*, *Bradyrhizobium*, *Actinoallomurus*, and unclassified taxa among the most abundant (Figure 5). After oil addition and incubation, the unbioaugmented control shifted toward *Nocardia*, *Paraburkholderia*, and *Nevskia*, whereas bioaugmented soils showed pronounced increases in *Rhodanobacter* and *Mycolicibacterium* (Figure 5; Table S5). Specifically, at day 30, the relative abundances of *Nocardia* (18.45%) and *Nevskia* (12.57%) in the control increased by 52% and 99% relative to day 0, respectively. In contrast, bioaugmented soils were enriched in *Rhodanobacter* (18.00% and 24.05% in CB, 19.71% and 23.74% in NS, and 16.84% and 22.90% in TC at days 30 and 60, respectively), whereas the control maintained low *Rhodanobacter* abundance (2.76% and 3.14%). The consistent enrichment of *Rhodanobacter* across all three bioaugmented treatments, relative to the unbioaugmented control, reflects a strong treatment-associated increase in its relative abundance during bioaugmentation.

Mycolicibacterium was a second major genus distinguishing the bioaugmented from the unbioaugmented microcosms. It remained low in the control (2.11% and 3.23% at days 30 and 60) but rose sharply in bioaugmented soils (28.08% and 24.20% in CB, 16.22% and 6.98% in NS, and 25.82% and 17.56% in TC at days 30 and 60, respectively). Higher *Mycolicibacterium* abundance in CB and TC than in the control was consistent with the inclusion of *Mycolicibacterium fluoranthenivorans* CN3-95 in the CB and TC inocula. However, because the 16S rRNA gene amplicon data do not provide strain-specific resolution, the observed enrichment cannot be attributed exclusively to the inoculated CN3-95 strain and may also include indigenous *Mycolicibacterium* populations.

Treatment-associated enrichment was also observed for several genera represented in the inocula (Table S5). *Gordonia* increased in NS and TC at day 30 (1.61% in NS; 0.94% in TC) compared with the control (0.02%) and CB (0.06%), consistent with the presence of *Gordonia* VNQ95 in NS and TC. Likewise, *Stenotrophomonas* increased in CB and TC (1.21% and 1.11%) but was low in NS (0.09%) and undetected in the control, consistent with *Stenotrophomonas maltophilia* CN5-326 being included in CB and TC. Agrobacterium was detected at day 60 in CB and TC but not in the corresponding control and NS samples, consistent with the inclusion of *Agrobacterium pusense* VNQ86 in CB and TC. Although these genus-level distributions are consistent with

365 treatment-specific enrichment of taxa represented in the respective inocula, they cannot confirm
366 the persistence or establishment of individual inoculated strains. Collectively, the results show
367 that bioaugmentation was associated with substantial restructuring of the soil bacterial community
368 and with the enrichment of several taxa during crude-oil remediation.

369 **3.3. Bioaugmentation enhanced the removal of recalcitrant hydrocarbon fractions,**
370 **particularly cyclic compounds**

371 GC–MS chromatograms (Figure 6) showed substantial changes in residual hydrocarbon profiles
372 over time, with differences in the extent of removal among treatments. Relative to the
373 unbioaugmented control, bioaugmented soils exhibited a marked reduction in residual n-alkanes
374 (C11–C33) and methyl-substituted alkanes, indicating enhanced hydrocarbon removal following
375 bioaugmentation.

376 Quantitative biodegradation efficiencies at days 30 and 60 are summarized in Figure 7 and Tables
377 S6–S7. n-Undecane (C11) was completely degraded by day 30 in both the unbioaugmented
378 control and all bioaugmented treatments, indicating that indigenous communities alone were
379 capable of removing this compound efficiently under these conditions. In contrast, long-chain
380 C25–C33 n-alkanes were degraded much more effectively in bioaugmented soils than in the
381 unbioaugmented control at day 30: 88.58–97.89% in CB, 88.67–96.88% in NS, and 92.83–98.30%
382 in TC, compared with 61.35–78.44% in the unbioaugmented control. By day 60, removal of C25–
383 C33 n-alkanes increased to 94.50–98.86% in CB, 94.58–98.68% in NS, and 96.29–98.86% in TC,
384 whereas the corresponding values in the unbioaugmented control ranged from 83.50% to 93.06%.
385 Methyl-substituted alkanes were also removed more efficiently after bioaugmentation. At day 30,
386 removal of the methyl-substituted alkanes listed in Table S7 ranged from 90.28% to 100% across
387 the bioaugmented treatments, compared with 65.97–76.31% in the unbioaugmented control. The
388 branched isoprenoids pristane and phytane similarly showed higher removal following
389 bioaugmentation. At day 30, pristane removal reached 97.18%, 91.73%, and 96.61% in CB, NS,
390 and TC, respectively, compared with 75.43% in the unbioaugmented control. Phytane removal
391 reached 96.79%, 90.06%, and 96.88% in CB, NS, and TC, respectively, compared with 66.49%
392 in the control. By day 60, pristane and phytane removal exceeded 97% in all three bioaugmented
393 treatments.

394 The strongest treatment differences at day 30 were observed for cyclic compounds. In the
395 unbioaugmented control, removal of Tricyclo(4.3.1.1^{2,5})undecane-10-ol was only 4.07% at day
396 30 and increased to 40.85% at day 60. In contrast, bioaugmented soils showed substantially higher
397 removal, reaching 83.86% and 94.99% in CB, 60.62% and 93.82% in NS, and 83.40% and 95.05%

in TC at days 30 and 60, respectively. Similarly, drimane removal in the unbioaugmented control was 10.87% at day 30 and 52.36% at day 60, compared with 83.88% and 96.49% in CB, 72.16% and 96.45% in NS, and 85.68% and 95.97% in TC, respectively. At day 30, removal of these two cyclic compounds was approximately 6.6–20.6-fold higher in the bioaugmented treatments than in the unbioaugmented control, representing the most pronounced enhancement associated with bioaugmentation among the hydrocarbon fractions examined. By day 60, removal efficiencies for both compounds had largely converged among the three bioaugmented treatments (>93%).

Comparisons among consortia showed that increasing inoculum complexity from four strains (CB or NS) to eight strains (TC) provided only limited additional improvement in hydrocarbon removal. For the long-chain C25–C33 n-alkanes at day 30, removal ranged from 88.58–97.89% in CB and 88.67–96.88% in NS, compared with 92.83–98.30% in TC. Although TC generally showed high removal of these long-chain n-alkanes, it did not consistently outperform both four-strain consortia across all compounds. A similar pattern was observed for the cyclic compounds. At day 30, CB and TC showed comparable removal of Tricyclo(4.3.1.1^{2,5})undecane-10-ol (83.86% and 83.40%, respectively), both exceeding NS (60.62%). For drimane, TC showed the highest day-30 removal (85.68%), followed closely by CB (83.88%), whereas NS showed lower removal (72.16%). By day 60, differences among the three bioaugmented treatments were small, with removal of Tricyclo(4.3.1.1^{2,5})undecane-10-ol ranging from 93.82% to 95.05% and drimane from 95.97% to 96.49%. These results indicate that increasing consortium complexity from four to eight strains did not consistently result in proportionally greater hydrocarbon removal under the experimental conditions.

4. Discussion

Petroleum pollution causes a series of problems in water, air, soil and the ecological environment and can reduce or destroy soil fertility (Hoang et al. 2021). Hydrocarbon inputs also disrupt nutrient cycling by constraining N and P availability (e.g., limiting nitrification and P turnover), thereby suppressing plant growth and increasing the risk of secondary contamination of groundwater and air (Galitskaya et al. 2021). Bioaugmentation with microbial consortia has therefore been widely investigated, with reports of both success (Laothamteep et al. 2022; Brzeszcz et al. 2020) and failure (Ali et al. 2022; Radwan et al. 2019), likely reflecting context dependence related to soil physicochemical properties, indigenous community structure, contaminant composition, and inoculum survival. Here, we combined isolation and screening of crude-oil-degrading bacteria with soil bioaugmentation, 16S rRNA gene amplicon profiling, and GC–MS analysis to determine whether defined bacterial consortia enhanced hydrocarbon

removal beyond intrinsic attenuation and whether increasing consortium complexity improved remediation performance.

In this study, 370 isolates were recovered from three oil-impacted regions, including 142 hydrocarbonoclastic candidates, and three consortia (CB, NS, and TC) were constructed for soil bioaugmentation. The 16S rRNA amplicon sequencing results showed that bacterial richness and diversity declined markedly during the first 30 days across all treatments. The Shannon index decreased in the control at days 30 and 60 relative to day 0, indicating that crude oil exposure and subsequent incubation were associated with reduced bacterial diversity even without bioaugmentation. The greater day-30 declines observed in several bioaugmented treatments suggest additional community restructuring following inoculation, potentially involving interactions and competition between introduced and indigenous populations. Bray–Curtis PCoA supported this pattern: day-0 samples clustered together, whereas after 30 and 60 days the unbioaugmented control separated from the bioaugmented treatments. Within the bioaugmented treatments, NS separated from CB, while TC occupied an intermediate position, consistent with its composition as a combination of the CB and NS consortia. Together, these results indicate substantial temporal and treatment-associated restructuring of the soil bacterial communities.

Similar responses have been observed in previous studies. Chaudhary et al. (2021) reported reduced bacterial diversity following bioaugmentation of diesel-contaminated soil microcosms, while Laothamteep et al. (2022) similarly demonstrated that crude-oil pollution and addition of an oil-degrading consortium altered soil bacterial diversity. Partial recovery of richness and diversity by day 60 in the bioaugmented soils indicates continued community restructuring during the later stage of incubation, consistent with previous reports that petroleum stress and bioaugmentation can transiently suppress bacterial diversity (Li et al. 2021; Mafiana et al. 2021; Martirosyan et al. 2025). This recovery may reflect changing environmental conditions and substrate availability as remediation progresses.

At the genus level, the day-0 communities showed broadly similar composition across treatments, whereas pronounced taxonomic shifts emerged during incubation. *Paraburkholderia*, *Rhodanobacter*, *Nevskia*, *Nocardia*, and *Mycolicibacterium* increased in relative abundance in different treatments, while *Actinoallomurus* and *Fontisphaera* declined. Several of these genera have previously been associated with degradation of petroleum hydrocarbons or other organic contaminants (Brzeszcz et al. 2024; Li et al. 2024; Azadi and Shojaei 2020; Pugazhendi et al. 2015; Chooklin et al. 2013). These shifts are consistent with selective changes in bacterial community composition under petroleum-contaminated conditions (Ali et al. 2022).

Bioaugmentation can alter microbial community structure through interactions between introduced and indigenous microorganisms (Liu et al. 2022). In the present study, the bioaugmented treatments showed pronounced enrichment of *Mycolicibacterium*, *Rhodanobacter*, and *Aquirhabdus*. *Rhodanobacter* has previously been reported in association with aromatic-hydrocarbon degradation (Bacosa et al. 2012) and diesel bioremediation (Chaudhary et al. 2021). Its substantially greater abundance in bioaugmented than unbioaugmented soils indicates that conditions associated with bioaugmentation favored its enrichment beyond that observed with crude oil alone. *Aquirhabdus* also increased markedly; however, because its hydrocarbon-degrading capacity has not yet been established, its enrichment should be interpreted as an association with petroleum-affected community succession rather than evidence of direct participation in hydrocarbon degradation.

Mycolicibacterium remained low in the control but was abundant in bioaugmented treatments, particularly CB and TC, both of which contained *Mycolicibacterium fluoranthenivorans* CN3-95. Previous studies have shown that *Mycolicibacterium* can degrade both n-alkanes and aromatic hydrocarbons (Laothamteep et al. 2022; Brzeszcz et al. 2024). Thus, its enrichment is consistent with an increased abundance of hydrocarbon-associated bacteria in the bioaugmented microcosms. However, because genus-level 16S rRNA gene amplicon data cannot distinguish CN3-95 from indigenous *Mycolicibacterium*, these results do not demonstrate strain-specific establishment or directly attribute hydrocarbon removal to the inoculated strain.

Treatment-associated enrichment was also observed for genera corresponding to several members of the inocula, including *Gordonia* VNQ95, *Stenotrophomonas maltophilia* CN5-326, and *Agrobacterium pusense* VNQ86. These genera have also been reported to degrade crude oil (Delegan et al. 2019; Sharma et al. 2019; Gargouri et al. 2017). Their enrichment in treatments containing the corresponding inoculated taxa is consistent with treatment-specific persistence or stimulation of members of these genera, although strain-specific establishment cannot be confirmed from the present amplicon data.

In contrast, *Sphingomonas*, *Acinetobacter*, *Paenibacillus*, and *Pseudomonas* did not show comparable enrichment despite the corresponding strains being included in the inocula. The genera *Sphingomonas*, *Acinetobacter*, and *Paenibacillus* are well known for their hydrocarbon-degrading potential and have been applied to contaminated soils (Zhou et al. 2016; Czarny et al. 2020; Shibulal et al. 2017). Likewise, several *Pseudomonas* strains isolated from oil-contaminated soils can utilize crude oil (Wu et al. 2023; Brzeszcz et al. 2024; Liu et al. 2023). Although *Pseudomonas aeruginosa* KR1-3 showed high crude-oil removal in liquid culture, no

corresponding genus-level enrichment of *Pseudomonas* was evident in the soil microcosms. This contrast illustrates that strong degradation performance under liquid-culture screening conditions does not necessarily result in comparable taxonomic enrichment after inoculation into soil. Environmental compatibility, competition, substrate availability, and physicochemical constraints may all influence bioaugmentation performance. Importantly, however, the absence of genus-level enrichment cannot be interpreted as evidence that an inoculated strain failed to persist or contribute to degradation.

The GC–MS results provide the clearest evidence of the functional benefit of bioaugmentation. Many C11–C24 n-alkanes showed high removal within 30 days in both bioaugmented and unbioaugmented soils, indicating substantial intrinsic degradation capacity toward relatively readily degradable n-alkanes. Shorter-chain n-alkanes are generally more readily biodegraded than longer-chain homologues, for which increasing chain length can reduce bioavailability and biodegradation efficiency (Behera et al. 2021; Varjani and Upasani 2017). The benefit of bioaugmentation became substantially more pronounced for long-chain n-alkanes (C25–C33), branched alkanes and branched isoprenoids, and particularly the cyclic compounds drimane and Tricyclo(4.3.1.1^{2,5})undecane-10-ol. Removal of the two cyclic compounds at day 30 was approximately 6.6–20.6-fold higher in bioaugmented than unbioaugmented soils. Thus, the principal benefit of inoculation was not uniformly greater removal of all petroleum components, but selective enhancement of fractions that persisted more strongly under intrinsic attenuation.

This preferential enhancement of more persistent hydrocarbon fractions is consistent with previous studies showing that exogenous strains or defined microbial consortia can improve petroleum hydrocarbon biodegradation (Laothamteep et al. 2022; Dai et al. 2020). Petroleum-degrading bacteria introduced through bioaugmentation may provide additional degradation capacity when they remain metabolically active under soil conditions (Nwankwegu et al. 2022). In the present study, enhanced hydrocarbon removal coincided with substantial restructuring of the bacterial community and enrichment of taxa such as *Mycolicibacterium* and *Rhodanobacter*.

A second important finding was that increasing consortium complexity did not consistently improve remediation performance. CB, NS, and TC achieved broadly comparable hydrocarbon removal, and although the eight-strain TC consortium generally performed well, it did not consistently outperform both four-strain consortia across the hydrocarbon fractions examined. This limited additional benefit may reflect functional redundancy among consortium members, competition for available resources, or environmental constraints on microbial activity, although these mechanisms were not directly tested. These findings suggest that increasing the number of

strains in a consortium does not necessarily produce a proportional increase in remediation efficiency and that consortium composition may be more important than inoculum complexity alone.

Overall, the results reveal a complementary pattern between intrinsic attenuation and bioaugmentation. Indigenous soil communities efficiently removed the more readily degradable hydrocarbon fractions, whereas the greatest additional benefit of bioaugmentation occurred for the more recalcitrant fractions, particularly long-chain n-alkanes and cyclic compounds. This enhanced removal coincided with pronounced restructuring of the bacterial community and enrichment of several hydrocarbon-associated taxa, although strain-specific persistence and taxon-specific contributions could not be resolved by 16S rRNA gene amplicon sequencing. Importantly, increasing consortium complexity from four to eight strains provided limited additional benefit, demonstrating that greater inoculum diversity does not necessarily translate into proportionally greater remediation performance. Together, these findings support the use of carefully selected defined bacterial consortia to complement intrinsic attenuation, particularly when persistent petroleum fractions limit remediation efficiency.

5. Conclusion

This study presents a practical framework for selecting and evaluating hydrocarbon-degrading bacteria for soil bioaugmentation, combining culture-based screening, soil microcosm evaluation, GC–MS analysis, and 16S rRNA gene amplicon profiling. From 370 isolates obtained from oil-impacted soils across three countries, 142 isolates showed crude-oil-utilizing potential, and representative strains were assembled into three defined consortia. In 2.5% (w/w) crude-oil-contaminated soil microcosms, bioaugmentation consistently enhanced the removal of recalcitrant fractions, particularly long-chain n-alkanes (C25–C33), branched alkanes, branched isoprenoids, and cyclic compounds, compared with the unbioaugmented control. While intrinsic attenuation efficiently removed more readily degradable n-alkanes, the largest additional benefits of bioaugmentation were observed for the cyclic compounds drimane and Tricyclo(4.3.1.1^{2,5})undecane-10-ol, whose removal at day 30 was approximately 6.6–20.6-fold higher in the bioaugmented treatments than in the unbioaugmented control.

16S rRNA gene amplicon profiling showed pronounced temporal and treatment-associated restructuring of the bacterial communities, characterized by an initial decline in richness and diversity followed by partial recovery in the bioaugmented soils. Several hydrocarbon-associated genera, particularly *Mycolicibacterium* and *Rhodanobacter*, were strongly enriched following bioaugmentation, while treatment-associated enrichment of *Gordonia*, *Stenotrophomonas*, and

Agrobacterium was also observed. However, genus-level amplicon data could not confirm the persistence or functional contribution of individual inoculated strains. The combined eight-strain consortium (TC) did not consistently outperform the four-strain CB and NS consortia, indicating that increasing consortium complexity alone does not necessarily provide proportional gains in hydrocarbon removal. Overall, these results support the use of carefully selected defined bacterial consortia to complement intrinsic attenuation, particularly for petroleum fractions that are less efficiently removed by indigenous soil communities, while demonstrating that greater consortium complexity does not necessarily translate into greater remediation performance.

Author contributions: Thi Tuyet Nhan Le: Methodology (microbial isolation and screening), Investigation (microcosm experiments), Data curation, Formal analysis (16S rRNA amplicon sequencing and community analysis), Writing – original draft, Writing – review and editing. Jin Jun: Investigation (bacterial screening and cultivation), Methodology (consortium construction and inoculum preparation), Data curation. Hosun Aum: Methodology (chemical analysis), Investigation (residual hydrocarbon extraction and GC–MS analysis). Jihoon Jung: Investigation (GC–MS analysis), Formal analysis (GC–MS data). Jaisoo Kim: Conceptualization, Supervision, Project administration, Resources, Funding acquisition, Writing – review and editing.

Acknowledgments: This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (No. 2022R1A2C1010877).

Conflict of interest statement: The authors declare no conflicts of interest.

Data availability statement: Raw 16S rRNA gene amplicon sequences are reported under NCBI BioProject PRJNA1237751.

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FIGURE LEGENDS

Table 1. Physical and chemical properties of the soil used in the microcosm experiment

Physical and chemical soil properties	Value
Soil texture (%)	
Sand	69.8
Silt	23.8
clay	6.4
Soil characteristics	
pH	5.3
Total N (%)	0.286
Available P (mg/kg)	75.29
Organic Carbon (%)	6.70

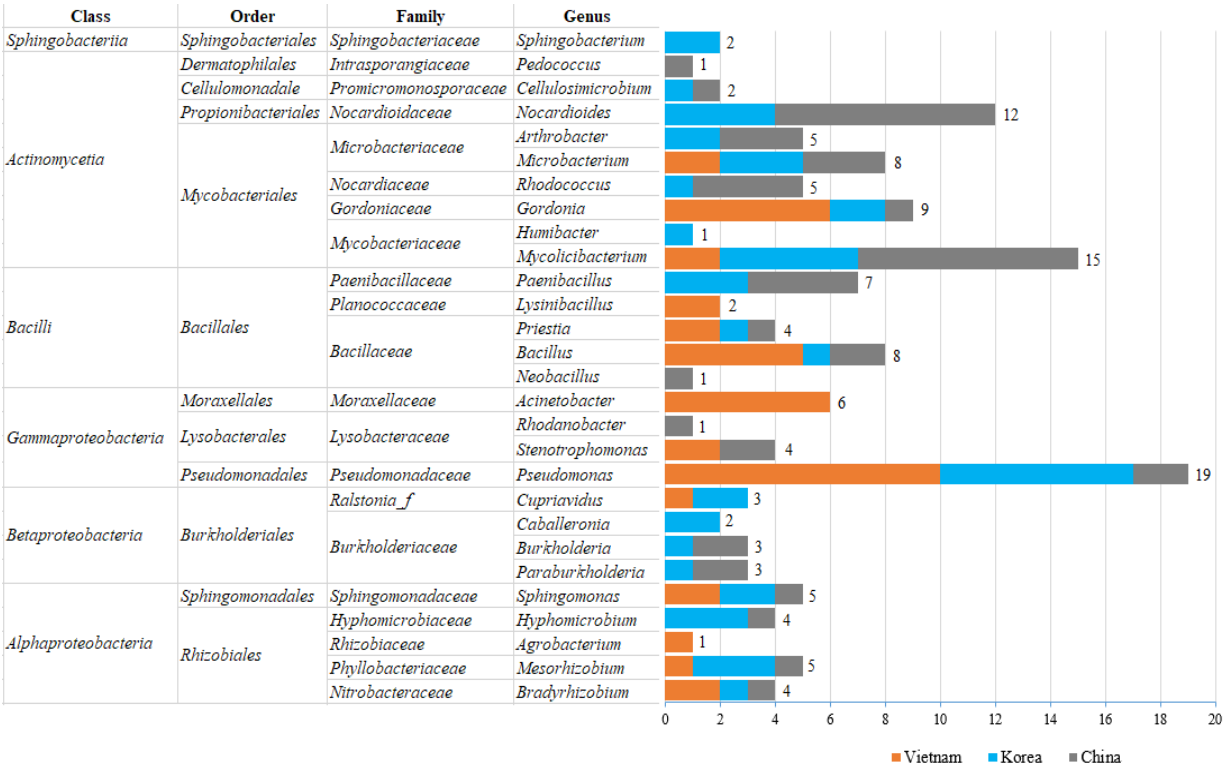


Figure 1. Hydrocarbonoclastic bacterial communities in the three studied soil samples

Table 2. Taxonomic distribution and alpha-diversity index in different soil samples. The symbols A1, A2, and A3 represent the control group, which was evaluated on days 0, 30 and 60. Similarly, the experimental groups supplemented with different bacterial consortia were

738 evaluated at the same time intervals, using the corresponding symbols: CB (B1, B2, B3), NS
739 (C1, C2, C3), and TC (D1, D2, D3).

	Control			Exp1 (CB)			Exp2 (NS)			Exp3 (TC)		
	A1	**A2**	**A3**	**B1**	**B2**	**B3**	**C1**	**C2**	**C3**	**D1**	**D2**	**D3**
Diversity indices												
Number of valid reads	47810	36690	31686	21154	25378	26372	36011	36534	51189	36183	21243	29061
ASVs	1395	739	701	741	284	350	1051	467	659	1190	294	438
Ace	1424	749	714	743	288	352	1063	474	677	1218	295	443
Chao 1	1441	750	718	742	288	351	1067	476	683	1216	294	441
Shannon diversity	9.45	7.47	7.73	8.72	5.79	6.12	9.02	6.57	7.09	9.16	6.03	6.52
Simpson's diversity	1.00	0.97	0.97	1.00	0.94	0.94	1.00	0.97	0.98	1.00	0.95	0.97
Good's coverage (%)	99.57	99.84	99.83	99.96	99.94	99.97	99.81	99.89	99.76	99.65	99.98	99.93
Taxonomic distribution												
Phylum	20	18	19	18	11	15	21	13	18	18	13	17
Class	48	40	39	44	22	30	45	26	36	48	24	33
Order	95	77	72	79	43	59	89	57	70	97	50	64
Family	163	118	111	132	66	96	143	94	123	167	78	109
Genus	291	194	191	225	117	152	263	167	218	283	126	174

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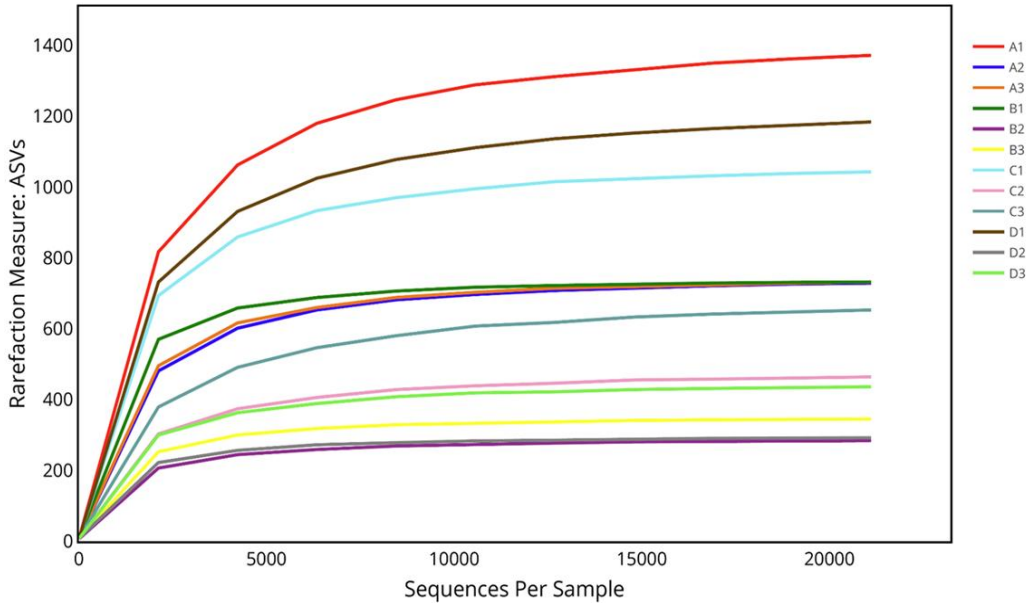


Figure 2. Rarefaction curves showing observed species richness in samples. The symbols A1, A2, and A3 represent the control group, which was evaluated on days 0, 30 and 60. Similarly, the experimental groups supplemented with different bacterial consortia were evaluated at the same time intervals, using the corresponding symbols: CB (B1, B2, B3), NS (C1, C2, C3), and TC (D1, D2, D3).

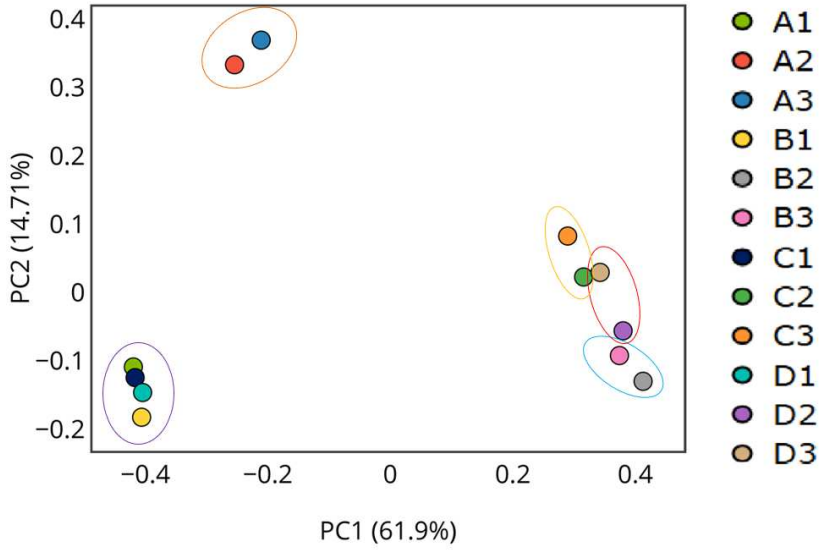
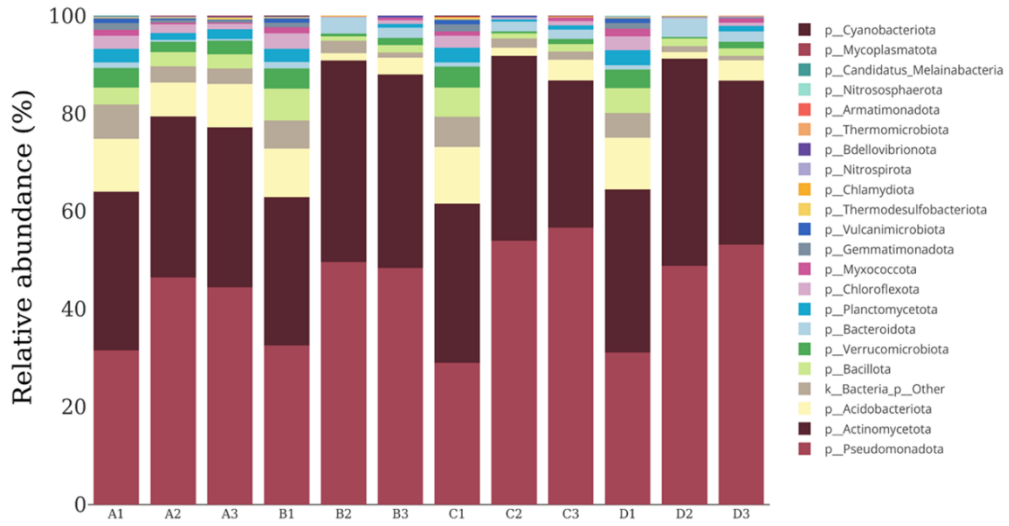


Figure 3. Principal coordinates analysis (PCoA) plot based on Bray-Curtis indices. The symbols A1, A2, and A3 represent the control group, which was evaluated on days 0, 30 and 60. Similarly, the experimental groups supplemented with different bacterial consortia were

753 evaluated at the same time intervals, using the corresponding symbols: CB (B1, B2, B3), NS (C1,
754 C2, C3), and TC (D1, D2, D3).
755
756



757 **Figure 4.** Relative abundance of microbial community composition at the phylum level.
758 The symbols A1, A2, and A3 represent the control group, which was evaluated on days 0, 30 and
759 60. Similarly, the experimental groups supplemented with different bacterial consortia were
760 evaluated at the same time intervals, using the corresponding symbols: CB (B1, B2, B3), NS (C1,
761 C2, C3), and TC (D1, D2, D3).
762

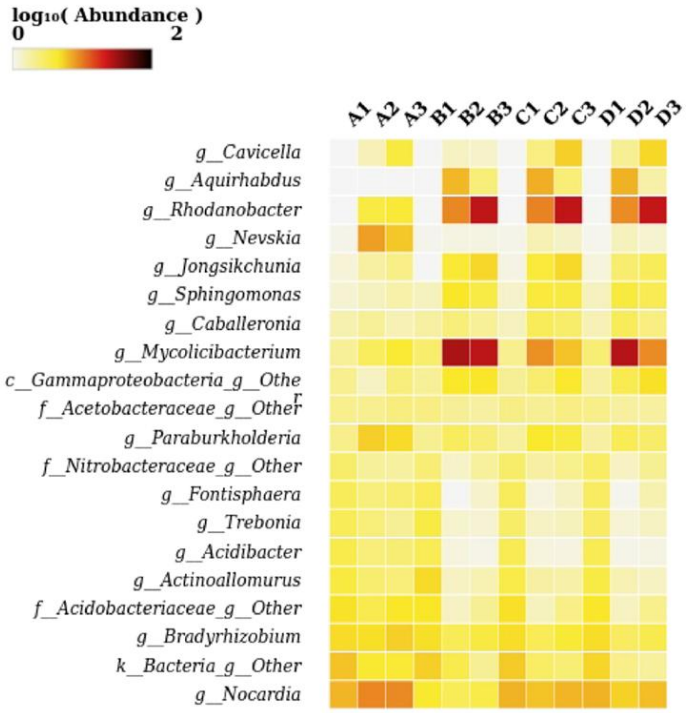


Figure 5. Heat map of the top 30 most abundant microbial genera. The symbols A1, A2, and A3 represent the control group, which was evaluated on days 0, 30 and 60. Similarly, the experimental groups supplemented with different bacterial consortia were evaluated at the same time intervals, using the corresponding symbols: CB (B1, B2, B3), NS (C1, C2, C3), and TC (D1, D2, D3).

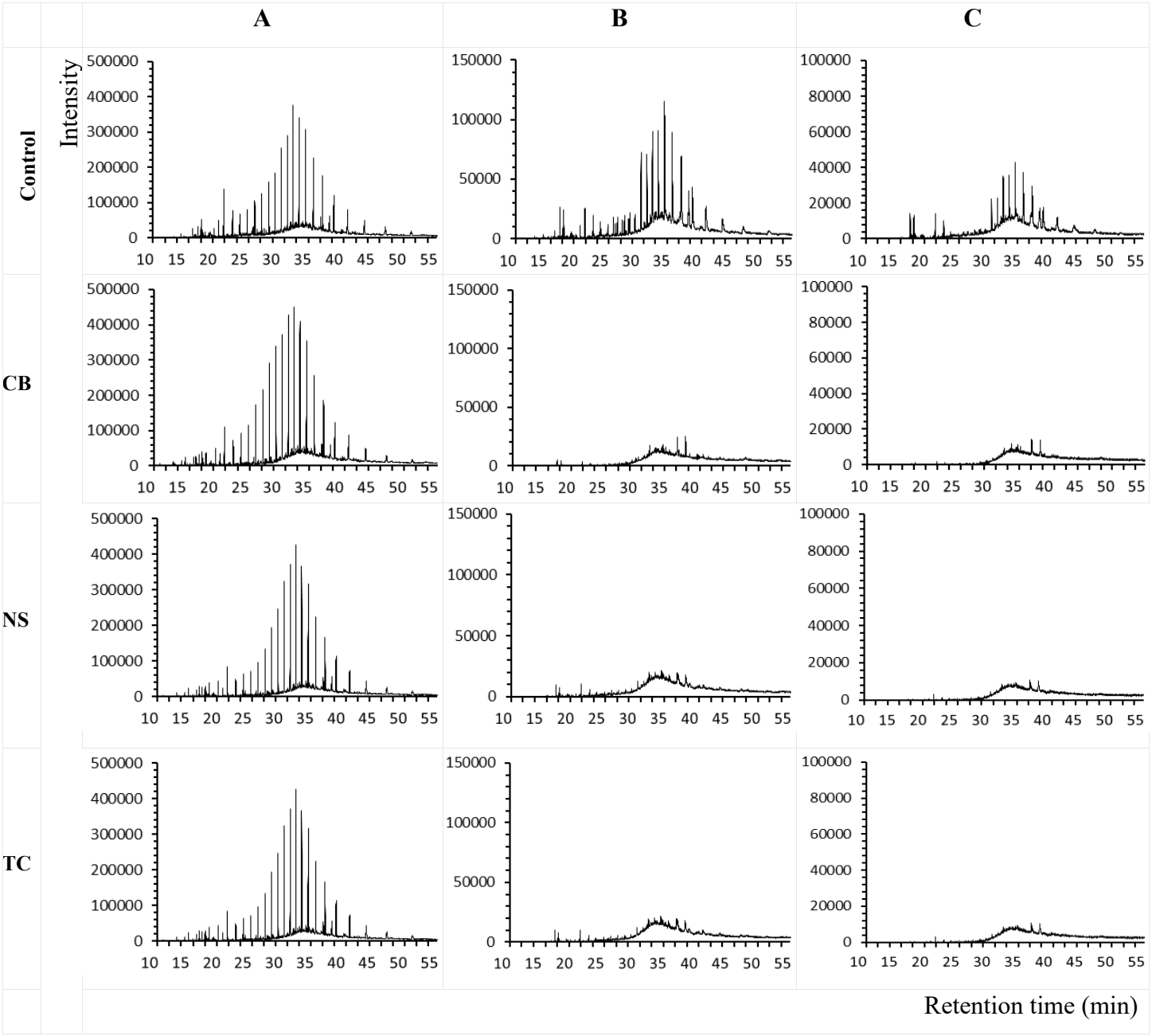


Figure 6. GC-MS chromatograms of unbioaugmented (control) and bioaugmented soil samples CB, NS, and TC: (A) Day 0, (B) Day 30, and (C) Day 60.

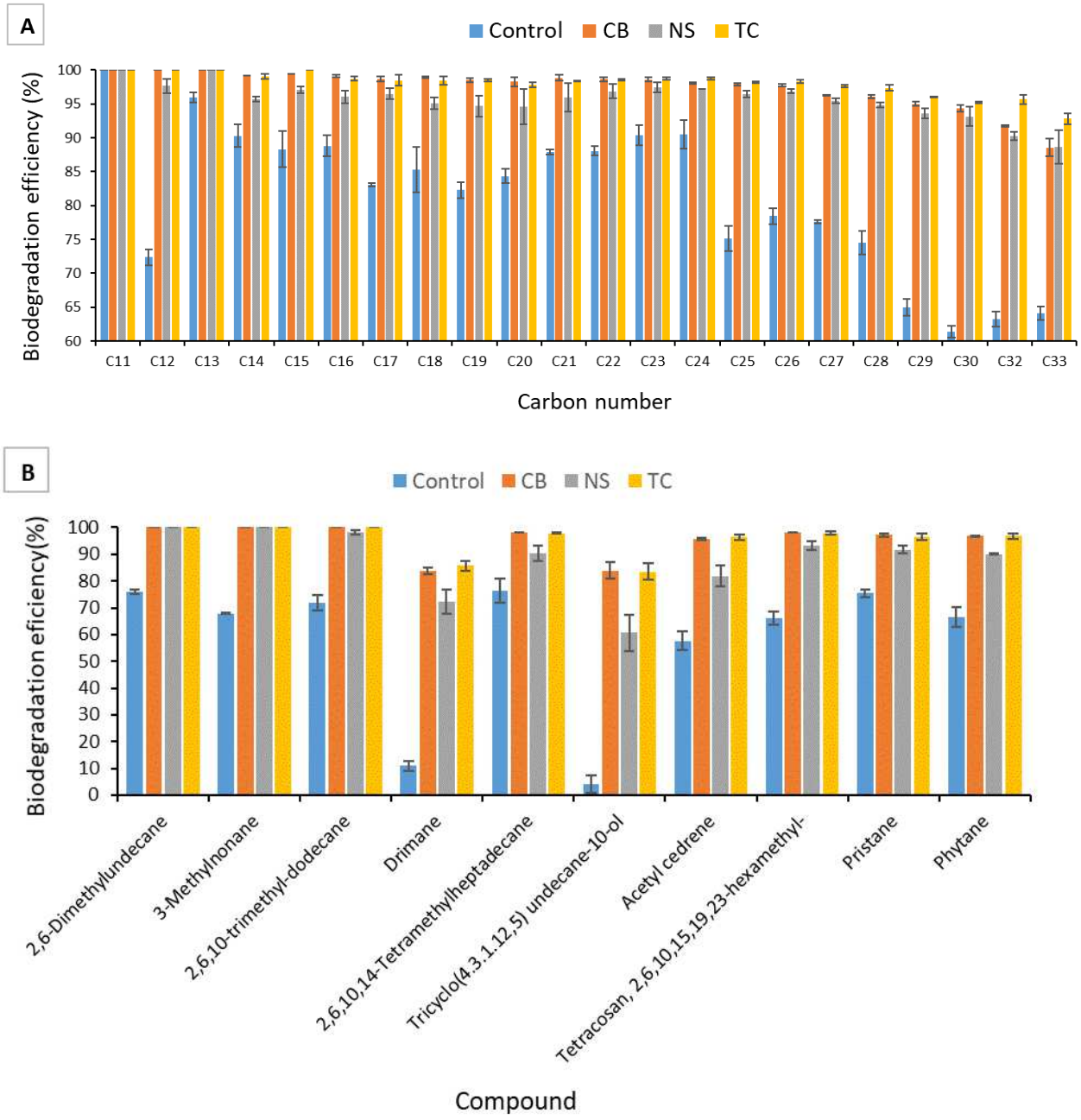


Figure 7. Biodegradation efficiencies of selected crude-oil components in the unbioaugmented control and soils bioaugmented with the CB, NS, and TC bacterial consortia after 30 days of incubation. (A) n-Alkanes (C11–C33) and (B) selected branched alkanes, branched isoprenoids, and cyclic compounds. Biodegradation efficiency was calculated as the percentage decrease in each compound relative to its initial value at day 0. Values are presented as mean ± SD of three soil subsamples.

Supplementary Files

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