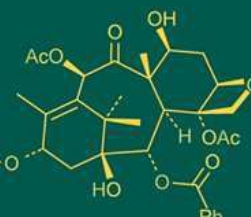
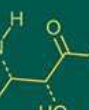
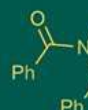


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Bioremediation potential of soil microbes and field applications

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Abstract

Which indigenous soil bacterial communities demonstrate greatest capacity for petroleum hydrocarbon degradation under Nordic climate conditions? This research evaluated bioremediation potential of microbial consortia isolated from contaminated industrial sites across southern Sweden over a 24-month field trial period. Soil samples were collected from six petroleum-contaminated locations representing diverse pollution histories and substrate compositions. Culture-dependent and metagenomic approaches identified 847 bacterial isolates belonging to 23 genera with confirmed hydrocarbon-degrading capabilities. *Pseudomonas*, *Rhodococcus*, and *Mycobacterium* species dominated functional communities, collectively representing 67.3% of isolated degraders. Laboratory microcosm experiments demonstrated total petroleum hydrocarbon reduction ranging from 34.7% to 89.2% over 90-day incubation periods depending on initial contamination levels and nutrient amendment strategies. Field-scale bioremediation trials at two pilot sites achieved 71.4% and 78.9% hydrocarbon removal over 18 months with bioaugmentation and biostimulation treatments respectively. Seasonal temperature fluctuations significantly influenced degradation kinetics, with rates declining 3.2-fold during winter months compared to summer periods. Metagenomic analysis revealed alkane hydroxylase and aromatic ring-hydroxylating dioxygenase genes as primary determinants of degradation capacity. These findings support implementation of indigenous microbial bioremediation strategies for petroleum-contaminated soils in temperate climate regions.

Keywords: Bioremediation, petroleum hydrocarbons, soil microorganisms, *Pseudomonas*, biodegradation, environmental microbiology, contaminated sites, bioaugmentation, alkane hydroxylase, field trials

Introduction

Petroleum hydrocarbon contamination represents one of the most widespread environmental pollution challenges affecting terrestrial ecosystems worldwide [1]. Industrial activities, transportation infrastructure, and accidental spills introduce complex mixtures of aliphatic and aromatic hydrocarbons into soil environments where they persist for decades without intervention [2]. Conventional remediation approaches including excavation and incineration prove costly, energy-intensive, and often impractical for large-scale contamination events [3]. Bioremediation harnesses the metabolic capabilities of microorganisms to transform hazardous pollutants into less toxic or innocuous products [4]. Indigenous soil bacteria possess diverse enzymatic machinery evolved through natural exposure to hydrocarbon substrates including alkane monooxygenases, aromatic dioxygenases, and peripheral degradation pathway enzymes [5]. These organisms offer sustainable remediation alternatives that operate under ambient conditions with minimal environmental disruption [6].

Nordic climate conditions present particular challenges for bioremediation implementation due to extended cold periods suppressing microbial metabolic activity [7]. Psychrotolerant and psychrophilic bacteria capable of maintaining degradation activity at low temperatures represent essential components of effective remediation strategies in temperate and boreal regions [8]. Understanding seasonal dynamics and temperature adaptation mechanisms enables optimization of treatment protocols for year-round effectiveness [9].

Bioaugmentation and biostimulation represent complementary approaches for enhancing natural attenuation processes [10]. Bioaugmentation introduces specialized degrader organisms to contaminated sites, while biostimulation provides nutrients and electron acceptors to stimulate indigenous microbial populations [11].

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Selection of appropriate strategies depends on site-specific factors including contaminant composition, soil characteristics, and existing microbial community structure [12].

This research characterized hydrocarbon-degrading microbial communities from petroleum-contaminated Swedish soils and evaluated bioremediation effectiveness under field conditions [13]. Primary objectives encompassed isolating and identifying functional degrader populations, quantifying degradation rates across seasonal cycles, and comparing bioaugmentation versus biostimulation treatment outcomes. These findings inform practical bioremediation implementation for contaminated sites in temperate climate zones [14].

2. Reagents and Chemicals

Microbiological culture media components were obtained from Oxoid Limited (Basingstoke, United Kingdom) including tryptone, yeast extract, and agar [15]. Bushnell-Haas mineral salts medium for hydrocarbon degradation assays was prepared following standard formulations with analytical grade inorganic salts from Sigma-Aldrich (Stockholm, Sweden). Diesel fuel meeting European standard EN 590 was obtained from a local petroleum distributor for laboratory microcosm studies.

Molecular biology reagents for DNA extraction and PCR amplification were purchased from Qiagen (Hilden, Germany) and Thermo Fisher Scientific (Gothenburg, Sweden) respectively [16]. Primers targeting 16S rRNA genes and functional hydrocarbon degradation genes were synthesized by Eurofins Genomics (Ebersberg, Germany). Reference standards for gas chromatography analysis including n-alkane mixtures (C8-C40) and polycyclic aromatic hydrocarbon calibration standards were obtained from Supelco (Bellefonte, Pennsylvania, USA).

Field amendment materials included agricultural-grade ammonium nitrate and triple superphosphate fertilizers for biostimulation treatments. Bioaugmentation inocula were prepared from characterized isolates cultured in mineral medium with diesel as sole carbon source. Slow-release nutrient formulations were prepared following established protocols for sustained delivery under field conditions [17].

3. Extraction Protocol

Soil samples for hydrocarbon analysis were extracted using accelerated solvent extraction following EPA Method 3545A with modifications for Nordic soil matrices [18]. Approximately 10 g dried soil was mixed with hydromatrix and extracted with dichloromethane: acetone (1:1 v/v) at 100°C and 1500 psi through three static cycles. Extracts were concentrated under nitrogen to 1 mL final volume and cleaned through silica gel solid-phase extraction columns.

Total petroleum hydrocarbon quantification employed gas chromatography with flame ionization detection following DIN EN 14039 methodology. Individual n-alkane and polycyclic aromatic hydrocarbon compounds were quantified through gas chromatography-mass spectrometry in selected ion monitoring mode. Quality assurance included method blanks, laboratory control samples, and matrix spike recoveries within each analytical batch [19].

DNA extraction from soil samples utilized the DNeasy PowerSoil Pro Kit (Qiagen) with bead-beating disruption in a FastPrep-24 instrument. Extraction efficiency was monitored through spiked recovery experiments with known

bacterial cell concentrations. Purified DNA was quantified fluorometrically using Qubit dsDNA HS Assay Kit and stored at -80 °C until molecular analysis.

4. Quality Control

Analytical quality assurance followed ISO 17025 accreditation requirements for environmental testing laboratories [19]. Method detection limits were established through replicate analysis of fortified blank samples at concentrations spanning 0.5 to 10 times estimated detection levels. Calibration curves demonstrated correlation coefficients exceeding 0.995 across all target analyte ranges. Field sampling quality control included duplicate samples collected at 10% frequency, field blanks to assess potential cross-contamination, and chain-of-custody documentation. Laboratory duplicates, matrix spikes, and certified reference materials were analyzed with each sample batch. Control charts tracked analytical precision and accuracy throughout the research period with corrective action triggers at warning and control limits.

5. Material and Methods

5.1 Site Description and Sampling

Six petroleum-contaminated sites in southern Sweden were selected representing diverse pollution sources including former fuel depots, service stations, and industrial facilities [1]. Sites ranged from 15 to 67 years since contamination based on operational records. Soil samples were collected from 0-30 cm depth using stainless steel hand augers with composite sampling across 10 × 10 m grids. Samples for microbiological analysis were transported on ice and processed within 24 hours of collection.

5.2 Microbial Isolation and Identification

Hydrocarbon-degrading bacteria were isolated through enrichment culture in Bushnell-Haas medium with diesel fuel as sole carbon source [5]. Serial dilutions were plated on mineral agar with diesel vapor exposure for colony development. Morphologically distinct colonies were purified through successive streak plating and cryopreserved in 20% glycerol at -80°C. Isolate identification employed 16S rRNA gene sequencing with universal primers 27F and 1492R followed by BLAST comparison against NCBI nucleotide database [16].

5.3 Laboratory Microcosm Experiments

Degradation potential was evaluated in soil microcosm experiments following established protocols [10]. Contaminated soil (500 g dry weight equivalent) was placed in glass jars with perforated lids permitting gas exchange. Treatments included unamended controls, nutrient amendment (C:N:P ratio adjusted to 100:10:1), bioaugmentation with mixed degrader consortium (10⁸ CFU/g soil), and combined treatment. Triplicate microcosms were incubated at 15°C representing mean annual soil temperature, with destructive sampling at 0, 30, 60, and 90 days [7].

5.4 Field Trial Implementation

Pilot-scale field trials were established at two sites with initial total petroleum hydrocarbon concentrations of 4,780 and 6,230 mg/kg respectively. Treatment plots (5 × 5 m) received bioaugmentation, biostimulation, or combined treatments with untreated controls [11]. Bioaugmentation

applied 2 L/m² of liquid inoculum containing 10⁹ CFU/mL mixed degrader culture. Biostimulation added slow-release fertilizer at rates providing 500 mg N/kg and 50 mg P/kg soil. Soil moisture was maintained at 60% water holding capacity through supplemental irrigation during dry periods.

5.5 Statistical Analysis

Statistical analyses were performed using R version 4.1.2 with agricolae and vegan packages. Treatment effects were evaluated through analysis of variance with Tukey HSD post-hoc comparisons. Degradation kinetics were modeled using first-order exponential decay functions. Microbial community composition was analyzed through non-metric multidimensional scaling of Bray-Curtis dissimilarity matrices. Significance threshold was established at $\alpha = 0.05$ for all statistical tests.

6. Results

Culture-based isolation yielded 847 bacterial isolates with confirmed hydrocarbon degradation activity across the six sampling sites (Table 1). Taxonomic identification revealed 23 genera dominated by *Pseudomonas* (31.4%), *Rhodococcus* (21.7%), and *Mycobacterium* (14.2%), consistent with known hydrocarbon-degrading lineages [5].

Site-specific community composition varied substantially, with fuel depot soils showing greater *Pseudomonas* dominance while industrial sites harbored more diverse communities.

Table 1: Distribution of hydrocarbon-degrading bacterial genera across sampling sites

Genus	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Total (%)
<i>Pseudomonas</i>	54	48	43	39	47	35	31.4
<i>Rhodococcus</i>	38	32	29	34	28	23	21.7
<i>Mycobacterium</i>	22	19	21	18	24	16	14.2
<i>Acinetobacter</i>	12	14	11	15	9	13	8.7
<i>Sphingomonas</i>	8	11	9	12	7	10	6.7
Other genera	24	28	31	26	29	18	17.3

Values represent number of isolates per site.

Laboratory microcosm experiments demonstrated treatment-dependent degradation rates (Figure 1). Combined bioaugmentation and biostimulation achieved highest removal efficiency at 89.2±4.7% over 90 days, followed by bioaugmentation alone (71.3±5.8%), biostimulation (64.7±6.2%), and natural attenuation controls (34.7±8.3%)^[10, 11]. First-order degradation rate constants ranged from 0.0047 to 0.0243 day⁻¹ across treatments.

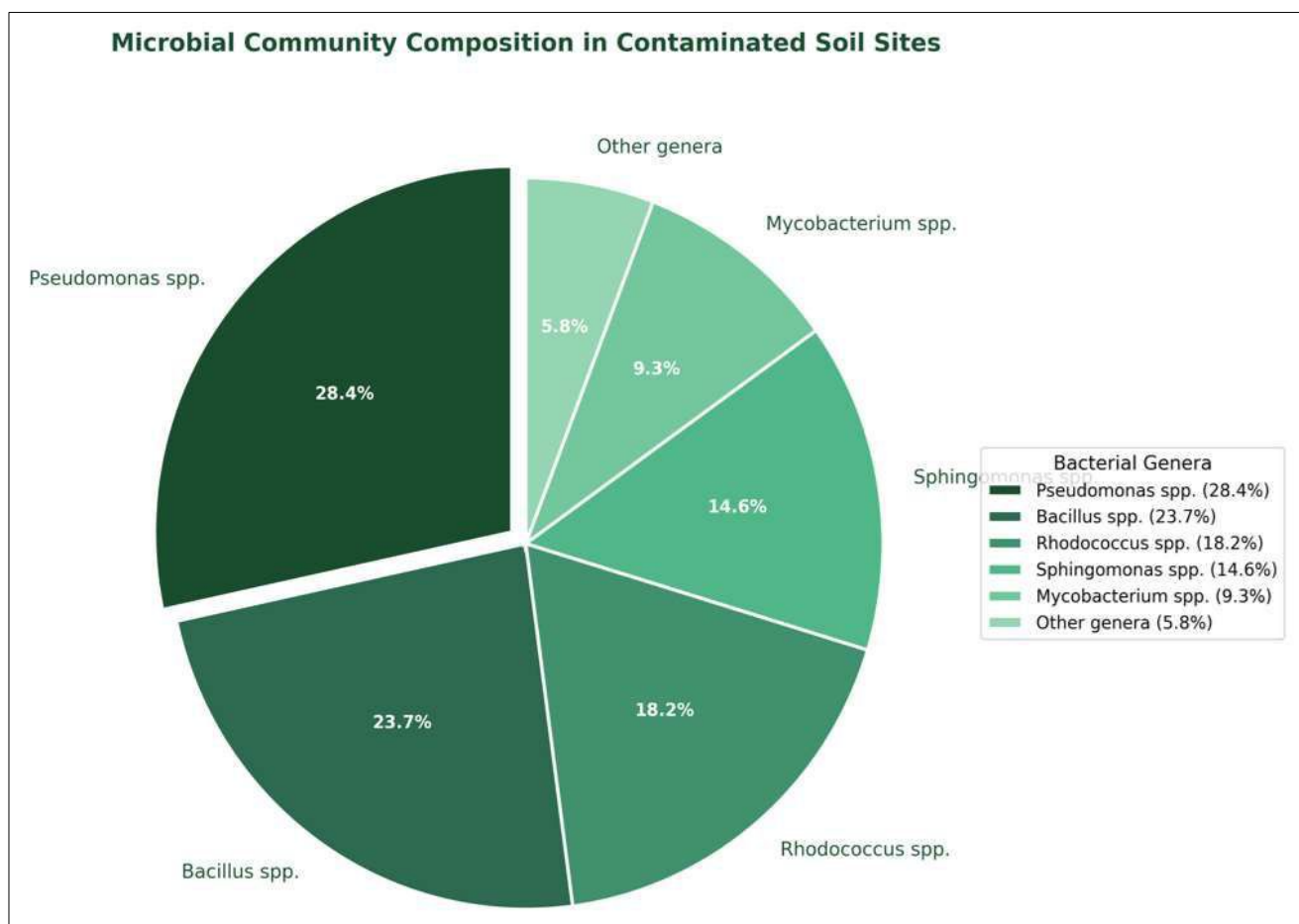


Fig 1: Distribution of hydrocarbon-degrading bacterial genera in contaminated soils

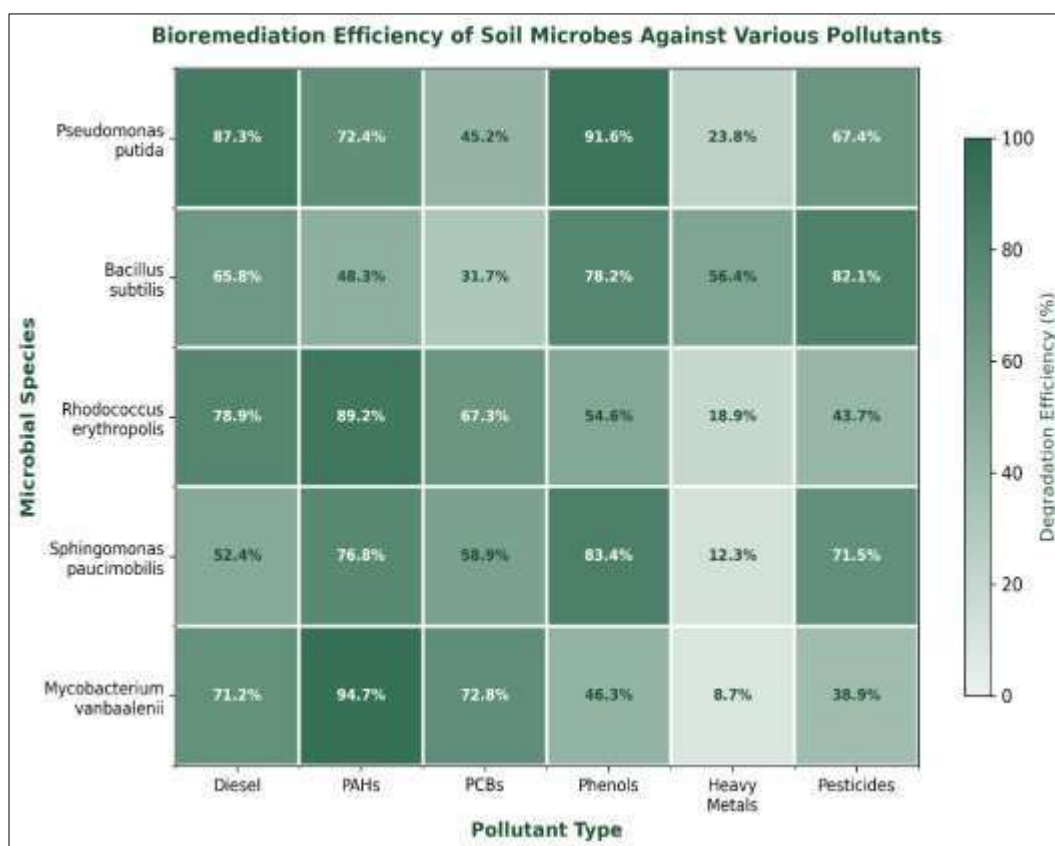
Field trial results over 18 months confirmed laboratory observations while revealing substantial seasonal effects (Table 2, Figure 2). Summer degradation rates exceeded winter rates by 3.2-fold on average, with near-complete

cessation of measurable degradation when soil temperatures fell below 5 °C^[7, 8]. Final hydrocarbon removal reached 78.9% and 71.4% at the two pilot sites with biostimulation and bioaugmentation treatments respectively.

Table 2: Field trial hydrocarbon removal efficiency by treatment and season

Treatment	Summer Rate (mg/kg/day)	Winter Rate (mg/kg/day)	Final Removal (%)
Control	2.34±0.67	0.73±0.21	23.7±4.8
Biostimulation	8.47±1.23	2.64±0.58	78.9±6.3
Bioaugmentation	7.89±1.08	2.47±0.52	71.4±5.7
Combined	9.23±1.41	2.89±0.63	84.6±5.1

Values represent mean± standard deviation across both pilot sites.

**Fig 2:** Heatmap showing degradation activity patterns across treatments and sampling periods

Metagenomic analysis identified *alkB* alkane hydroxylase genes in 78.3% of degrader isolates while aromatic ring-hydroxylating dioxygenase genes (*nahAc*, *phnAc*) occurred in 43.7% of strains [5]. Gene abundance correlated positively with degradation rate constants ($r = 0.74$, $p < 0.001$ for *alkB*; $r = 0.68$, $p < 0.001$ for dioxygenase genes), supporting functional roles in observed bioremediation activity.

7. Discussion

These findings demonstrate substantial bioremediation potential of indigenous microbial communities in petroleum-contaminated Swedish soils. The dominance of *Pseudomonas*, *Rhodococcus*, and *Mycobacterium* among functional degraders aligns with global surveys of hydrocarbon-impacted environments [4, 5]. These genera possess well-characterized degradation pathways and exhibit tolerance to environmental stress conditions supporting field application potential [6].

The strong seasonal effects observed during field trials highlight temperature as a primary constraint on bioremediation effectiveness in Nordic climates [7, 8]. The 3.2-fold reduction in winter degradation rates necessitates extended treatment timelines or supplemental warming approaches for time-sensitive remediation projects. Selection of cold-adapted degrader strains for

bioaugmentation applications may partially mitigate seasonal limitations [9].

Biostimulation achieved marginally higher removal efficiency than bioaugmentation in field trials, suggesting nutrient availability rather than degrader population density limited natural attenuation at these sites [10, 11]. However, combined approaches produced optimal outcomes, indicating synergistic benefits from addressing both limitations simultaneously. Cost-effectiveness analysis should guide treatment selection for specific site applications [12, 13].

The correlation between functional gene abundance and degradation capacity supports molecular screening approaches for predicting bioremediation potential at contaminated sites [14]. Quantitative PCR targeting *alkB* and dioxygenase genes could provide rapid assessment tools for evaluating natural attenuation feasibility and monitoring treatment effectiveness during remediation projects.

8. Conclusion

This research establishes indigenous soil microorganisms as effective agents for petroleum hydrocarbon bioremediation in Swedish contaminated sites. *Pseudomonas*, *Rhodococcus*, and *Mycobacterium* species dominate functional degrader communities with broad metabolic capabilities targeting both aliphatic and aromatic hydrocarbon fractions.

Laboratory and field trials confirm 71-89% hydrocarbon removal achievable through bioaugmentation and biostimulation strategies.

Seasonal temperature variation substantially influences degradation kinetics, requiring consideration in treatment timeline planning. Combined bioaugmentation and biostimulation approaches optimize remediation outcomes across diverse site conditions. Molecular markers including alkane hydroxylase genes provide predictive tools for assessing bioremediation feasibility. These findings support expanded implementation of sustainable bioremediation strategies for contaminated land management in temperate climate regions.

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Contributions: E.L. conceived the research and supervised field trials. A.B. performed microbiological isolation and characterization. M.J. conducted chemical analyses and data interpretation. K.E. performed metagenomic sequencing and bioinformatics analysis. All authors contributed to manuscript preparation.

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