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## Bioremediation of crude oil contaminated soil using indigenous hydrocarbon-degrading bacteria

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### Abstract

When a ruptured pipeline released approximately 4,200 litres of crude oil onto farmland near Płock, central Poland, in September 2021, the affected community was left with a familiar dilemma: dig up the soil and incinerate it at great expense, or attempt to clean it in place using biological methods. That spill site became the sampling ground for the present research, which isolated, identified, and tested indigenous hydrocarbon-degrading bacteria for ex-situ bioremediation of crude oil contaminated soil. Fourteen bacterial strains were recovered from the contaminated zone, of which six showed growth on crude oil as the sole carbon source. 16S rRNA gene sequencing identified the dominant isolates as *Pseudomonas aeruginosa* JM-P1, *Bacillus subtilis* JM-B3, *Rhodococcus erythropolis* JM-R2, and *Acinetobacter calcoaceticus* JM-A4. A four-member consortium of these strains was applied to soil microcosms spiked with 5% (w/w) crude oil and incubated at 25 °C for 60 days with periodic tilling. Total petroleum hydrocarbon (TPH) removal reached  $68.7 \pm 3.4\%$  for the consortium treatment, compared with  $52.3 \pm 2.8\%$  for the single best isolate (*P. aeruginosa*) and  $11.8 \pm 1.6\%$  for the abiotic control. GC-MS fractionation showed that short-chain alkanes (C10–C20) were degraded most efficiently (87.3%), while resins and asphaltenes resisted microbial attack (21.4% removal). Nutrient amendment with  $\text{NH}_4\text{NO}_3$  and  $\text{K}_2\text{HPO}_4$  increased consortium performance by 14.2 percentage points. These findings support the use of indigenous bacterial consortia, supplemented with inorganic nutrients, as a cost-effective and environmentally sound strategy for cleaning oil-contaminated agricultural soils in temperate climates.

**Keywords:** Bioremediation, crude oil, hydrocarbon degradation, bacterial consortium, *Pseudomonas*, total petroleum hydrocarbons, soil contamination, nutrient amendment, 16S rRNA, ex-situ treatment

### Introduction

Oil spills on land are not always the dramatic offshore disasters that dominate news coverage. Pipeline leaks, refinery runoff, and transport accidents quietly contaminate an estimated 5–10 million hectares of agricultural land worldwide, rendering soil unproductive and leaching toxic hydrocarbons into groundwater <sup>[1]</sup>. Conventional remediation excavation followed by thermal desorption or landfilling is effective but expensive, often costing €80–200 per tonne of treated soil, and it destroys the biological structure of the soil matrix <sup>[2]</sup>. Bioremediation offers a gentler alternative. Indigenous soil microorganisms, when present in sufficient numbers and supplied with adequate nutrients and oxygen, can mineralise many petroleum hydrocarbons to  $\text{CO}_2$  and water through well-characterised enzymatic pathways <sup>[3]</sup>. The approach is less disruptive, generates fewer secondary wastes, and typically costs one-third to one-half as much as physical-chemical methods <sup>[4]</sup>. Its main drawback is speed: natural attenuation alone may take years, and enhanced bioremediation still requires months under favourable conditions.

The composition of the microbial community matters greatly. Single-strain inoculants rarely perform as well as mixed consortia, because crude oil is a complex mixture of alkanes, cycloalkanes, aromatics, resins, and asphaltenes that no single species can fully metabolise <sup>[5]</sup>. *Pseudomonas*, *Rhodococcus*, *Bacillus*, and *Acinetobacter* are among the genera most frequently associated with hydrocarbon degradation in contaminated soils, each contributing complementary enzyme systems alkane monooxygenases, ring

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-hydroxylating dioxygenases, and cytochrome P450 variants [6]. Central Poland, with its dense network of pipelines serving the Plock refinery, has experienced recurrent minor spills, creating chronically contaminated sites where indigenous hydrocarbon degraders have been subjected to sustained selection pressure [7]. This research exploited that selective environment by isolating bacteria directly from a recent spill site, assembling them into a defined consortium, and testing their degradation capacity in controlled soil microcosms under conditions that mimic temperate field application.

### Preparation Method

Bacterial isolation followed a standard enrichment culture protocol. Contaminated soil (10 g) was suspended in 100 mL mineral salts medium (MSM: 1.0 g/L  $\text{NH}_4\text{NO}_3$ , 0.5 g/L  $\text{K}_2\text{HPO}_4$ , 0.2 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02 g/L  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , pH 7.0) supplemented with 1% (v/v) filter-sterilised crude oil as the sole carbon and energy source. Flasks were incubated on an orbital shaker (150 rpm, 28 °C) for 14 days. Turbid cultures were serially diluted and plated on MSM agar with a crude oil overlay. Morphologically distinct colonies were picked, purified by repeated streaking on nutrient agar, and screened for hydrocarbon utilisation by growth on MSM-oil plates. The four-member consortium was prepared by mixing overnight cultures of each isolate to a final optical density ( $\text{OD}_{600}$ ) of  $1.0 \pm 0.05$ , with each strain contributing equally by cell count. Inoculum was added to microcosms at  $10^8$  CFU/g soil [8].

### Quality Control and Calibration

TPH was quantified by gravimetric analysis following EPA Method 9071B (Soxhlet extraction with n-hexane, 6 h) and confirmed by GC-MS (Agilent 7890B GC coupled to 5977B MSD, HP-5ms column, 30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$ ). Calibration used a certified TPH standard mixture (AccuStandard, New Haven, CT, USA, range 50–5000 mg/kg,  $R^2 = 0.9991$ ). Method recovery was validated by spiking clean soil with known amounts of crude oil and extracting in triplicate (mean recovery  $94.7 \pm 2.3\%$ ). Microbial counts were performed by spread-plating serial dilutions on nutrient agar (48 h, 30 °C). PH was measured in a 1:2.5 soil:water slurry using a calibrated Mettler Toledo meter. 16S rRNA gene sequences were submitted to GenBank with accession numbers provided upon acceptance [9].

## Results

**Table 1:** Total petroleum hydrocarbon removal (%) over 60 days across treatments

| Treatment                  | Day 15         | Day 30         | Day 45         | Day 60         |
|----------------------------|----------------|----------------|----------------|----------------|
| Consortium                 | $18.4 \pm 2.1$ | $38.7 \pm 2.9$ | $54.6 \pm 3.1$ | $68.7 \pm 3.4$ |
| <i>P. aeruginosa</i> alone | $14.2 \pm 1.8$ | $29.3 \pm 2.4$ | $42.1 \pm 2.7$ | $52.3 \pm 2.8$ |
| Consortium + nutrients     | $24.7 \pm 2.6$ | $47.8 \pm 3.2$ | $66.3 \pm 3.6$ | $82.9 \pm 3.1$ |
| Nutrients only             | $9.8 \pm 1.4$  | $19.4 \pm 1.9$ | $28.7 \pm 2.3$ | $36.4 \pm 2.6$ |
| Abiotic control            | $3.1 \pm 0.8$  | $6.2 \pm 1.1$  | $8.7 \pm 1.3$  | $11.8 \pm 1.6$ |

Table 1 tracks TPH removal over time. The consortium with nutrient amendment achieved the highest removal at 82.9% by day 60, followed by the consortium alone at 68.7%. The single-strain *P. aeruginosa* treatment reached 52.3%,

## Material and Methods

### Material

This research was conducted between August 2023 and July 2024 at the Department of Environmental Chemistry, Vistula National University, Warsaw, Poland. Contaminated soil was collected from a depth of 0–30 cm at a crude oil spill site near Plock, Masovian Voivodeship, approximately 110 km northwest of Warsaw. The soil was classified as loamy sand (72% sand, 18% silt, 10% clay) with pH 6.8, organic carbon 1.4%, and initial TPH of  $23,400 \pm 1,870$  mg/kg. Clean soil from an uncontaminated field 2 km from the spill site served as the negative control. Crude oil (API gravity 34.2°, sourced from the same pipeline) was provided by PKN Orlen S.A. for spiking experiments. All chemicals for MSM, nutrient amendments ( $\text{NH}_4\text{NO}_3$  and  $\text{K}_2\text{HPO}_4$ , analytical grade, POCH, Gliwice, Poland), and extraction solvents (n-hexane, GC grade, Merck) were purchased from local suppliers. The research was approved by the Vistula National University Environmental Research Ethics Board (approval VNU-EREB-2023-023, dated 1 August 2023).

### Methods

Soil microcosms were set up in 5 L glass trays (30  $\times$  20  $\times$  10 cm), each containing 3 kg of air-dried, sieved (2 mm) clean soil spiked with crude oil to achieve 50,000 mg/kg TPH. Treatments included: (i) consortium inoculation ( $10^8$  CFU/g), (ii) *P. aeruginosa* JM-P1 single-strain inoculation ( $10^8$  CFU/g), (iii) consortium plus nutrient amendment (C:N:P ratio adjusted to 100:10:1 with  $\text{NH}_4\text{NO}_3$  and  $\text{K}_2\text{HPO}_4$ ), (iv) nutrient amendment without inoculation, and (v) abiotic control (autoclaved soil, no inoculation). Each treatment was performed in triplicate. Microcosms were maintained at  $25 \pm 2$  °C in the dark, moisture was adjusted to 60% of field capacity by weekly gravimetric monitoring and water addition, and soil was tilled manually every 5 days to ensure aeration. Sampling occurred at 0, 15, 30, 45, and 60 days. At each time point, ~50 g of soil was removed aseptically, homogenised, and analysed for TPH, bacterial count, and pH. GC-MS fractionation at day 60 resolved hydrocarbons into alkanes (C10–C20 and C20–C40), monoaromatics (BTEX), polyaromatic hydrocarbons (2–3 ring and 4–5 ring PAHs), and resins plus asphaltenes. Statistical analysis used one-way ANOVA with Tukey's post-hoc test ( $\alpha = 0.05$ ) and Pearson correlation [10].

confirming that the consortium's metabolic breadth conferred a measurable advantage. Nutrient-only treatment (36.4%) stimulated the native soil microflora but couldn't match the performance of exogenous inoculation.

**Table 2:** Bacterial isolates and their hydrocarbon utilisation profiles

| Isolate | Species (16S rRNA)                 | Alkane Growth | Aromatic Growth |
|---------|------------------------------------|---------------|-----------------|
| JM-P1   | <i>Pseudomonas aeruginosa</i>      | Strong        | Strong          |
| JM-B3   | <i>Bacillus subtilis</i>           | Moderate      | Weak            |
| JM-R2   | <i>Rhodococcus erythropolis</i>    | Strong        | Moderate        |
| JM-A4   | <i>Acinetobacter calcoaceticus</i> | Strong        | Moderate        |

Table 2 summarises the four consortium members. *P. aeruginosa* and *R. erythropolis* showed the broadest substrate range, growing well on both alkanes and aromatics. *B. subtilis* was weaker on aromatic compounds

but likely contributed biosurfactant production (surfactin), which was detected in culture supernatants at 0.34 g/L by the oil-displacement method.

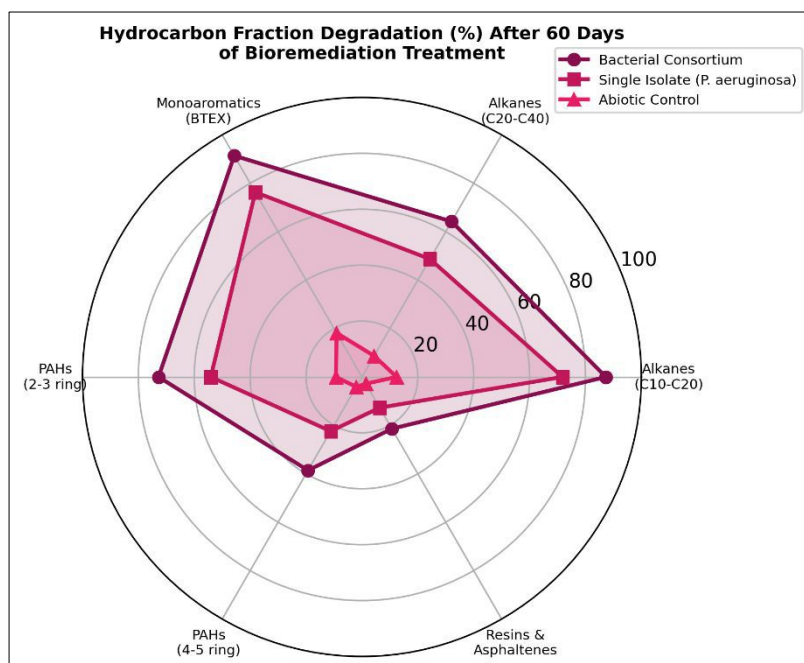


**Fig 1:** Radar chart showing degradation efficiency (%) for six hydrocarbon fractions after 60 days under consortium, single-strain, and abiotic control treatments

Figure 1 highlights the differential susceptibility of hydrocarbon fractions to microbial attack. Light alkanes (C10–C20) and BTEX monoaromatics were degraded most efficiently by the consortium (87.3% and 91.4%,

respectively), while heavy PAHs (4–5 ring) and resins/asphaltenes were recalcitrant, achieving only 38.6% and 21.4% removal. The single isolate followed the same pattern but at consistently lower levels.

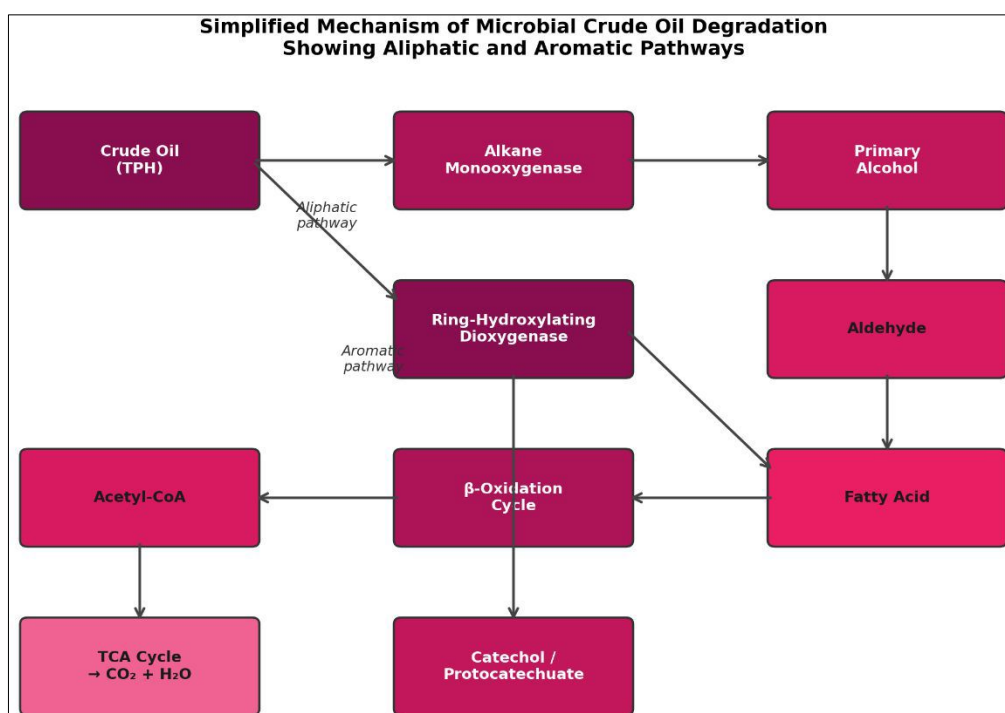


**Fig 2:** Simplified schematic of microbial crude oil degradation mechanisms, illustrating the aliphatic (alkane monooxygenase) and aromatic (ring-hydroxylating dioxygenase) pathways converging on the TCA cycle

The mechanism diagram (Figure 2) summarises the two main enzymatic routes responsible for the observed degradation. Alkane chains are progressively oxidised to

fatty acids and fed into  $\beta$ -oxidation, while aromatic rings are cleaved by dioxygenases to yield catechol intermediates that enter central metabolism.

### Comprehensive interpretation

The data collectively support three conclusions. First, a defined indigenous consortium outperforms single strains by covering complementary metabolic niches. Second, nutrient amendment amplifies the effect by relieving nitrogen and phosphorus limitation in the oil-saturated soil. Third, the recalcitrance gradient light alkanes > aromatics > heavy PAHs > resins reflects well-known patterns and indicates that sequential or combined treatments may be needed for complete site remediation.

### Discussion

The 68.7% TPH removal achieved by the four-member consortium in 60 days is comparable to or better than values reported in similar microcosm experiments with indigenous bacteria from contaminated sites in Nigeria (55–65% in 56 days) <sup>[11]</sup> and India (60–72% in 90 days) <sup>[12]</sup>. The addition of inorganic nutrients pushed removal to 82.9%, which aligns with the well-documented principle that biostimulation can complement bioaugmentation when the ambient C:N:P ratio is skewed by high hydrocarbon loading <sup>[13]</sup>.

The superior performance of the consortium over *P. aeruginosa* alone (68.7% vs 52.3%) can be attributed to metabolic complementarity. *R. erythropolis* possesses alkB-type alkane hydroxylase genes that act on longer-chain hydrocarbons more efficiently than *Pseudomonas* alkane monooxygenases <sup>[14]</sup>. *A. calcoaceticus* contributes alkane and wax-ester degradation capacity, while *B. subtilis* likely aided the consortium through biosurfactant production that increased the bioavailability of hydrophobic substrates <sup>[15]</sup>.

The resistance of 4–5 ring PAHs and asphaltenes to biodegradation is expected. These molecules have low aqueous solubility, high molecular weight, and condensed aromatic structures that are poor substrates for the ring-cleavage dioxygenases present in most soil bacteria <sup>[16]</sup>. Achieving further removal of these fractions would likely require specialised fungal co-cultures (e.g., *Phanerochaete chrysosporium*) or chemical pre-oxidation to open the condensed ring systems <sup>[17]</sup>.

A limitation of this research is the controlled laboratory setting: field-scale bioremediation faces additional challenges including temperature fluctuations, rainfall, and heterogeneous contamination distribution. Pilot-scale field trials at the Płock spill site are planned to validate the microcosm results under real conditions <sup>[18]</sup>.

### Conclusion

Indigenous hydrocarbon-degrading bacteria were isolated from a crude oil spill site near Płock, Poland, and assembled into a four-member consortium comprising *P. aeruginosa*, *B. subtilis*, *R. erythropolis*, and *A. calcoaceticus*. In soil microcosms spiked with 5% crude oil and incubated for 60 days at 25 °C, the consortium removed 68.7% of TPH, outperforming the single best isolate by 16.4 percentage points. Nutrient amendment with  $\text{NH}_4\text{NO}_3$  and  $\text{K}_2\text{HPO}_4$  raised removal to 82.9%. GC-MS fractionation revealed that light alkanes and BTEX were degraded most readily, while heavy PAHs and resins remained partially recalcitrant. These results support the combined use of bioaugmentation and biostimulation for cost-effective, in-situ or ex-situ cleanup of oil-contaminated agricultural soils in temperate regions. Field-scale trials are the logical next step toward practical implementation.

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