Original Research Article

Bioremediation of Petroleum Hydrocarbon-Contaminated Soil using Activated Sludge Derived from *Vitellaria paradoxa* Seed Wastewater

ABSTRACT

Contamination of soil with petroleum hydrocarbons (HCs) is a serious environmental challenge, posing severe immediate and long-term impacts as many of their constituents are toxic, mutagenic and/or carcinogenic. In this study, activated sludge derived from *Vitellaria paradoxa* nut wastewater was evaluated for its most effective component in bioenhancement of a sandy soil that is low in nutrients and endogenous microbial affinity for HCs (<10% of HC concentrations converted to CO_2 in 30 d). Soils contaminated with aviation fuel, kerosene, petrol, diesel and crude oil ($1\%_{w/w}$ and $2\%_{w/w}$) were bioenhanced with (i) HC-adapted mixed-culture degraders derived from activated sludge (bioaugmentation only), (ii) sterilised sludge (biostimulation only), and (iii) activated sludge (combined bioaugmentation and biostimulation). Mineralization, substrate-induced respiration, microbial biomass-C and PAH-degrading *Pseudomonas* population were monitored in the HC-contaminated soils over 30 d using standard methods. A consortium of HC-adapted degraders (applied at 1.2×10^6 cfu·g_{soil}^{-1}) consistently produced significantly ($p < 0.05$) greatest extents of HC mineralization (362–450 *vs.* 58–87 mgCO_2·$10g_{soil}^{-1}$ in non-amended HC-contaminated soils). There was no much difference in the enhancement using sterilised sludge or activated sludge (199–376 *vs.* 204–312 mgCO_2·$10g_{soil}^{-1}$ respectively). Increases in PAH-degrading Pseudomonas number from 3.4×10^4 in control soil to x.x–y.y $\times 10^6$ cfu·g_{soil}^{-1} in HC-contaminated soils were observed. Ecophysiological indices (e.g. microbial metabolic quotient qCO_2, microbial respiratory efficiency MRE, and microbial respiratory coefficient Q_R) showed that the augmented degraders were well-adapted to the HC-contaminated soils with strong biodegradability, particularly to heavier HCs diesel and crude oil.

Keywords: activated sludge, bioaugmentation, bioremediation, biostimulation, petroleum hydrocarbon, soil, *Vitellaria paradoxa*

INTRODUCTION

The microbial degradation of petroleum hydrocarbon contaminants is very efficient in contaminated land clean-up. The use of mixed microbial cultures over pure culture has been found to be more effective in bioremediation of hydrocarbon contaminated soil (Elshafei *et al*., 2024).

Biodegradation has been established as a very cost-effective method employed to mop up hydrocarbons contaminating the environment.

In Nigeria, there is an average of 300 oil spills every year. Environmentalists consider the Niger Delta region of the country as one of the world's most polluted regions (Sojinu *et al.*, 2010; Eseoghene *et al.,* 2022)

Most of the terrestrial oil spills in this region occur in areas with difficult terrain, which hinders *in situ* biodegradation studies. These concerns influenced the need to research and to develop an effective but affordable bioremediation strategy to assist in managing contaminated soils.

Microbial remediation of a hydrocarbon-contaminated site is accomplished with the help of a diverse group of microorganisms, particularly the indigenous bacteria present in soil. These

microorganisms can degrade a wide range of target constituents present in oily sludge (Barathi *et al.,* 2001). A large number of *Pseudomonas* strains capable of degrading PAHs have been isolated from soil and aquifers (Esin Eraydin Erdoğan *et al.,* 2012)

A critical factor in deciding whether bioremediation is the appropriate clean-up remedy for a site is whether the contaminants are susceptible to biodegradation by the organisms at the site (or by organisms that could be successfully added to the site). Although existing microorganisms can detoxify a vast array of contaminants, some compounds are more easily degraded than others. Bioaugmentation also solves the difficulty of lack of microorganisms with the microbial capabilities of degrading hydrocarbons in the contaminated sites, a major limiting factor in bioremediation.

Properties that reflect the biomass, activity and diversity of microbial community populations in the soil serve as useful indicators of the impact of outside disturbances (including HC contamination) on soil health. The qCO_2 and Q_R can also be used to evaluate the metabolic efficiency of the soil microbial community (Ashraf *et al.,* 2022)

MATERIALS AND METHODS

2.1 Materials

Soil, sampled from the Ah horizon (5–25 cm), was collected from a conserved section of the experimental plot in the botanical garden of the University Ilorin, Nigeria. The plot of land from which the soil was sample has never been exposed to chemical fertiliser or pesticide use and no history of contamination with petroleum hydrocarbons. The plot had not been tilled or planted with crops in the past 25 years. Activated sludge was obtained after anaerobic digestion of wastewater effluent from butter processing of shea nuts (*Vitellaria paradoxa*). The sludge material was grinded with mortar and pestle to provide homogenous powder for ease of amendment to soil. Both the soil and activated sludge samples were analysed for physiochemical and microbiological characteristics. Standard methods used for these analyses have been described elsewhere (Caravaca and Roldán, 2003; APHA 2023). Petroleum hydrocarbons including kerosene (specific gravity 0.82 and C-content 84%), petrol (specific gravity 0.76 and C-content 83%) and diesel (specific gravity 0.85 and C-content 87%) were purchased from commercial fuel stations in Ilorin, while aviation fuel (specific gravity 0.72 and C-content 88%) was donated by CITA Petroleum Limited, Lagos, Nigeria. Bonny medium crude oil (specific gravity 0.87 and C-content 85%) was obtained from Warri Refining and Petrochemical Industry (WRPC), Warri, Nigeria. The microbiological culture media used in this experiment include nutrient broth (NB), nutrient agar (NA), MacConkey agar (MCA), eosin methylene blue agar (EMB), salmonella-shigella agar (SSA), sorbitol MacConkey agar supplemented with cefixime-tellurite (CT-SMAC), pseudomonas agar supplemented with antibiotics centrimide-fucidine-cephalosporin (pseudomonas-CFC agar), Bushnell Hass broth (BHB), and mineral salt medium (MSM). NB, NA, MCA, EMB, SSA, CT-SMAC and pseudomonas-CFC agar were products of Oxoid Ltd., UK. Chemicals used to constitute BHB and MSM as well as all other reagents were of analytical grades and obtained from Sigma Aldrich, UK. The culture media were prepared according to manufacturers' instructions or following established protocols (APHA 2023). Where necessary, sterilization was by autoclaving at 121^{o}C for 15 minutes.

2.2 Soil contamination with petroleum hydrocarbons

Before contamination, stones and large particle materials were hand-picked from the soil, and it was then sieved through a 2 mm mesh. Subsamples of soil were air dried at 25^{o}C for 48 h to *ca*. 60% of the water holding capacity (WHC), sieved (≤2 mm) and conditioned at 25 ± 1°C in the dark for 5 d to allow microbial activity to stabilize (Kemmitt et al., 2008). Various amounts of the petroleum hydrocarbons were spiked into 600 g of soil following the single-step amendment procedure as described by Doick *et al*. (2003) to give final concentrations of 1%$_{w/w}$ and 2%$_{w/w}$ (10 and 20 g_{oil} $kg^{-1}{}_{soil}$ DW, respectively). The established spiking technique

guaranteed thorough mixing and even distribution of hydrocarbons in soil. Moisture content of the HC-contaminated soil subsamples was adjusted to its 60% water holding capacity (WHC). Each HC-contaminated soil subsample was divided equally (200 g) into brown wide mouth bottles and covered with perforated aluminum foil and placed in the dark (25^{o}C, 70% humidity). The HC-contaminated soils were analysed for basal respiration, substrate-induced respiration, microbial biomass, and Pseudomonad number as described below.

2.3 Soil and sludge pre-treatments

Portions of the soil and sludge were sterilized over three cycles of autoclaving with intermediate 2-d incubation at favourable temperature to allow germination of the spores. This is to ensure completely destruction of both the vegetative and spores of microorganisms present in the sample.

2.4 Enrichment and recovery of hydrocarbon-degrading bacteria in sludge

Mixed-culture of bacteria capable of degrading hydrocarbons was recovered from the sludge through sequential enrichment technique using BHB, a selective medium for isolation of hydrocarbon-degrading bacteria. Compositions of BHB (g/l): $MgSO_4$ (0.20), $CaCl_2$ (0.02), K_2HPO_4 (1.00), KH_2PO_4 (1.00), NH_4NO_3 (1.00), Fe_2Cl_3 (0.05); final pH at 25^{o}C (7.0). Portions (5 g) of the activated sludge were introduced into flasks containing 100 ml of BHB and spiked with 0.5 ml of petrol. The flasks were placed on orbital shaker–incubator (150 rpm, 37^{o}C). After 4 d incubation, 10 ml aliquot of the culture broth was sub-cultured into fresh 100 ml BHB and spiked 1 ml of petrol as the source of carbon. This process was repeated four times to select hydrocarbon-degrading species with high biodegradability.

2.5 Harvest and standardisation of bacterial mixed-culture

Cell cultures were harvested by centrifugation (7000 ×*g* for 15 minutes) to recover nutrient-free cell pellets. Cell washing in phosphate buffer saline (PBS) by centrifugation was repeated three times to obtain pure cell pellets which were later suspended in PBS and stored at 4^{o}C until use and not more than 2 d. PBS compositions (g/l): NaCl (8), KCl (0.2), Na_2HPO_4 (1.44), KH_2PO_4 (0.24); pH adjusted to 6.98. The density of the cell suspension was compared with the McFarland equivalence turbidity standard and cell number estimated on a spectrophotometer (OD_{600nm}) to approximately 1.2 x 10^8 cells per ml.

2.6 Quantification of endogenous and substrate-induced mineralization in HC-contaminated soils treated with different amendments

For quantification of endogenous biodegradation, a measured volume (30 ml) of pre-sterilised MSM (0.3 g NaCl, 0.6 g $(NH_4)_2SO_4$, 1.5 g $MgSO_4.7H_2O$, 0.6 g KNO_3, 0.25 g KH_2PO_4, 0.75 g Na_2HPO_4 in one litre of deionized water) was added to 10 g of the uncontaminated control (SC) or HC-contaminated soils (SH) in modified 250-ml Schott respiratory bottles with Teflon®-lined screw caps, in triplicates (Fenlon *et al*., 2011). A set of respiratory bottles containing only MSM and HCs without soil was setup as negative control blanks to correct for the abiotic release of CO_2. The MSM contained both N and P sources to ensure that these nutrients were not limiting microbial growth and metabolism of HCs in the soil. A plain bottle containing 2 ml of 1 M NaOH was suspended in the respiratory bottle to trap the CO_2 evolved during mineralization. The respiratory bottles were incubated on an orbital shaker–incubator (100 rpm, 30^{o}C) and sampled at defined intervals for 30 d. The NaOH solution was precipitated with 3 N $BaCl_2$ and the amount of CO_2 trapped was determined by Warder titrimetric method using standardized 1 M HCl and quantifying the acid consumed between pH 8.3 and 3.7 .

To evaluate the effect of sludge amendment on biodegradation of HCs in soil, three regimens were used: (i) mixed-culture inoculum derived from the activated sludge (i.e. bioaugmentation; SHIN), (ii) sterilised activated sludge (i.e. biostimulation; SHSS), or (iii) untreated activated sludge (combined bioaugmentation and biostimulation; SHAS). For both SHSS and SHAS amendments, 1 g of the sludge material was spiked into the respiratory bottles. For the SHIN

amendment, 0.1 ml of the hydrocarbon-degrading cell suspension was added. All treatment microcosms, set up in triplicates, were further handled as described above.

For the substrate-induced respiration (SIR), carried out after 1 d and 30 d following soil contamination with HCs, the setup was slightly modified from that described above. Soil sample (10g) was suspended in 10 ml of sterilised MSM which was augmented with 5.6 $mg \cdot g^{-1}$ glucose (the saturation concentration was determined in a preliminary test). Only SHIN amendment was evaluated for the SIR experiment. The respiratory bottles were placed on an orbital shaker–incubator (100 rpm, 30°C) and sampled at 2, 4, 8, 12, 24, 48, 72, 96 and 120 h. The CO_2 evolved was quantified as previously described.

2.7 Estimation of soil microbial biomass-carbon in amended soils

Soil microbial biomass carbon (SMB-C: an indicator of the overall size of the soil microbial community) was estimated by the substrate-induced respiration method initially described in Domsch and Anderson (1978) with little modification. Substrate-induced respiration, developed to measure the response of the 'metabolically active' component of the soil microbial community , reflects the size of the potentially active microbial biomass since it evaluates the maximum potential respiratory activity, not the actual activity. This technique has been widely applied to determine SMB-C in agricultural and contaminated soils. The setup for the substrate-induced mineralization assay was also employed with the data obtained after the 2–4 h mineralization employed to calculate the SMB-C using the formula proposed by. This method is based on the initial maximal respiratory response of microbial cells after the introduction of easily-degradable C-substrate like glucose to soil. SMB-C was determined in the uncontaminated and HC-contaminated soils after 1 and 30 d following contamination with the different HCs.

2.8 Determination of pseudomonad number in HC-contaminated soils

After 1 and 30 d of soil contamination with HCs, 1 g of soil was sampled to determine the number of pseudomonad species. The suspension of the soil sample was serially diluted (up to 10^{-4} dilution) and appropriate diluents (0.1 ml) were inoculated by spread plate technique on pseudomonas-CFC agar. The agar plates, prepared in duplicates, were incubated at 37°C and for 48–72 h before enumeration of distinct colonies.

Statistical analysis

Assessment of the candidate models with different hypotheses indicated that a model with uptake and mineralization of hydrocarbon in both the aqueous and soil-sorbed phases will adequately fit the biodegradation data. The choice of the best model from the pool of suitable candidate models was based on a battery of analyses (including the *F*-test method, r^2 values and scaled root mean squared error, RMSE) performed by nonlinear regression GLM program of SigmaStat statistical software version 3.5 (IBM SPSS Software Inc., Chicago, IL, USA). A modified Gompertz model which described the two-compartment, three-parameter biphasic mineralization kinetics was selected and expressed mathematically as:

$$(1) \qquad A = A_0 \cdot (exp \cdot (-exp \cdot [-(t - t_0) / k_{aq}) + k_{ss} \cdot t,$$

$$(2) \qquad K_{aq} = A_0 / \mu_{max} \cdot e$$

where A is the extent of HC mineralization to CO_2 ($mgCO_2 \cdot 10g_{soil}^{-1}$) as a function of time, t; A_0 is the asymptotic yield of CO_2 evolved in the aqueous phase ($mgCO_2 \cdot 10g_{soil}^{-1}$); k_{aq} is the first-order rate constant (d^{-1}) of the mineralization in soil-sorbed phase; t_0 is the time (d) prior to inflection point; k_{ss} is zero-order rate constant ($mgCO_2 \cdot 10g_{soil}^{-1} \cdot d^{-1}$) in soil-sorbed phase, and μ_{max} maximum mineralization rate in the aqueous phase, ($mgCO_2 \cdot 10g_{soil}^{-1} \cdot d^{-1}$)respectively. Effects of HC type and concentration on endogenous and substrate-induced mineralization as well as influence of amendments on HC mineralization were analysed using SigmaStat 3.5 and significance reported at P <0.05. All experiments were conducted in triplicate, except otherwise stated, and the mean values reported.

RESULTS AND DISCUSSION

3.1 Physicochemical and microbiological characteristics of soil and activated sludge

The soil used is classified as sandy, with soil organic matter (SOM) content of 4.2%, high pH 9.4, low C:N ratio of 4.5 and a low bacterial population of 8.6×10^5 cfu·g^{-1}. Petroleum hydrocarbons and heavy metals were not detected in the soil. Detailed characteristics of the soil are shown in Table 1. The poor profiles of the soil indicate that additional sources of nutrients and microbial loads may be required for bio-enhancement in the event of HC contamination. The activated sludge derived from wastewater effluent from *V. paradoxa* seeds is a dried matured digestate of heterogenous texture with very low moisture content (3.4%) and high organic matter content (89.7%). The properties of the activated sludge are also provided in Table 1. The high nitrogen and phosphorus contents as well as the low electrical conductivity show that it may be useful for soil nutrient amendment. The mean total bacteria number was 5.3×10^5 cfu·g^{-1}, total coliform number averaged 3.8×10^1 cfu·g^{-1} and *Salmonella-Shigella* count averaged 4.8×10^1 cfu·g^{-1}. There is no *E. coli* or *E. coli* O157:H7 cells recovered in the activated sludge. Although it has relatively low microbial load, it is safe to handle as it is free of pathogens. Absence of enteric pathogens like *E. coli* and *E. coli* O157:H7 may be due to the source of the sludge which is shea butter wastewater. It is also possible that the treatment applied might have eliminated many organisms and pathogens from the activated sludge. However, presence of other heterotrophic bacteria predominantly *Pseudomonas* species was observed.

Table 1: Physicochemical and microbiological characteristics of soil and activated sludge

Soil Characteristic	Value	Sludge characteristic	Value
Classification		Texture	Heterogeneous materials
Particle analysis (%)		Colour	Brown to black
Sand	89.0	Odour	No foul smell
Silt	5.4	Maturity	Mature
Clay	5.6	Bulk density (g·cm^{-3})	0.87
Texture	Sandy	Moisture (%)	3.4
pH (dH_2O)	9.4	pH (1:2 $CaCl_2$)	8.8
Elemental analysis		Organic matter (%; LOI)	89.7
Soil organic matter (%; LOI)	4.2	Total organic carbon, $C_{org.}$ (g·kg^{-1})	86.4
Total organic carbon, $C_{org.}$ (g·kg^{-1})	24.1	Total nitrogen (g·kg^{-1})	32.7
Total nitrogen (g·kg^{-1})	5.4	NH_4-N (%)	26.2
NO_3 (%)	2.4	Total phosphorus (mg·kg^{-1})	187.2
Total phosphorus (mg·kg^{-1})	95.0	PO_4-P (%)	12.8
PO_4-P (%)	23.3	Conductivity mS·cm^{-1}	1.8
Exchangeable base (C·mol^{-1}·kg^{-1})		Total bacteria (cfu·g^{-1})	5.3×10^3
Ca	16.0	PAH degrader (cfu·g^{-1})	
Mg	19.1	Total coliform (cfu·g^{-1})	3.8×10^1
Na	0.8	*Salmonella-Shigella* (cfu·g^{-1})	0
K	0.8	*Escherichia coli* (cfu·g^{-1})	0
H+Al	0.6	*E. coli* O157:H7 (cfu·g^{-1})	0
ECEC	36.1		
Total bacteria (cfu·g^{-1})	8.6×10^5		
PAH degrader (cfu·g^{-1})			

3.3 Endogenous and enhanced biodegradation of petroleum hydrocarbon-contaminated soils

The mineralization kinetics (measured as $mgCO_2 \cdot 10g_{soil}^{-1}$) of the different HC-contaminated soils are presented in Figure 1. There was microbial respiratory activity in the uncontaminated control soil over the 30 d period. An increased amount of CO_2 was produced in the HC-contaminated soils than the control soil, indicating biodegradation of HCs leading to their mineralization. However, amendments significantly ($p < 0.05$) increased microbial respiratory activities with markedly higher CO_2 evolution (199–495% increase over non-amended HC-contaminated soils). This indicates that extensive biodegradation of HCs in soil was significantly enhanced ($p < 0.05$) by the various treatment regimens. The mineralization kinetics exemplified a typical microbial-associated dissipation with initial rapid rates of CO_2 evolution in the first 10 d, followed by decelerating hydrocarbon removal rates.

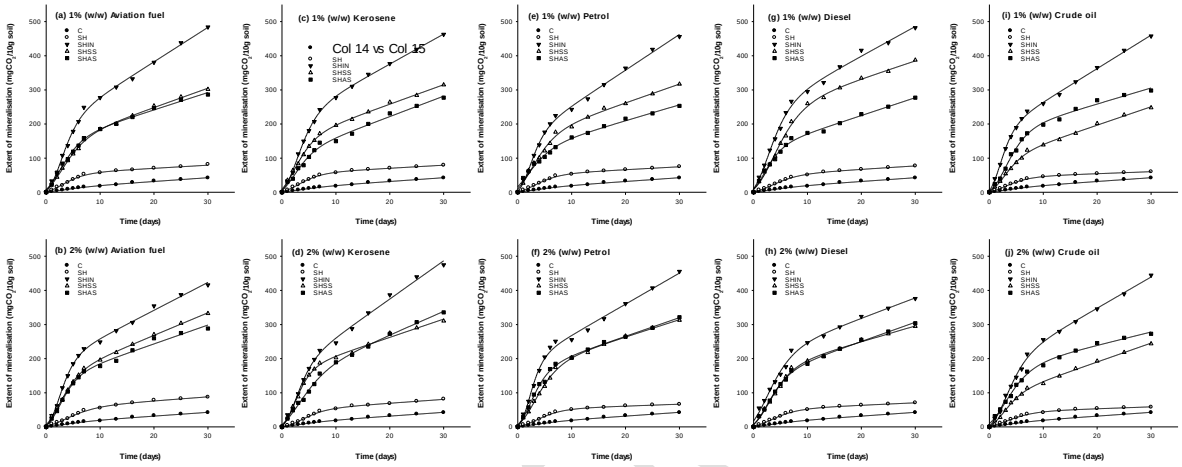

Figure 1: Mineralization in (●) uncontaminated soil (SC), (○) HC-contaminated soils and amended with (▼) HC-adapted degraders (SHIN), (Δ) sterilised sludge (SHSS) and (■) activated sludge (SHAS). Contamination with aviation fuel (a, b), kerosene (c, d), petrol (e, f), diesel (g, h) and crude oil (i, j) at $1\%_{w/w}$ and $2\%_{w/w}$.

Significant CO_2 evolution ($p < 0.05$) was observed in all the HC-contaminated soils as compared to the control soil; additional 22.8–44.0 and 20.6–49.6 $mgCO_2 \cdot 10g_{soil}^{-1}$ were released after 30 d in the 1% and $2\%_{w/w}$ HC-contaminated soils, respectively (Table 2). This indicates presence of HC-degrading microbes in the native soil. Both the type and concentration of HCs influenced the extents of mineralization by the endogenous microbiota in the native soil. Mineralization of the heavier HCs petrol, diesel and crude oil was markedly inferior to the lighter HCs aviation fuel and kerosene, being evidently significant ($p < 0.05$) at the higher contaminant level. Greater mineralization recorded in soils contaminated with the lighter HCs is attributed to presence of larger proportion of easier-to-degrade aliphatic constituents (*n*-paraffins and naphthalenes) as against the heavier HCs with more of recalcitrant asphaltenes, aromatics and PAHs (Pires *et al*., 2018); Microorganisms are known to be more adaptable to, and possess greater biodegradability to aliphatic HCs than PAHs (Varjani 2017). Higher concentrations of heavier HCs may exert greater toxicity against HC-sensitive microbial species thereby inducing greater reduction in microbial population and species diversity with mainly the degraders and tolerant groups thriving in the impacted soils (Gałązka et al., 2018; Hou et al., 2018; Xu et al., 2018)

Further, Table 2 highlights that the amendments enhanced the extent of mineralization, being significantly ($p < 0.05$) higher in SHIN soils than in SHSS and SHAS soils. Higher CO_2 evolution in SHIN soils is remarkable particularly when it is expected that portions of CO_2 released in SHSS and SHAS soils would have come also from degradation of the sludge materials. This implies that bioaugmentation is superior to biostimulation for the

bioremediation of HCs in this particular soil. Notably, supplementation with activated sludge (i.e. supply of both active microbial community and nutrients) did not proof to be a better option to stimulation with sterilised sludge (i.e. additional sources of nutrient only). Thus, in soils with low endogenous microbial metabolic activity, such as the native soil used in this study, introduction of microbial consortium with high biodegradability should be the main strategy for bioremediation of HCs. Meanwhile, the influence of HC concentration was more pronounced in the SHSS and SHAS soils. Biostimulation with sterilized sludge promoted significantly ($p < 0.05$) higher mineralization at the lower contaminant level for heavier HCs diesel and crude oil, but had less influence for lighter HCs. Whereas, bio-enhancement with activated sludge gave superior mineralization at the higher contaminant level, being significant ($p < 0.05$) for all HCs except aviation fuel. This suggests that in heavily contaminated soils, aside improving nutrient availability, greater microbial load is required to achieve substantial degradation activity (Eman Koshlaf, Andrew S Ball 2017). Previous studies have shown evidence that initial degrader population is critical for the early onset and rapid mineralization of HCs in soils (Towell MG et al.2011). Microbial populations of 10^7–10^8 cells·g_{soil}^{-1} are required to establish degradation activity in recently contaminated soils (Eman Koshlaf, Andrew S Ball 2017), and approximately 10^6 cells·g_{soil}^{-1} of HC-adapted microbiota is considered sufficient to degrade PAHs in long-term impacted soils. The activated sludge used in this present study contained much lower microbial cell densities (10^3 cells·g_{soil}^{-1}), and it is possible that a higher inoculum would have a better influence on the degradation process by decreasing the lag phase and also the PAH dissipation time relative to the native microbiota performance.

Table 2: Summary of the extent of mineralization (ΣCO_2–C $mgCO_2 \cdot 10g_{soil}^{-1}$) in HC-contaminated soils with different amendments (different superscript letters indicate means of amendment regimens are significantly different at $p < 0.001$)

Hydrocarbon	Conc. (%$_{w/w}$)	Amendments			
		SH	SHIN	SHSS	SHAS
Aviation fuel	1	81.4^{a}	484.0^{c}	331.4^{b}	286.5^{b}
Aviation fuel	2	87.0^{a}	415.8^{d}	332.2^{c}	288.2^{b}
Kerosene	1	79.2^{a}	462.6^{d}	314.6^{c}	277.2^{b}
Kerosene	2	81.4^{a}	474.9^{c}	310.2^{b}	335.6^{b}
Petrol	1	74.8^{a}	456.2^{d}	316.8^{c}	253.0^{b}
Petrol	2	66.0^{a}	455.5^{c}	312.4^{b}	321.2^{b}
Diesel	1	77.0^{a}	481.8^{d}	387.2^{c}	277.2^{b}
Diesel	2	70.4^{a}	376.2^{c}	294.6^{b}	303.6^{b}
Crude oil	1	60.2^{a}	457.6^{d}	298.2^{b}	247.5^{c}
Crude oil	2	58.0^{a}	444.4^{c}	243.6^{b}	273.0^{b}
Mean		73.54^{a}	450.90^{d}	314.12^{c}	285.30^{b}
SEM		3.05	10.39	11.35	8.76

3.4 Kinetics of endogenous and enhanced mineralization in HC-contaminated soil

Modelled kinetic parameters for the endogenous and enhanced mineralization of HCs in soil are presented in Table 3. These provide additional information on the biodegradation of HCs in soil . Substantial portions (61–70 and 57–75% respectively) of ΣCO_2–C released (60.20–81.40 and 58.00–87.00 $mgCO_2 \cdot 10g_{soil}^{-1}$ respectively) in 1%$_{w/w}$ and 2%$_{w/w}$ HC-contaminated soils were from the aqueous phase (A_0: 42.16–52.52 and 42.83–52.54 $mgCO_2 \cdot 10g_{soil}^{-1}$ respectively). However, in control soil only 23% of ΣCO_2–C released was from the aqueous phase (A_0: 9.65 $mgCO_2 \cdot 10g_{soil}^{-1}$). Meanwhile, analysis of the mineralization kinetics data indicated that 32–53, 33–59 and 36–59% of ΣCO_2–C released in SHIN, SHSS and SHAS soils were in the aqueous phase (A_0: 148.38–224.83, 79.47–226.93 and 88.84–164.87 $mgCO_2 \cdot 10g_{soil}^{-1}$ respectively). As expected, mineralization was markedly higher in the aqueous phase of SHIN soils than of SHSS and SHAS soils.

The maximum mineralization rates attained in the aqueous phase of HC-contaminated soils (μ_{max}: 5.37–7.95 $mgCO_2 \cdot 10g_{soil}^{-1} \cdot d^{-1}$) were about 5–7.5-magnitudes higher than the steady rate of the control soil (μ_{max}: 1.06 $mgCO_2 \cdot 10g_{soil}^{-1} \cdot d^{-1}$). This suggests presence of sizeable HC degrader-population in the native soil. Noteworthy, while the times to attain maximum mineralization rates, t_o, in the aqueous phase were not significantly different, the maximum mineralization rates were significantly ($p < 0.05$) higher in the SHIN soils as compared to the other two amendments. The introduced degraders are well adapted to HCs, hence, their turnover rates of HC in the soil-sorbed phase (k_{ss}) resulted in consistently ($p < 0.05$) higher rates of mineralization in SHIN soils (k_{ss}: 26.46–45.66 $mgCO_2 \cdot 10g_{soil}^{-1} \cdot d^{-1}$) than the SHSS and SHAS soils (k_{ss}: 14.03–32.33 and 15.18–28.45 $mgCO_2 \cdot 10g_{soil}^{-1} \cdot d^{-1}$ respectively). The model used to describe the two-phase mineralization kinetics indicated a very good fit ($0.992 \geq R^2 \geq 0.999$).

Table 3: Parameters of modified Gompertz kinetics model fitted to the measured mineralization data for uncontaminated soil and HC-contaminated soils with different amendments (different letters down the column for each hydrocarbon type indicate significant difference at $p < 0.0001$)

Hydrocarbon	Conc. (%w/w)	Treatment	A_0 ($mgCO_2 \cdot 10g_{soil}^{-1}$)	μ_{max} ($mgCO_2 \cdot 10 g_{soil}^{-1} \cdot d^{-1}$)	t_o	k_{ss} ($mgCO_2 \cdot 10g_{soil}^{-1} \cdot d^{-1}$)	R^2
		SC	9.65 (2.31) a	1.06 (0.17)a	3.31 (1.10)a	1.10 (0.10)a	0.995
Aviation fuel	1	SH	50.76 (2.66)b	7.95 (0.43)b	3.02 (0.15)a	0.99 (0.12)a	0.997
		SHIN	180.65 (10.37)d	33.91 (1.68)d	2.80 (0.23)a	10.09(0.50)c	0.998
		SHSS	135.35 (7.33)c	18.77 (0.77)c	2.92 (0.24)a	5.66 (0.33)b	0.998
		SHAS	139.70 (8.61)c	21.40 (1.06)c	2.52 (0.27)a	5.09 (0.40)b	0.997
	2	SH	52.54 (4.37)b	5.97 (0.34)b	3.68 (0.37)a	1.19 (0.19)a	0.999
		SHIN	178.21 (7.83)d	38.30 (1.62)d	2.41 (0.18)a	8.18 (0.39)b	0.998
		SHSS	136.08 (5.24)c	22.79 (0.74)c	2.43 (0.17)a	6.61 (0.25)b	0.998
		SHAS	132.72 (9.93)c	24.63 (1.63)c	2.50 (0.31)a	5.53 (0.48)b	0.995
Kerosene	1	SH	52.52 (1.64)b	7.75 (0.19)b	2.97 (0.13)a	0.90 (0.08)a	0.999
		SHIN	200.03 (7.17)e	34.34 (1.02)e	2.81 (0.15)a	8.78 (0.34)c	0.999
		SHSS	144.16 (6.75)d	23.32 (0.92)d	2.28 (0.21)a	5.70 (0.32)b	0.998
		SHAS	102.43 (10.82)c	17.05 (1.53)c	2.35 (0.46)a	5.97 (0.52)b	0.993
	2	SH	46.75 (2.60)b	6.68 (0.27)b	3.70 (0.23)a	1.13 (0.12)a	0.998
		SHIN	150.95 (11.69)d	30.19 (2.12)e	2.65 (0.31)a	11.19 (0.57)d	0.997
		SHSS	155.42 (7.24)d	32.33 (1.43)e	2.35 (0.19)a	5.35 (0.36)b	0.997
		SHAS	130.83(12.20)cd	15.84 (1.04)c	3.27 (0.42)a	6.95 (0.53)b	0.997
Petrol	1	SH	49.12 (2.39)b	6.95 (0.25)b	3.15 (0.21)a	0.85 (0.11)a	0.997
		SHIN	148.38 (9.34)cd	32.62 (2.02)e	2.32 (0.26)a	10.47 (0.47)c	0.997
		SHSS	151.49(12.22)cd	21.22 (1.32)d	2.63 (0.36)a	5.52 (0.56)b	0.995
		SHAS	116.30 (11.53)c	15.98 (1.27)c	1.81 (0.50)a	4.67 (0.53)b	0.992
	2	SH	49.68 (2.53)b	6.81 (0.25)b	3.49 (0.22)a	0.56 (0.11)a	0.997
		SHIN	177.45 (10.02)d	45.66 (2.85)f	2.17 (0.23)a	9.11 (0.52)c	0.996
		SHSS	161.79 (8.45)d	22.74 (0.89)d	3.15 (0.23)a	5.08 (0.38)b	0.998
		SHAS	155.88 (7.78)cd	28.45(1.26) e	2.38 (0.21)a	5.48 (0.38)b	0.997
Diesel	1	SH	47.15 (2.05)b	6.09 (0.19)b	3.46 (0.19)a	1.00 (0.09)a	0.999
		SHIN	224.83 (15.48)d	34.93 (1.93)d	2.68 (0.30)a	8.70 (0.72)c	0.996
		SHSS	226.93 (14.53)d	26.88 (1.18)c	3.69 (0.28)a	5.27 (0.63)b	0.998
		SHAS	120.30 (8.30)c	22.16 (1.41)c	1.93 (0.31)a	5.23 (0.41)b	0.995
	2	SH	49.03 (2.42)b	6.41 (0.23)b	3.22 (0.22)a	0.72 (0.11)a	0.997
		SHIN	198.88(17.38)cd	26.46 (1.77)c	2.46 (0.41)a	5.97 (0.79)b	0.994
		SHSS	156.68 (10.31)c	22.77 (1.16)c	2.84 (0.29)a	4.64 (0.47)b	0.996
		SHAS	137.82 (6.50)c	21.67 (0.83)c	2.53 (0.21)a	5.63 (0.31)b	0.998
Crude oil	1	SH	42.83 (1.40)b	6.12 (0.15)b	3.01 (0.14)a	0.58 (0.06)a	0.999
		SHIN	166.41 (5.32)d	38.74 (1.31)f	1.98 (0.13)a	9.80 (0.27)c	0.999
		SHSS	164.87 (11.15)d	25.93 (1.37)e	3.07 (0.26)a	4.69 (0.52)b	0.996
		SHAS	88.84 (5.63)c	15.18 (0.80)c	2.80 (0.26)a	5.39 (0.27)b	0.998
	2	SH	42.16 (2.37)b	5.37 (0.22)b	3.21 (0.25)a	0.56 (0.11)a	0.997

SHIN	165.26 (10.51)d	26.88 (0.27)e	3.17 (0.26)a	9.13 (0.49)c	0.998
SHSS	79.47 (4.68)c	14.03 (0.70)c	2.74 (0.24)a	5.54 (0.22)b	0.998
SHAS	160.85 (10.88)d	21.08 (1.06)d	2.90 (0.30)a	3.91 (0.49)b	0.996

A_0: asymptotic CO_2 yield; μ_{max}: maximum mineralization rate; t_o: time at inflection to maximum rate; k_{ss}: slowly-occurring mineralization of microbially-incorporated or microbially-bound C.

3.5 SIR in HC-contaminated soil

Figure 2 presents the SIR in HC-contaminated soils after 1 d and 30 d of contamination. The mineralization kinetics are typical of readily-utilizable substrates in soils, with over 60% of ΣCO_2–C_{SIR} evolution within the first 24 h of a 5-d course. Appreciable CO_2 evolution was recorded after lag time of <6 h in all HC-contaminated soils, irrespective of contaminant level. Microbial respiratory activity was repressed in HC-contaminated soils after 1 d as higher ΣCO_2–C_{SIR} was recorded in the control soil. At 1%$_{w/w}$ contaminant level, the effect was significant for lighter HCs (Figure 2a) and at 2%$_{w/w}$ contaminant level, the effect was significant for all HCs except crude oil (Figure 2b). It means this repressive effect is pronounced in soils contaminated with lighter HCs aviation fuel and kerosene than heavier HCs petrol, diesel and crude oil. In contrast, after 30 d the effect became stimulatory on microbial respiratory activity. At both contaminant levels, ΣCO_2–C_{SIR} was significantly ($p < 0.05$) higher in the HC-contaminated soils than the control soil. More ΣCO_2–C_{SIR} was evolved in soils with heavier HCs than lighter HCs (Figures 2c and d). Overall, ΣCO_2–C_{SIR} was significantly lower in the HC-contaminated soils after 1 d than 30 d of contamination.

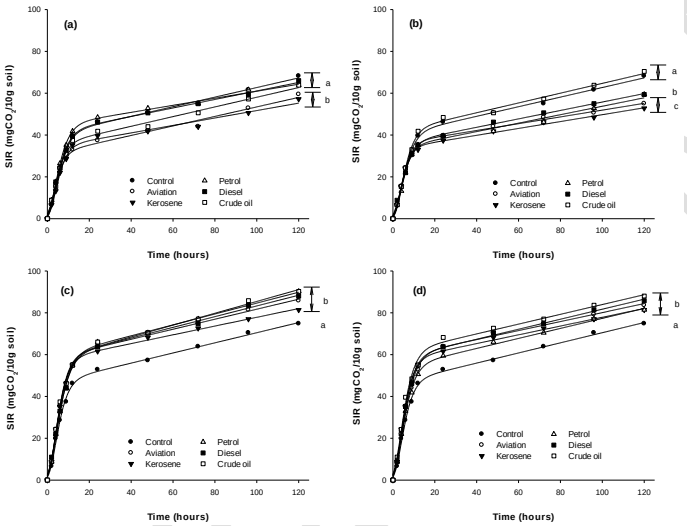

Figure 2: Substrate-induced respiration in (●) uncontaminated soil and HC-contaminated soils (○) aviation fuel, (▼) kerosene, (∆) petrol, (■) diesel and (□) crude oil applied at (a) 1%$_{w/w}$ and (b) 2%$_{w/w}$ contaminant levels after 1 d and at (c) 1%$_{w/w}$ and (d) 2%$_{w/w}$ contaminant levels after 30 d of contamination

At 1 d of contamination, while ΣCO_2–C_{SIR} by HC-adapted degraders was not different for all HCs, at 1%$_{w/w}$ contaminant level (Figure 3a), it was significantly lower for aviation fuel at 2%$_{w/w}$ contaminant level (Figure 3b). After 30 d of contamination, ΣCO_2–C_{SIR} by HC-adapted degraders was still not different for all HCs at 1%$_{w/w}$ contaminant level but became significantly higher for lighter HCs aviation fuel and kerosene (Figures 2c and d). Microbial respiratory activity in the HC-contaminated soils was markedly stimulated with the addition of HC-adapted degraders as compared to their absence (Figures 2 and 3).

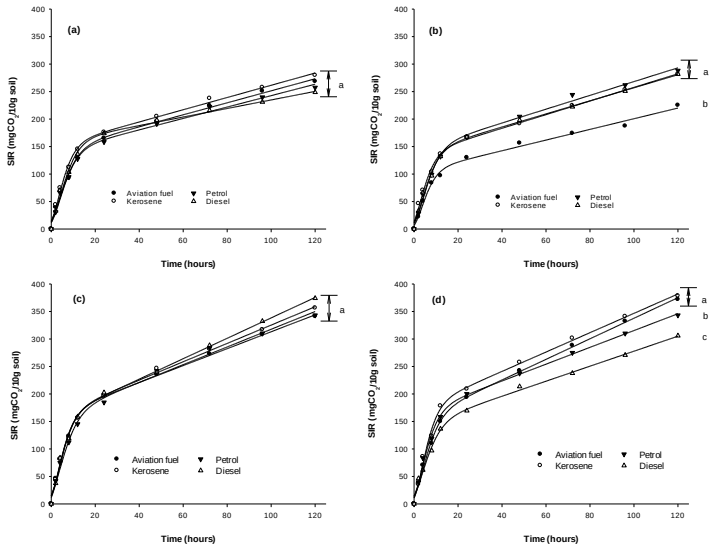

Figure 3: Substrate-induced respiration in HC-contaminated soils and amended with HC-adapted degraders (●) aviation fuel, (○) kerosene, (▼) petrol, and (Δ) diesel applied at (a) 1%$_{w/w}$ and (b) 2%$_{w/w}$ contaminant levels after 1 d and at (c) 1%$_{w/w}$ and (d) 2%$_{w/w}$ contaminant levels after 30 d of contamination

3.4 Microbial biomass-C in petroleum HC-contaminated soils

Soil microbial biomass-C (SMB-C) was maintained at 330–345 $\mu g \cdot g_{soil}^{-1}$ in the control soil for the 30 d incubation period (Figure 4a). Whereas significant increase in SMB-C was initially recorded in soils contaminated with the more-complex, heavier HCs petrol, diesel, and crude oil (335–570 $\mu g \cdot g_{soil}^{-1}$), SMB-C was maintained significantly higher ($p < 0.05$) in all the HC-contaminated soils (395–580 $\mu g \cdot g_{soil}^{-1}$) above the control soil after 30 d of contamination. This suggests the metabolism and incorporation of C from the HCs into the soil microbial biomass. SMB-C significantly ($p < 0.05$) increased at 1 d in HC-contaminated soils amended with HC-adapted degraders (2045–3022 $\mu g \cdot g_{soil}^{-1}$) and it was sustained for the duration of the 30 d contamination (2789–3578 $\mu g \cdot g_{soil}^{-1}$) (Figure 4b). Soil microbial biomass is thought to account for approximately 60% of the variation in soil microbial respiration (Colman and Schimel, 2013; Hou *et al*., 2019). SMB-C increased in a semiarid Mediterranean soil contaminated long-term with oil sludges at 7%$_{w/w}$ from 370 to 629 $\mu g \cdot g_{soil}^{-1}$ (Caravaca and Roldán, 2003). Studies have shown that the biomass weight of *Pseudomonas* sp. of approximately 2.4×10^8 cfu·ml^{-1} is equivalent to 2.085 mg·ml^{-1} (Kim *et al*., 2012).

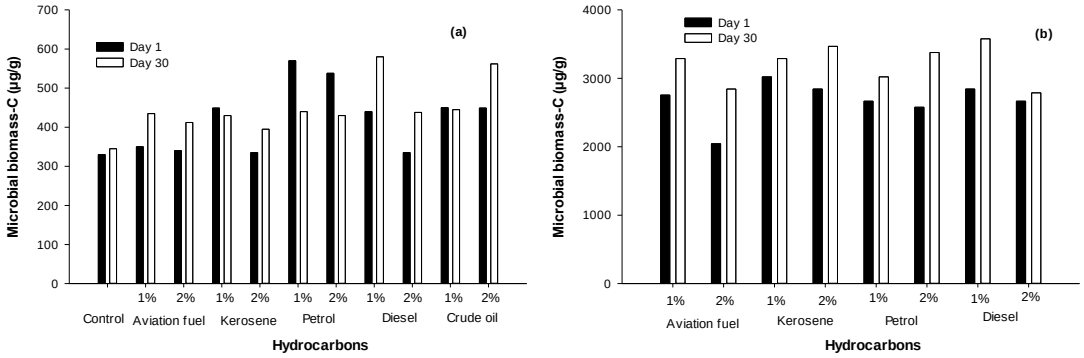

Figure 4: Soil microbial biomass-C (µg/g soil) in (a) uncontaminated soil and HC-contaminated soils and (b) HC-contaminated soils amended with HC-adapted degraders at (■) 1 d and (□) 30 d of contamination

3.4 Ecophysiological indices to evaluate HC stress on soil microbial community

From the three microbial parameters measured (basal biodegradation, substrate-induced respiration, and soil microbial biomass-C), ecophysiological indices which reflect the bioenergetic status of microbial biomass were calculated. The indices have been used as bioindicators of environmental stress and disturbance in soil microbial population and diversity (Anderson and Domsch 1993; Cheng *et al*., 1996) and, concomitantly, the soil health and recovery. These are microbial metabolic quotient (qCO_2), which is the ratio of basal respiration to soil microbial biomass-C ($qCO_2 = R_B$/SMB-C), microbial respiratory efficiency (MRE), which is the percentage of the total C metabolised that is incorporated into biomass-C (MRE = [SMB-C/(R_B + SMB-C)]) and microbial respiratory coefficient (Q_R), which is the ratio of substrate-induced respiration to basal respiration ($Q_R = R_{SIR}/R_B$). qCO_2 is an important parameter in understanding soil C cycling and has been widely used in assessing soil microbial metabolic status (Xu et al., 2017; Wardle and Ghani, 2018). MRE describes substrate utilization efficiency of microorganisms and provides further evidence on how C is partitioned in soil (Wang Y et. al 2022). QR, also referred to as microbial carbon limitation index (Hou *et al*., 2019), is a measure of the level of microbial respiratory response to fresh supply of readily available substrates (glucose is commonly used) above the endogenous soil microbial respiration. These microbial metabolic indices were affected by the interaction between type and concentration of HCs and changed markedly over the course of the incubation period (Table 3).

Initially, the qCO_2 was significantly ($p < 0.05$) higher in the HC-contaminated soils in comparison with the control soil (1.16–2.08 *vs.* 0.67 µg CO_2–C·µg^{-1} C_{mic}·d^{-1}) but levelled up at the end of 30 d in both HC-contaminated soils and control soil (0.19–0.31 *vs*. 0.22 µg CO_2–C·µg^{-1} C_{mic}·d^{-1}). The qCO_2 was also generally higher at 2%$_{w/w}$ contaminant level. The initial higher qCO_2 in the HC-contaminated soils may imply an initial stress on the degrader population or a disturbance in the soil microbial ecosystem caused by the HCs (Koshlaf E, Ball AS 2017; Kakde P, Sharma J.2024). The decrease in qCO_2 over time suggests adaptation of the endogenous microbiota (Eman Koshlaf, Andrew S Ball 2017). This may also result from the increase in microbial biomass with corresponding decrease in residual HCs available for biodegradation. Amendment with HC-adapted inoculum caused qCO_2 to decrease in the soils contaminated with lighter HCs and increase when heavier HCs were applied. This means that the augmented degraders are better adapted to lighter HCs while heavier HCs exerted more stress on them. This finding may not necessarily be interpreted as the effect of stress caused by heavier HCs on the HC-adapted degraders, when the efficiency of the microbial turnover (i.e. MRE) of the HCs is considered. A number of researchers have expressed cautions in interpreting qCO_2 as it may not necessarily infer stress alone but also reflects the maturity/stability of an ecosystem or a more respirable substrate. Despite its lower CO_2 evolution, substrate use efficiency was initially appreciably higher in the control soil than the HC-contaminated soils (60 *vs.* 34–46%) suggesting an initial toxic effect of HCs on the soil endogenous microbiota. However, MRE improved considerably by the end of 30 d in HC-contaminated soils (44 *vs.* 40–55%). In comparison, amendment with HC-adapted degraders resulted in improved efficiency of C incorporation into microbial biomass (50–67%). These results confirm that despite the relatively higher qCO_2 recorded, the HC-adapted degraders are more efficient in utilising the heavier HCs than the endogenous microbiota.

Normally, fresh supply of readily-available carbon substrates elevates microbial metabolic activities in soil. As much as 2–6-fold increases in soil respiration immediately (within the first 4 h) after glucose addition has been demonstrated in soils (Anderson and Domsch, 1978; Pang *et al*., 2015; Mingorance and Pena, 2016; Hou *et al*., 2019). Q_R was also affected by the interaction between type and concentration of HCs. Compared to the control soil, significantly ($p < 0.05$) lower Q_R values were obtained in HC-contaminated soils at the onset of biodegradation (21 *vs.* 3–7). However, Q_R values became higher (i.e. carbon limitation increased over time) in both control and HC-contaminated soils, being markedly greater in soils contaminated with heavier HCs at 1%$_{w/w}$ and with all HCs at 2%$_{w/w}$ after 30-d incubation (60 *vs.* 48–131). Meanwhile, amendment with HC-adapted degraders reduced the effect of carbon limitation in the HC-contaminated soils remarkably by 2–4-folds, with Q_R values

significantly lower than in the control soil (22–33 *vs.* 60). These findings imply that, whereas endogenous metabolic activity was limited by bioavailability in soils contaminated with heavier HCs, the HC-adapted degraders improved bioavailability as well as possess greater ability to access soil-bound HCs; thus resulting in the observed bioenhanced mineralization of HCs. This submission is substantiated by the modelled mineralization parameters that show that mineralization increased by 4–7-folds in the aqueous phase and by 7–17-folds in the soil-sorbed phase when HC-adapted degraders were amended to the HC-contaminated soils.

Table 4: Metabolic quotient (qCO_2), substrate utilization efficiency (MRE) and respiratory coefficient (Q_R) in uncontaminated soil (SC) and HC-contaminated soils amended without (SH) and with HC-adapted degraders (SHIN)

Hydrocarbon	Treatment	qCO_2 (μg CO_2–C·μg^{-1} C_{mic}·d^{-1})#				MRE (%)				Q_R			
		1 d		30 d		1 d		30 d		1 d		30 d	
		1%	2%	1%	2%	1%	2%	1%	2%	1%	2%	1%	2%
Control	SC	0.67		0.22		60		44		21		60	
Aviation fuel	SH	1.76	1.81	0.30	0.22	41	34	40	46	6	5	48	73
	SHIN	1.20	1.61	0.28	0.20	55	62	58	50	5	4	21	33
Kerosene	SH	1.67	1.97	0.31	0.22	40	43	49	50	6	5	48	71
	SHIN	1.09	1.24	0.27	0.20	52	55	57	50	5	5	22	29
Petrol	SH	1.16	1.34	0.20	0.20	46	45	50	55	7	6	75	87
	SHIN	1.40	1.45	0.25	0.29	58	59	55	59	4	5	25	21
Diesel	SH	1.40	1.84	0.23	0.20	35	34	49	55	7	6	69	94
	SHIN	1.55	2.06	0.26	0.23	61	67	57	54	4	3	23	29
Crude oil	SH	1.28	1.58	0.25	0.19	41	34	52	51	6	7	127	131
	SHIN	nd	nd	nd	nd	nd	nd	nd	nd	Nd	nd	nd	nd

#: qCO_2 and Q_R values are always significantly higher at 1 d than at 30 d; nd: not determined

3.5 Pseudomonas population in HC-contaminated soils

Pseudomonas sp. were predominant in the uncontaminated soil (3.4×10^4 cfu·g_{soil}^{-1}), and increased by several folds immediately after contamination in soil, irrespective of type and concentration of HCs (Figure 5). Further increases were recorded in most of the HC-contaminated soils after 30 d. It was noted that in soils that *Pseudomonas* sp. numbers decreased after 30 d (e.g. petrol at 1%$_{w/w}$ and 2%$_{w/w}$ and kerosene at 1%$_{w/w}$), the initial increases were massive. The increase in *Pseudomonas* population HC-contaminated soils implies their adaptation to, and growth using HCs as substrates (Gałązka et al., 2018). This bacterial group would have been involved in HC mineralization, although it appears the species present in this native soil have low biodegradability as they are not yet well adapted to HCs. Pseudomonads commonly recovered in HC-contaminated soils are capable of utilizing HCs as sole sources of carbon and energy (Das and Chandran 2011) and many of the species have demonstrated strong biodegradability to complex HCs (Eman Koshlaf, Andrew S Ball 2017). HC contamination is known to impact negatively species richness, evenness and phylogenetic diversity. This selective bias of the HC toxicity often favours *Pseudomonas* species dominance in heavily HC-contaminated soils (Labud et al., 2007; Overholt et al., 2015; Hou et al., 2018).

Bioenhancement of HC-contaminated soils using bacteria with high degradative capabilities may overcome the catabolic limitations of endogenous microflora. Review of studies posited that bioaugmentation is particularly suited for sites with significant proportions of HC contamination and for recently polluted soils which do not have an adapted microbial population (Lebeau, 2011; Tyagi *et al*., 2011;). Many bench-scale studies that demonstrated the success of bioaugmentation have also been scaled up to field trials for the clean-up of soil from petroleum hydrocarbon sites (Agnello AC et al. 2016). Some of these efforts showed positive outcomes while others could not replicate results reported in the laboratory. A major drawback to the field application of bioaugmentation is the challenge of viability and

adaptability of exogenous HC-degrader under specific prevailing conditions in a given contaminated soil (Andreoni and Gianfreda, 2007; Das and Chandran, 2011; Shekhar et al., 2015). A solution is to isolate and optimise microorganisms from the same site (Atlas, 1995; Bento et al., 2005).

Some researchers have argued that biostimulation of indigenous microorganisms with additional nutrients can better improve biodegradation of organic contaminants in soils than bioaugmentation with exogenous microorganisms (Thompson et al., 2005; Chang et al., 2010; Karamalidis et al., 2010; Ai-Kindi and Abed, 2016). Some other authors have argued to the contrary, especially when the HC-degraders' biodegradability has been enhanced through either adaptation or genetic modifications (Kim et al., 2005; Jimenez et al., 2006; Garcia-Blanco et al., 2007; Karamalidis et al., 2010; Zhao et al., 2017). The latter notion is supported by the results from this present study.

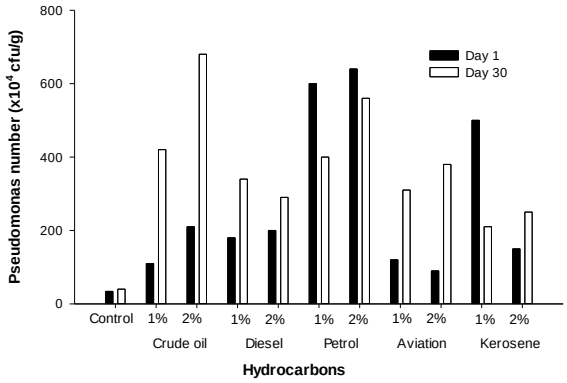

Figure 5: Populations (cfu·g^{-1}) of PAH-degrading *Pseudomonas* sp. in uncontaminated soil and HC-contaminated soils at (■) 1 d and (□) 30 d of contamination

CONCLUSION

Soils with low endogenous microbial affinity for HCs can be bioenhanced by applying HC-adapted degraders to achieve improved mineralization of various HCs. In this study, a microbial consortium whose biodegradability to HCs has been enhanced proved to be more effective in bioremediation of HC-contaminated soils than the activated sludge of *V. paradoxa* from which it was derived. Application of the activated sludge (i.e. bioaugmentation and biostimulation) did not demonstrate superiority over the sterilised sludge (i.e. biostimulation only) confirming the notion that low loads of non-adapted microorganisms cannot confer any additional bio-enhancement of biodegradation in HC-contaminated soils. Hence, there may be need to first enhance the biodegradability of microbiomes in activated sludge materials before their application for bioremediation of HC-contaminated soils.

REFERENCES

1. Elshafei, A.M., Mansour, R. Microbial bioremediation of soils contaminated with petroleum hydrocarbons. *Discov. Soil* **1**, 9 (2024). https://doi.org/10.1007/s44378-024-00004-5

2. Sojinu, O.S.S., Wang, J., Sonibare, O.O. and Zang, E.Y. (2010). Polycyclic aromatic hydrocarbons in sediments and soil from oil exploration areas of the Niger Delta, Nigeria. *Journal of Hazardous Materials* 174: 641-647.

3. Eseoghene S. Olaifa , Evans S. Osabuohien, Haruna Issahaku , Oluwatoyin Matthew. Oil Spillages and Captured Fish Production in the Niger Delta Region of Nigeria. International Journal of Energy Economics and Policy, 2022, 12(3), 175-183.

4. Barathi, S. and Vasudevan, N. (2001). Utilization of petroleum hydrocarbons by *Pseudomonas fluorescens* isolated from a petroleum-contaminated soil. *Environment International* 26(5-6): 413-416.

5. Esin Eraydin Erdoğan , Fikrettin Şahin and Ayten Karaca. Determination of petroleum-degrading bacteria isolated from crude oil-contaminated soil in Turkey. African Journal of Biotechnology Vol. 11(21), pp. 4853-4859, 13 March, 2012

6. M. N. Ashraf, M. A. Waqas, and S. Rahman. Microbial Metabolic Quotient is a Dynamic Indicator of Soil Health: Trends, Implications and Perspectives (Review). *Eurasian Soil Science, 2022, Vol. 55, No. 12, pp. 1794–1803.*

7. Caravaca F. and Roldan A. (2003). Assessing changes in physical and biological properties in a soil contaminated by oil sludges under semiarid Mediterranean conditions. *Geoderma* 117: 53-61.

8. APHA (2023) Standard methods for the examination of water and wastewater, 24th edn. American Public Health Association, Washington DC.

9. Kemmitt, S. J., Lanyon, C. V., Waite, I. S., Wen, Q., Addiscott, T. M., Bird, N. R. A., O'Donnell, A. G., & Brookes, P. (2008). Mineralization of native soil organic matter is not regulated by the size, activity or composition of the soil microbial biomass—a new perspective. *Soil Biology and Biochemistry*, *40*(1), 61-73. https://doi.org/10.1016/j.soilbio.2007.06.021

10. Kieron J Doick, Philip H Lee, Kirk T Semple. 2003. Assessment of spiking procedures for the introduction of a phenanthrene-LNAPL mixture into field-wet soil. Environmental Pollution, Volume 126, Issue 3, Pages 399-406, ISSN 0269-7491, https://doi.org/10.1016/S0269-7491(03)00230-6.

11. Katie A. Fenlon, Kostas Andreou, Kevin C. Jones, Kirk T. Semple. 2011. The formation of bound residues of diazinon in four UK soils: Implications for risk assessment. Environmental Pollution, Volume 159, Issue 3, Pages 776-781. ISSN 0269-7491. https://doi.org/10.1016/j.envpol.2010.11.039.

12. Anderson JPE, Domsch KH. 1978. A physiological method for the quantitative measurement of microbial biomass in soils. Soil Biology and Biochemistry 10(3):215–221 DOI 10.1016/0038-0717(78)90099-8.

13. Pires, A., Han, Y., Kramlich, J., and Garcia-Perez, M. (2018). Chemical composition and fuel properties of alternative jet fuels. *BioResources* 13(2): 2632-2657.

14. Varjani S. J. (2017). Microbial degradation of petroleum hydrocarbons. *Bioresour. Technol.* 223 277–286. 10.1016/j.biortech.2016.10.037 [DOI] [PubMed] [Google Scholar]

15. Gałązka A, Grządziel J, Gałązka R, Ukalska-Jaruga A, Strzelecka J and Smreczak B (2018) Genetic and Functional Diversity of Bacterial Microbiome in Soils With Long Term Impacts of Petroleum Hydrocarbons. Front. Microbiol. 9:1923. doi: 10.3389/fmicb.2018.01923

16. Hou, N., Zhang, N., Jia, T., Sun, Y., Dai, Y., Wang, Q., et al. (2018). Biodegradation of phenanthrene by biodemulsifier-producing strain Achromobacter sp. LH-1 and the study on its metabolisms and fermentation kinetics. *Ecotoxicol. Environ. Saf.* 163, 205–214. doi: 10.1016/j.ecoenv.2018.07.064.

17. Xu X., Liu W., Tian S., Wang W., Qi Q., Jiang P., Gao X., Li F., Li H. and Yu H. (2018). Petroleum hydrocarbon-degrading bacteria for the remediation of oil pollution under aerobic conditions: A perspective analysis. Frontiers in Microbiology Volume 9 Article 2885. doi.org/10.3389/fmicb.2018.02885.

18. Eman Koshlaf, Andrew S Ball. Soil bioremediation approaches for petroleum hydrocarbon polluted environments[J]. *AIMS Microbiology*, 2017, 3(1): 25-49. doi: 10.3934/microbiol.2017.1.25

19. Towell MG, Bellarby J, Paton GI, Coulon F, Pollard SJ, Semple KT. Mineralisation of target hydrocarbons in three contaminated soils from former refinery facilities. Environ Pollut. 2011 Feb;159(2):515-23. doi: 10.1016/j.envpol.2010.10.015. Epub 2010 Nov 20. PMID: 21095049.

20. Colman BP, Schimel JP. 2013. Drivers of microbial respiration and net N mineralization at the continental scale. Soil Biology and Biochemistry 60:65–76 DOI 10.1016/j.soilbio.2013.01.003.

21. Hou J, Dijkstra FA, Zhang X, Wang C, Lü X, Wang P, Han X, Cheng W. 2019. Aridity thresholds of soil microbial metabolic indices along a 3,200 km transect across arid and semi-arid regions in Northern China. PeerJ 7:e6712 DOI 10.7717/peerj.6712

22. Kim DJ, Chung SG, Lee SH and Choi JW (2012). Relation of microbial biomass to counting units for Pseudomonas aeruginosa. *African Journal of Microbiology Research* 6(21): 4620-4622. DOI: 10.5897/AJMR10.902

23. Anderson T, Domsch K. 1993. The metabolic quotient for CO2 (qCO2) as a specific activity parameter to assess the effects of environmental conditions, such as pH, on the microbial biomass of forest soils. Soil Biology Biochemistry 25(3):393–395 DOI 10.1016/0038-0717(93)90140-7.

24. Cheng WX, Zhang QL, Coleman DC, Carroll CR, Hoffman CA. 1996. Is available carbon limiting microbial respiration in the rhizosphere? Soil Biology and Biochemistry 28(10–11):1283–1288 DOI 10.1016/S0038-0717(96)00138-1.

25. Xu XF, Schimel JP, Janssens IA, Song X, Song CC, Yu GR, Sinsabaugh RL, Tang DD, Zhang XC, Thornton PE. 2017. Global pattern and controls of soil microbial metabolic quotient. Ecological Monographys 87(3):429–441 DOI 10.1002/ecm.1258.

26. Wardle DA, Ghani A. 2018. A tale of two theories, a chronosequence and a bioindicator of soil quality. Soil Biology and Biochemistry 121:A3–A7 DOI 10.1016/j.soilbio.2018.01.005.

27. Wang Y, Wilhelm RC, Swenson TL, Silver A, Andeer PF, Golini A, Kosina SM, Bowen BP, Buckley DH, Northen TR. 2022. Substrate Utilization and Competitive Interactions Among Soil Bacteria Vary With Life-History Strategies. Front Microbiol. 2022 Jun 9;13:914472. doi: 10.3389/fmicb.2022.914472. PMID: 35756023; PMCID: PMC9225577.

28. Kakde P, Sharma J. Microbial Bioremediation of Petroleum Contaminated Soil: Structural Complexity, Degradation Dynamics and Advanced Remediation Techniques. *J Pure Appl Microbiol*. 2024;18(4):2244-2261. doi: 10.22207/JPAM.18.4.28

29. Pang X, Zhu B, Lü X, Cheng W. 2015. Labile substrate availability controls temperature sensitivity of organic carbon decomposition at different soil depths. Biogeochemistry 126(1–2):85–98 DOI 10.1007/s10533-015-0141-0.

30. Mingorance MD, Pena A. 2016. A methodological approach to measure soil respiration. Journal of Geochemical Exploration 169:187–191 DOI 10.1016/j.gexplo.2016.07.019.

31. Das N and Chandran P (2011). Microbial degradation of petroleum hydrocarbon contaminants: An overview. *Biotechnology Research International* Volume 2011, Article ID 941810, 13 pages doi:10.4061/2011/941810.

32. Labud, V., Garcia, C., and Hernandez, T. (2007). Effect of hydrocarbon pollution on the microbial properties of a sandy and a clay soil. *Chemosphere* 66, 1863–1871. doi: 10.1016/j.chemosphere.2006.08.021.

33. Overholt, W.A., Marks, K.P., Romero, I.C., Hollander, D.J., Snell, T.W., and Kostka, J.E. (2015). Hydrocarbon degrading bacteria exhibit a species-specific response to dispersed oil while moderating ecotoxicity. *Appl. Environ. Microbiol.* 82, 518–527. doi: 10.1128/AEM.02379-15.

34. Lebeau, T. (2011). Bioaugmentation for in situ soil remediation: How to ensure the success of such a process *In*: Bioaugmentation, Biostimulation and Biocontrol (Singh *et al*. (eds.)). Soil Biology 28. pp 129-186. DOI 10.1007/978-3-642-19769-7_7.

35. Tyagi M, da Fonseca MM, de Carvalho CC. 2011. Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. Biodegradation. 2011 Apr;22(2):231-41. doi: 10.1007/s10532-010-9394-4. Epub 2010 Aug 3. PMID: 20680666.

36. Agnello AC, Bagard M, Van Hullebusch ED, et al. (2016) Comparative bioremediation of heavy metals and petroleum hydrocarbons co-contaminated soil by natural attenuation, phytoremediation, bioaugmentation and bioaugmentation-assisted phytoremediation. *Sci Total Environ* 563: 693-703

37. Andreoni, V., and Gianfreda, L. (2007). Bioremediation and monitoring of aromatic-polluted habitats. Appl. Microbiol. Biotechnol. 76, 287–308. doi: 10. 1007/s00253-007-1018-5.

38. Das N and Chandran P (2011). Microbial degradation of petroleum hydrocarbon contaminants: An overview. *Biotechnology Research International* Volume 2011, Article ID 941810, 13 pages doi:10.4061/2011/941810.

39. Shekhar, S., Sundaramanickam, A., and Balasubramanian, T. (2015). Biosurfactant producing microbes and their potential applications: a review. Crit. Rev. Environ. Sci. Technol. 45, 1522–1554. doi: 10.1080/10643389.2014. 955631.

40. Atlas, R. M. (1995). Petroleum biodegradation and oil spill bioremediation. Mar. Pollut. Bull. 31, 178–182. doi: 10.1016/0025-326X(95)00 113-2

41. Bento, F. M., Camargo, F. A. O., Okeke, B. C., and Frankenberger, W. T. (2005). Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresour. Technol. 96, 1049–1055. doi: 10.1016/j.biortech.2004.09.008

42. Thompson, I. P., van der Gast, C. J., Ciric, L., and Singer, A. C. (2005). Bioaugmentation for bioremediation: the challenge of strain selection. Environ. Microbiol. 7, 909–915. doi: 10.1111/j.1462-2920.2005.00804.x.

43. Chang, W. J., Dyen, M., Spagnuolo, L., Simon, P., Whyte, L., and Ghoshal, S. (2010). Biodegradation of semi- and non-volatile petroleum hydrocarbons in aged, contaminated soils from a sub-Arctic site: laboratory pilot-scale experiments at site temperatures. Chemosphere 80, 319–326. doi: 10.1016/j.chemosphere.2010. 03.055

44. Karamalidis, A. K., Evangelou, A. C., Karabika, E., Koukkou, A. I., Drainas, C., & Voudrias, E. A. (2010). Laboratory scale bioremediation of petroleum-contaminated soil by indigenous microorganisms and added Pseudomonas aeruginosa strain Spet. *Bioresource technology*, *101*(16), 6545-6552. https://doi.org/10.1016/j.biortech.2010.03.055

45. Ai-Kindi, S., and Abed, R. M. (2016). Effect of biostimulation using sewage sludge, soybean meal, and wheat straw on oil degradation and bacterial community composition in a contaminated desert soil. Front. Microbiol. 7:240. doi: 10.3389/ fmicb.2016.00240

46. Kim, S. J., Choi, D. H., Sim, D. S., and Oh, Y. S. (2005). Evaluation of bioremediation effectiveness on crude oil-contaminated sand. Chemosphere 59, 845–852. doi: 10.1016/j.chemosphere.2004.10.058.

47. Jimenez, N., Vinas, M., Sabate, J., Diez, S., Bayona, J. M., Solanas, A. M., et al. (2006). The Prestige oil spill. 2. Enhanced biodegradation of a heavy fuel oil under field conditions by

the use of an oleophilic fertilizer. Environ. Sci. Technol. 40, 2578–2585. doi: 10.1021/es052370z.

48. Garcia-Blanco, S., Venosa, A. D., Suidan, M. T., Lee, K., Cobanli, S., and Haines, J. R. (2007). Biostimulation for the treatment of an oil-contaminated coastal salt marsh. Biodegradation 18, 1–15. doi: 10.1007/s10532-005- 9029-3.

49. Zhao, H. M., Hu, R. W., Huang, H. B., Wen, H. F., Du, H., Li, Y. W., et al. (2017). Enhanced dissipation of DEHP in soil and simultaneously reduced bioaccumulation of DEHP in vegetable using bioaugmentation with exogenous bacteria. Biol. Fertil. Soils 53, 663–675. doi: 10.1007/s00374-017- 1208-y.