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Original Paper

Degradation properties of *Rhizobium petrolearium* on different concentrations of crude oil and its derivative fuels

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Abstract

The degradative efficiency of the recently identified species, *Rhizobium petrolearium*, on crude oil, diesel, petrol and kerosene was analysed in this study in order to assess its potential as a bioresource in environmental remediation and to investigate the effect of pollutant concentration on degradation efficiency. The identity of the isolate was confirmed by 16S rRNA sequencing and the variation in crude oil and fuel concentration during the biodegradation assay were measured using gas chromatography. Crude oil and the fuels were readily biodegradable at both single and tenfold concentrations, with petrol being the most degraded by the end of the study. Pollutant concentration was shown to affect degradation properties. At 1% concentration, the hydrocarbon compounds were almost completely degraded (99.3 to 99.6%) by day 5, but at the 10% concentration, the degradation level ranged from 31.8 to 63.8% on day 21. Crude oil and diesel oil showed the lowest biodegradation rates at 1% concentration and had half-lives of 0.68 and 0.64 days, respectively. Crude oil and kerosene were the most poorly degraded at 10% concentration with half-lives of 39.61 and 19.80 days, respectively. The $C_9 - C_{17}$ aliphatic fractions were generally the most readily utilised. This study presents a description of the biodegradation capabilities of *R. petrolearium* against crude oil and its derivative fuels and provides data regarding the possible role of this isolate in the development of bioaugmentation-focused bioremediation systems.

Key words: aliphatic hydrocarbons, biodegradation, bioremediation, bioresources, *Rhizobium*.
Abbreviations: CFU, colony forming units; ETPH, extractable total petroleum hydrocarbons.

Introduction

Oil spills are a major hazard associated with industrialisation and modern economy. They have been described as one of the foremost global environmental challenges as the environmental impact of these spills is far-reaching with consequences that continue long after the actual spill incident (Mansir, Jones, 2012). The key concern regarding the presence of these compounds in any ecosystem stems from their potential to disrupt ecosystem functionality. Ecosystem functions like respiration and nutrient cycling are often interrupted by the introduction of inordinate quantities of hydrocarbon compounds into the environment (Scott et al. 2014). For instance, the presence of petroleum in soil will change its elemental make-up and organic matter content. Increases in soil surface temperature have also been reported. In response to prolonged exposure to petroleum hydrocarbons and changes in environmental conditions, certain autochthonous microorganisms or their functional genes may become dormant while less resilient species may be totally wiped out. Groups with the capacity for petroleum hydrocarbon degradation will proliferate rapidly in response to the stimulus at the expense of non-hydrocarbon utilisers (Enujiugha, Nwanna 2004; Galazka et al. 2018; Galitskaya et al. 2021). All these responses diminish net ecosystem productivity and the capacity of the ecosystem to effectively sustain higher life forms. This is why the pursuit of effective, environmentally secure, and sustainable solutions to this menace via the exploitation of relevant bioresources lies at the forefront of research in modern environmental biotechnology.

Biodegradation is fundamental to the elimination of pollutant compounds from any environment and, therefore, cannot be excluded from investigations into environmental remediation interventions. Modern remediation technology is multifaceted, typically, with physical, chemical and biological dimensions. The biological approach to remediation is termed bioremediation and most commonly utilises the natural process of biodegradation and its microbial ecosystem drivers. This may sometimes entail deliberately supplementing engineered microorganisms into an environment to aid the biodegradation and pollutant removal process in a technique referred to as bioaugmentation. The feasibility of this technique

(bioaugmentation) is dependent on a distinct grasp of the catabolic capabilities of specific microorganisms against particular pollutant types in order to define their applicability as bioresources in pollution management and environmental biotechnology (Abdulsalam, Omale 2009; Adams et al. 2015; Andreolli et al. 2015). Crude oil and its derivative fuels have been identified as the most regularly occurring environmental pollutants (Gouda et al. 2007; Truskewycz et al. 2019; Ławniczak et al. 2020). The leading fuel derivatives of the fractional distillation of crude oil are petrol, kerosene and diesel; all of which are used as the main fuel sources for a variety of domestic and industrial purposes. These compounds frequently persist in the environment following a spill event (Gouda et al. 2007; Chaudhary et al. 2020).

The genus *Rhizobium* is reported to contain about 48 species. It comprises Gram negative, aerobic, rod-shaped motile bacteria having polar or lateral flagella. Rhizobia are of significant agricultural and environmental significance owing to their proven ability to establish symbiotic relationships with leguminous plants. They are an important plant-growth-promoting endophytic bacterial genus. *Rhizobium* spp. are, therefore, common to soil ecosystems where they may occur symbiotically in root nodules of legumes or non-symbiotically in the rhizosphere and in bulk soil. This genus of diazotrophs plays a vital role in nitrogen cycling as they are the key converters of atmospheric nitrogen to bioavailable ammonium in soil (Tan et al. 2001; Zhang et al. 2012). *Rhizobium* species have also been associated with the utilisation of complex hydrocarbon compounds like the polycyclic aromatic hydrocarbons as a carbon source (González-Paredes et al. 2013; Huang et al. 2016).

This study aimed to investigate the role of initial pollutant concentration on the biodegradation efficiency and degradative properties of *Rhizobium petrolearium*, a not so well-known species of this important group of soil microorganisms, on crude oil and its derivative fuels – petrol (gasoline), kerosene (dual purpose kerosene, closely related to paraffin oil) and diesel oil (automotive gas oil) to gain an understanding of its bioaugmentation potential. *R. petrolearium* was first described by Zhang et al. (2012). Only very limited studies have been carried out on the hydrocarbon degradative capacities of *R. petrolearium* with no known studies on its ability to utilise specific petroleum products.

Materials and methods

Soil, crude oil and fuel samples

The soil used in this study was obtained from the petroleum impacted Bomu area of Ogoniland (4.6340° N, 7.3559° E), in Rivers state in the Nigerian Niger Delta. Soil sample collection was done using a sterile auger and carried out from the soil surface to a depth of approximately 20 cm. Samples were collected at equidistant points within a 3 m^2 area and then merged to form composites.

The crude oil employed for investigation was Bonny light crude oil obtained from the Port Harcourt Refining Company, Eleme, Rivers state, Nigeria while petrol, diesel and kerosene fuels were procured from Lopec Petroleum, Alakahia, Port Harcourt, Nigeria. The crude oil and three fuel samples were filter sterilised using a 0.22 µm membrane filter while the media used were sterilised by autoclaving at 121 °C and 103.35 kPa for 30 min.

Isolation and preliminary characterisation of Rhizobium petrolearium

A 5 g portion of soil was suspended in sterile normal saline in a ratio of 1:10 w/v. The set up was shaken vigorously and then allowed to settle. The suspension was subjected to serial dilution and then aliquots from selected dilutions were aseptically inoculated unto sterile Bushnell Haas agar modified with 1% crude oil via the spread plate technique. The plates were incubated inverted 30 °C for 24 to 48 h. The discrete colonies obtained were purified using the streaking method on fresh agar plates (Cheesbrough 2006).

The pure isolates obtained were typified via extensive testing of their microscopic and biochemical qualities (Holt et al. 1994; Cheesbrough 2006). Following confirmation of tentative identity, isolates were maintained on yeast extract mannitol agar slants until required for further analysis.

Identification of R. petrolearium *via 16S rRNA gene sequence analysis*

Prior to DNA extraction, the test isolate was cultivated in Luria Bertani (LB) broth for 24 h, after which the 24 h axenic bacterial cells were harvested by centrifugation at 14000 rpm for 5 min.

A ZR fungal/bacterial DNA Miniprep kit (Zymo Research, USA) was utilised in the extraction of genomic DNA from the harvested bacterial cells as outlined by the manufacturer. The DNA obtained was assessed for purity using a Nanodrop 2000 spectrophotometer and then quantified and visualised on 1% w/v agarose gel with a UV transilluminator. The 16S region of the rRNA genes of the isolates was amplified using 27F and 1492R forward and reverse universal primers on an ABI 9700 thermal cycler (Applied Biosystems, USA) at a final volume of 50 µL. The PCR mix included: the X2 Dream taq Master mix (Inqaba, South Africa) (taq polymerase, DNTPs, $MgCl_2$), 0.4 M strength primers and the extracted DNA as the template. Sequencing was accomplished with a BigDye® Terminator 117 v3.1 kit on an ABI sequencer (Inqaba Biotechnological, South Africa) under the following PCR conditions: initial denaturation at 94 °C for 5 min, followed by 36 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, elongation at 72 °C for 45 s and final elongation at 72 °C for 7 min with a hold temperature of 10 °C. The amplified products obtained were resolved on a 1.5% agarose

gel at 120V for 15 min and then visualised using a UV transilluminator (Nasser et al. 2017).

The assembled high quality 16S rRNA sequence reads obtained were compared against the National Centre for Biotechnology Information database by basic local alignment search tool (BLAST) analysis. Blast hits with e-values closest to 0.0 were concluded to be closest to the isolate and were used for alignment and assembly of the phylogenetic tree.

Preparation of enrichment culture

A loopful of the test isolate was inoculated into sterile mineral salts broth medium supplemented with 1% contaminant (crude oil, petrol, diesel or kerosene) as the sole carbon source in Erlenmeyer flasks. The set-up was incubated with agitation at 30 °C for 48 h then 5 mL of the culture was introduced into fresh sterile contaminant-modified medium and incubated as above. This was done for three rounds.

Biodegradation assay

Mineral salts broth medium adapted with crude oil or the individual derivative fuel as the sole carbon source was used for growth and biodegradation studies (Okpokwasili and Okorie, 1988). The 24 h broth culture with an optical density of about 0.07 at 600 nm of *R. petrolearium* was employed in in vitro biodegradation studies to ensure that the cells used were in the exponential phase of growth. Testing was done with two replicates making three set-ups in total. Each set consisted of flasks each containing sterile medium with crude oil or the relevant fuel as the sole carbon source applied separately at 1 or 10 % v/v and inoculated with 1 mL of the 24 h broth culture of *R. petrolearium*. The pH was maintained at 7.5 based on the optimum pH levels for the isolate established by Zhang et al. (2012). The flasks were plugged with non-absorbent cotton wool and incubated at 30 °C with shaking. The negative control group consisted of uninoculated contaminant-modified medium to monitor possible non-biological losses in contaminant concentration and confirm the influence of the test isolate. For the 10% spike level, samples were drawn from each flask first after 24 h and then every 3 days for a period of 21 days while sampling was carried out every 24 h for 7 days in the case of the 1% spike concentration.

Quantification of growth

Growth of the test isolate was determined by both turbidimetry and plate counts. Turbidimetry entailed determination of the absorbance of the samples drawn from the flasks at 600 nm using a UV spectrophotometer (Shimadzu, Japan) standardised using uninoculated sterile media. Colony count investigations were conducted on yeast extract mannitol agar using the spread plate technique. Following incubation at 30 °C for 48 h, plates with visible colonies ranging from 30 to 300 were enumerated using an automated digital colony counter (Balance Instrument Co., China). The total viable counts were expressed as mean colony forming units per millilitre (CFU mL^{-1}).

Extractable total petroleum hydrocarbon content

The modified method outlined by USEPA (1996) was used to measure the extractable total petroleum hydrocarbon (ETPH) content by the liquid-liquid extraction method using a gas chromatograph fitted with a flame ionisation detector (Agilent 6890N, USA). For higher contaminant concentrations, sampling for determination of ETPH content was carried out at weekly intervals for 21 days while for the lower concentration, collection of samples for ETPH analysis stopped at day 5.

The residual hydrocarbon pollutant was extracted from the samples by shaking in a separatory funnel with about 30 mL dichloromethane with regular venting to release any extra pressure. The sample was then left to sit and settle for about 1 h to allow for separation of the water phase from the organic layer. This was followed by filtration and then concentration via evaporation using rotary evaporators overnight.

Sample clean-up entailed mixing the concentrate with cyclohexane and transferring it into a plugged glass column with addition of a slurry containing a combination of silica gel and dichloromethane and then anhydrous sodium sulphate and subsequently pentane. Elution of the extract was done with 10 mL pentane. The eluted sample was evaporated in a fume cupboard overnight at about 28 °C.

The micro-syringe of the gas chromatograph was cleaned thrice with dichloromethane and then rinsed before a 3 µL aliquot of the concentrated eluted sample of ETPH was injected into the gas chromatograph column to allow for separation of the constituent compounds. The separated compounds were then passed through the fitted flame ionisation detector with hydrogen as the carrier gas employed at 27.58 kPa.

Biodegradation kinetics

The biodegradation kinetics including removal efficiency, prediction coefficient, mathematical model, degradation constant and half-life for the different treatments were determined via a first order kinetics linear model applied to the ETPH data obtained at regular intervals from each set-up.

The degradation efficiency of total petroleum hydrocarbon in the crude oil and fuel samples was determined using equation:

$$\text{Degradation Efficiency (\%)} = (C_0 - C_t) / C_0 \times 100,$$

where C_0 is nitial ETPH concentration (mg kg^{-1}), C_t is residual ETPH concentration at time t (mg kg^{-1}), t is remediation time (days).

The biodegradation data were defined by pseudo first order kinetics linear regression model as described by Prasad and Suresh (2015) using the following equations:

$$\ln C_t / C_0 = -k_t,$$
$$t½ = (\ln 2) / k,$$

where C_0 is nitial ETPH concentration (mg kg^{-1}), C_t is residual ETPH concentration at time t (mg kg^{-1}), t is remediation time (days), k is biodegradation rate constant (day – 1), t is time (days), $t½$ is biodegradation half-life.

Data analysis

All the collected data were subjected to standard statistical distribution analysis. Relationships (within groups and between groups) between the degradation efficiencies of *R. petrolearium* against the test compounds at single fold and tenfold initial concentrations were defined via one-way and multiway analysis of variance at a 95% confidence interval. The analyses were done using SPSS 23.0.

Results

Phylogenetic tree analysis alongside the analysis of the 16S rRNA sequence obtained for the isolate with isolate code A6 established the identity of the test isolate as *Rhizobium petrolearium*. The phylogenetic tree representing the evolutionary distance between the test isolate and its closest genomic neighbours is shown in Fig. 1. The representative nucleotide sequence for the isolate is catalogued under accession number MF547450 at the National Centre for Biotechnology Information GenBank®.

The determination of growth via variations in the total viable bacterial counts and optical density of the test isolate, *R. petrolearium*, in the presence of 1 and 10 % concentrations of the hydrocarbon contaminants studied, revealed that for 10% contaminant concentration, total viable counts peaked between days 12 and 15 and the isolate entered into the death phase of the growth cycle by day 15, while for 1% spike levels, a steady decline in total viable bacterial counts from about days 1 – 2 was seen (Fig. 2, Fig. 3). Somewhat akin to the decline in counts as the study progressed, the optical density of the growth media exhibited an initial persistent rise with increasing contact time for both contaminant spike levels after which a decline was observed (Fig. 2, Fig. 3).

Except for diesel, maximum cell density levels (0.23 – 0.36 OD_{600}) were attained between days 3 and 5 at 1% spike. Diesel, oddly, showed a steady rise in optical density at 1% v/v in spite of the drop in corresponding viable counts. For the 10% spike, cell density reached a maximum level (1.24

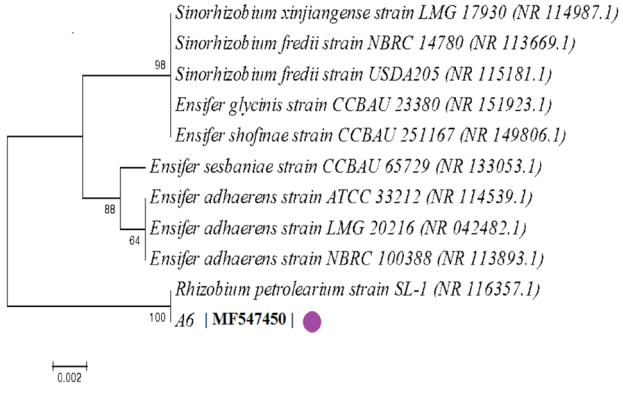

Fig. 1. Phylogenetic tree of the isolate A6 from the present study (indicated by the purple circle) based on partial sequences of 16S rRNA compared with closely related sequences from the GenBank database. The GenBank accession number is shown after the isolate code. The isolate was identified as *Rhizobium petrolearium*.

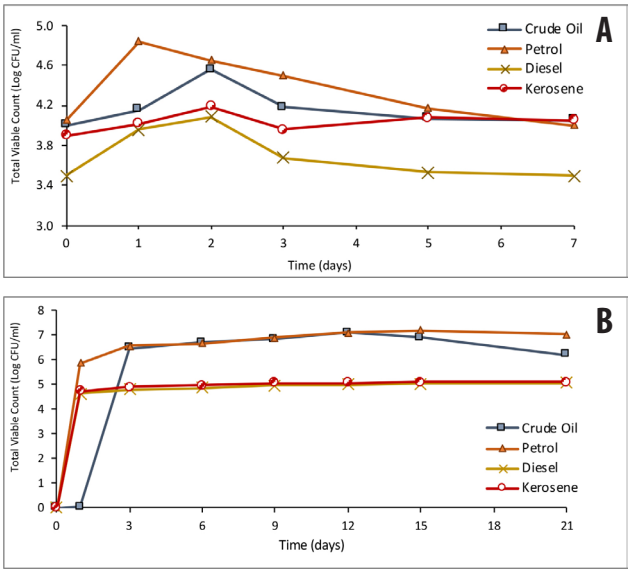

Fig. 2. Growth profile of *R. petrolearium* on the petroleum compounds at 1% (A) and 10% (B) concentration. Values are means of triplicates.

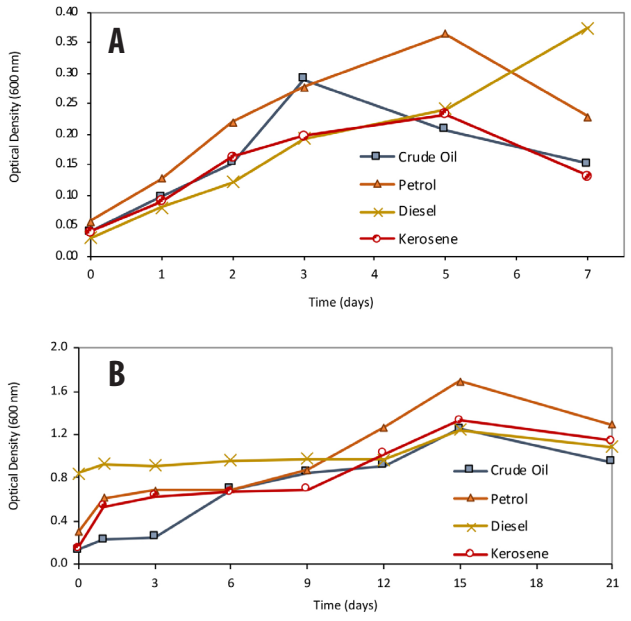

Fig. 3. Changes in cell density of *R. petrolearium* on the petroleum compounds at 1% (A) and 10% (B) concentration. Values are means of triplicates.

– 1.69 OD_{600}) at around day 15. Overall, the growth of the bacterium, *R. petrolearium*, was greater at 10% contaminant concentration than at 1%. The control cells showed zero counts and no demonstrable variation in optical density.

The test isolate was able to effectively utilise crude oil and its derivative fuels as a source of carbon for their growth. The test contaminants were almost completely eliminated from the growth medium by day 5 of the study at 1% spike. Crude oil, generally, fared poorly at high concentrations and proved to be the most recalcitrant hydrocarbon contaminant with removal levels of 31.79 and 99.25 % at 10% and 1% spike concentrations, respectively (Fig. 4). It was followed closely by diesel (99.48%) at 1% spike and kerosene (52.31%) at 10% spike. At 1% concentration, degradation levels over 99% were achieved within 5 days for the four compounds tested. There were statistically significant differences ($p \leq 0.05$) in degradation efficiencies between the single fold and tenfold initial concentration applied. Within groups, degrees of degradation obtained at 1 % v/v were not significantly different at $p \leq 0.05$ for the four test compounds; however, at 10 % v/v, statistically significant differences were seen in the degradation efficiencies of *R. petrolearium* against the test hydrocarbon complexes at 95% confidence interval.

The variation in concentrations of the aliphatic fractions obtained from the ETPH across the four hydrocarbon compounds tested at 10% contamination is shown in Fig. 5. Examination of the initial and residual total petroleum hydrocarbon fractions showed C_{13}, C_{18} and C_{21} to be the recalcitrant fractions at 1% concentration (Table 1). At the higher concentration of 10%, the volume of residual fractions was greater compared to 1% with the shorter chain compounds (C_9 – C_{17}) being the most readily utilised by the test isolate and longer chains particularly in the range C_{22} – C_{30} were the most recalcitrant. This is based on percentage removal of individual fractions. The ETPH levels in the control studies (not shown) remained relatively unaltered.

The biodegradation data fit into the pseudo-first order kinetic model as shown in Table 2. The results from the kinetic model established that microbial degradation was highest at lower concentrations of crude oil and its derivative fuels with a marked difference in biodegradation half-lives between the 1% initial concentration and 10% initial concentration. Crude oil had the greatest half-life across the board. For crude oil, the tenfold increase in concentration brought about an almost 98% drop in its biodegradation half-life while for its derivative fuels, the reduction in half-lives with the tenfold increase in concentration was in the range of 95.65 to 96.96%.

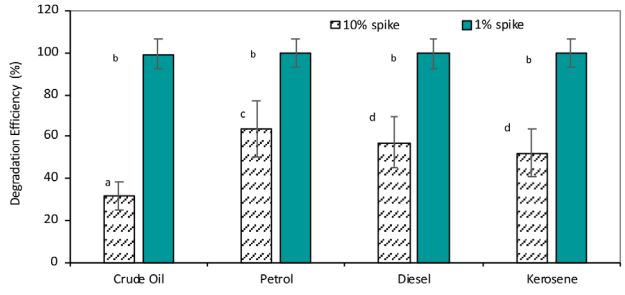

Fig. 4. Degradation efficiency of *R. petrolearium* on the test hydrocarbon compounds by the end of the study at 1 and 10% concentrations. Different letters indicate significant differences at 95 % confidence interval. The bars represent the standard deviation from the mean. The degradation levels obtained for the set-ups with 1% spike were over 99 % by day 5 for all the compounds studied.

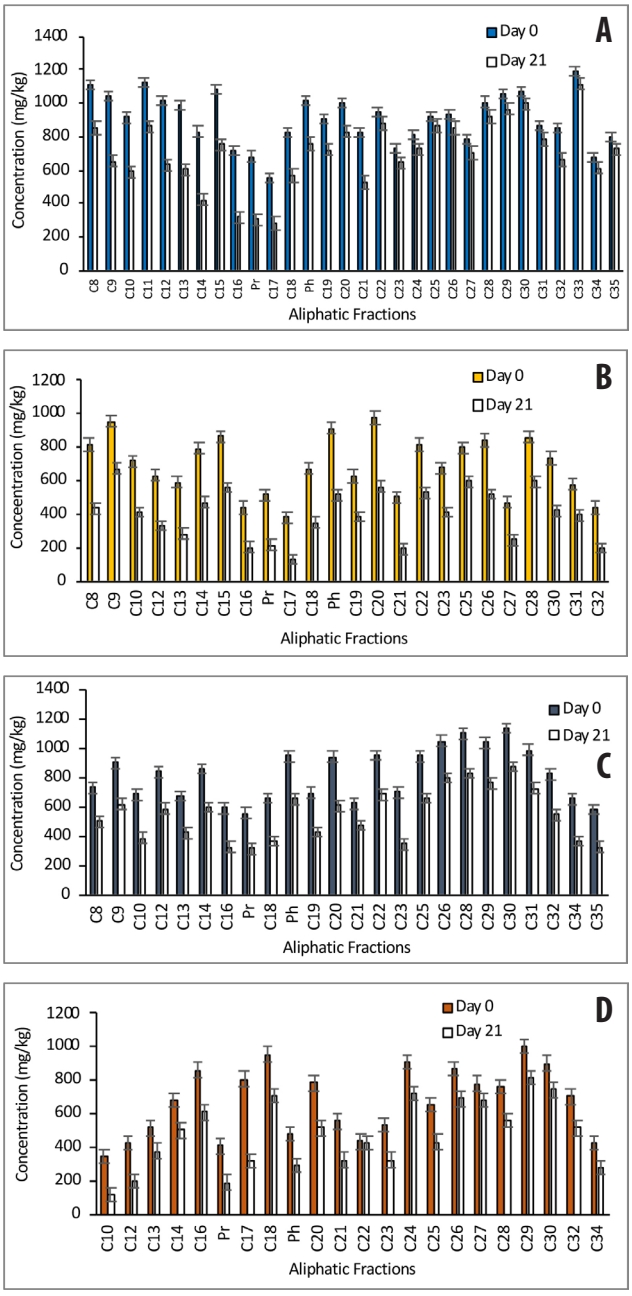

Fig. 5. Initial and residual aliphatic hydrocarbon fractions for crude oil (A), petrol (B), diesel oil (C) and kerosene (D) at 10% spike. The aliphatic compounds are represented by the length of their carbon chains. Bars represent standard error.

Table 1. Initial and residual (day 5) saturated hydrocarbon fraction concentrations obtained for the test compounds at 1% concentration

Fraction	Crude oil		Petrol		Diesel oil		Kerosene	
	Initial (mg kg^{-1})	Residual (mg kg^{-1})	Initial (mg kg^{-1})	Residual (mg kg^{-1})	Initial (mg kg^{-1})	Residual (mg kg^{-1})	Initial (mg kg^{-1})	Residual (mg kg^{-1})
C8	1075.53	–	805.42	–	728.15	–	–	–
C9	1012.14	–	941.89	–	896.89	–	–	–
C10	864.78	–	698.72	–	684.13	–	336.04	–
C11	1101.36	–	–	–	–	–	–	–
C12	963.89	–	615.42	–	835.57	–	405.61	–
C13	926.51	21.51	565.35	6.15	671.81	13.31	511.15	6.25
C14	799.63	–	778.45	–	857.11	–	663.13	–
C15	1027.17	–	842.86	–	–	–	–	–
C16	681.71	–	416.25	–	590.15	–	850.52	–
Pristane	649.69	–	503.71	–	552.16	–	398.78	–
C17	515.25	–	369.42	–	–	–	795.41	–
C18	783.22	11.43	657.43	4.05	659.19	–	942.04	–
Phytane	992.51	–	899.75	–	947.91	–	467.97	–
C19	859.52	–	602.19	–	695.49	–	–	–
C20	983.12	–	962.85	–	938.52	–	753.45	–
C21	726.52	5.19	483.48	–	623.18	8.06	518.19	8.90
C22	929.17	–	838.75	–	952.72	–	476.21	–
C23	701.89	–	694.42	–	696.16	–	576.85	–
C24	782.34	–	–	–	–	–	945.28	–
C25	912.93	–	812.53	–	943.71	–	702.13	–
C26	911.19	–	898.19	–	1049.72	–	915.36	–
C27	748.26	–	485.15	–	–	–	824.71	–
C28	986.82	–	913.72	–	1100.78	–	821.89	–
C29	1013.62	–	–	–	1039.94	–	1042.16	–
C30	1045.90	–	772.61	–	1134.77	–	935.88	–
C31	844.13	–	589.20	–	984.84	–	–	–
C32	818.36	–	476.83	–	822.10	–	739.83	–
C33	1149.63	–	–	–	–	–	–	–
C34	636.82	–	–	–	653.97	–	427.75	–
C35	771.15	–	–	–	581.92	–	–	–

Discussion

The growth profile of *R. petrolearium* on the different pollutants revealed that the compounds were somewhat readily utilised by the isolate especially at single fold concentrations although there was a steady decline in counts and cell density following an initial rise as the study proceeded. This decline in abundance coincided with the drop in contaminant concentrations. The number of bacterial species growing on a substrate relative to time when it is available as the sole carbon source has been pinpointed as indicative of the ease of the substrate's biodegradation with biodegradation levels considered greatest when counts are highest (Okpokwasili, Olisa 1991). The variation in biodegradation levels obtained in the current study are likely mainly due to differences in the structure and composition of the different compounds and differences in concentration. Crude oil and its derivative fuels are complex mixtures of different equally complex aliphatic and aromatic hydrocarbon compounds with crude oil containing a larger number of these complex compounds compared to petrol, diesel and kerosene. These hydrocarbon compounds vary in their chemical structure and molecular weights which, in turn, impact on their predisposition to breakdown by microorganisms.

As with the present study, several studies have demonstrated that the more complex the constituent compounds or the hydrocarbon structure, the less susceptible the hydrocarbon complex is to biodegradation (Huang et al. 2004; Haritash, Kaushik 2009). Compounds with a greater branched methyl substituent content or presence of condensed aromatic rings are commonly not as readily biodegraded as their less complex counterparts (Ubalua 2011). Likewise, the light polar structure of resins mean that they are more prone to microbial degradation (Spiecker et al. 2003). Typically, the ease of biodegradation of petroleum constituent compounds proceeds in the order, *n*–alkanes > branched alkanes > low molecular weight

Table 2. Biodegradation kinetics of the test contaminants at the two contaminant levels investigated during the study

Level	Parameter	Crude Oil	Petrol	Diesel	Kerosene
1%	Prediction coefficient (R^2)	0.9049	0.9083	0.9192	0.9122
	Biodegradation model	y = –1.0161x + 9.1019	y = –1.1228x + 8.3482	y = –1.0899x + 8.8844	y = –1.1527x + 8.8944
	Biodegradation half-life (days)	0.6822 (16.373 h)	0.6173 (14.815 h)	0.6360 (15.264 h)	0.6013 (14.432 h)
10%	Prediction coefficient (R^2)	0.9173	0.8388	0.8997	0.8855
	Biodegradation model	y = –0.0175x + 10.238	y = –0.0488x + 9.9098	y = –0.041x + 10.058	y = –0.035x + 9.7296
	Biodegradation half-life (days)	39.608	14.204	16.906	19.804

aromatics > cycloalkanes > heavy aromatics > asphalthenes, with saturates and light aromatics faring best (Macaulay 2015; Vaidehi, Kulkarni 2012). It has been stated that for most microorganisms, *n*-alkanes are the preferred carbon source and where they are available, the degradation of more complex unsaturated compounds becomes greatly reduced (Kniemeyer et al. 2005). The aliphatic portions of petrol, kerosene and diesel oil typically consist of *n*-alkanes with chain lengths ranging from C_4 to C_{10}, C_{10} to C_{20} and C_{15} to C_{40}, respectively (Onwurah et al. 2007). The ease of biodegradation for petrol at the two concentration levels seen in the current study may, therefore, be due to its relatively less complex composition and its constituent shorter chain n-alkanes compared to kerosene, diesel and crude oil. One report noted that diesel oil is known to consist of a high quantity of aliphatic hydrocarbons (Militon et al. 2010).

Similarly to the results of the present study, it was shown that petrol was more readily biodegraded than diesel oil with an intrinsic biodegradation by soil microorganisms of 96% compared to 60 to 73% seen with diesel oil (Marchal et al. 2003). In contrast to the findings at 1% spike levels and comparable to the results from the 10% spike in the current study, however, diesel demonstrated greater biodegradation levels than kerosene and engine oil against 10 bacterial isolates (including species of *Listeria, Bacillus, Pseudomonas, Citrobacter* and *Staphylococcus aureus*) at a 0.2% spike in a study describing petroleum hydrocarbon degrading strains from ship-breaking yards (Ahamed et al. 2010). They further found that diesel was degraded by 84.8 to 96.8% while kerosene was degraded by only about 50.0 to 78.3%;. These values are surprisingly lower than found in the present study at 1% initial concentration, which is higher than the 0.2% applied in the investigation by Ahamed et al. (2010). Biodegradation rates and effectiveness of microbial uptake are also typically comparable to the concentration of the compound, barring other influences. This relationship is often somewhat directly proportional up to specific threshold concentrations after which further escalation in concentration impacts negatively biodegradation and uptake rates (Allamin et al., 2020). This could be the case in the present study at the tenfold contaminant concentrations. Other studies have corroborated that after a certain concentration level, there is generally a corresponding drop in biodegradation efficiency as substrate concentration rises (Jørgensen 2008; Huang et al. 2016; Ławniczak et al. 2020). This is consistent with results from a degradation study in a marine ecosystem where elimination efficiency was seen to decline with increasing crude oil concentration (Chen et al. 2017). A total loss of microbial degradation at concentrations of over 50% for petroleum hydrocarbon compounds was also reported (Shayanfar et al. 2011).

It is reported that elevated concentrations of petroleum hydrocarbons may greatly inhibit growth of microorganisms, even those with proven petroleum hydrocarbon degradation capacity (Xu et al. 2018). A study on microbial breakdown of oily sludge confirmed that biodegradation rates were most efficient between concentrations of 1.25 and 5% with optimum biodegradation observed at 5%. Oil concentrations over 5% resulted in a drop in relative abundance within the microbial community, resulting in a decline in removal rates (Cerqueira et al. 2011). In addition, it was confirmed that concentration played a significant role in utilisation of diesel oil by *Bacillus* sp. (Raju et al. 2017). Turbidity measures of bacterial cell density were found to increase with increasing diesel concentration from 0.5 to 2.0%, but at 2.5% concentration, cell density declined. The impact of petroleum hydrocarbon concentration on biodegradation may be attributed to cell membrane toxicity exerted by petroleum hydrocarbon compounds particularly at high concentrations. Furthermore, increased levels of these xenobiotics have been known to destabilise C:N:P ratios within the ecosystem (Chaillan et al. 2006), which would, in turn, impact negatively the activity of degrading species. Poor degradability at higher concentrations could speak to the significance of a microbial consortium in biodegradation and biofilm formation to combat toxicity effects.

Shorter aliphatic fractions within hydrocarbon compounds are normally the first groups attacked by microorganisms during biodegradation (Gouda et al. 2007). In tandem with the findings in the present study, the fractions in the range C_{18} to C_{22} have been identified as not being readily degradable compared to other aliphatic fractions (Bajagain et al. 2018). The C_{18} to C_{22} *n*-alkane fractions were also noted as the most recalcitrant during a study on biodegradation of diesel oil (Chaudhary et al. 2020). This decreased susceptibility to microbial degradation has been attributed to their low solubility, which could translate to lower bioavailability, their lower

polarity and greater stearic hindrance. Fractions that are more bioavailable tend to be more readily biodegraded with degradation rates proportional to their concentration in the system (Stroud et al. 2007; Shayanfar et al. 2011; Tarr et al. 2016). Unlike *R. petrolearium* as seen in the current study, *Rhodococcus* sp. was found to show a proclivity for the C_{14} to C_{19} aliphatic fractions in crude oil (Binazadeh et al. 2009).

The degradative efficiency of the test isolate could be further strengthened by prior contact with petroleum hydrocarbons. The past exposure of the *R. petrolearium* isolate investigated in the current study is crucial to its observed prowess in petroleum hydrocarbon degradation. Previous exposure of a microbial community to hydrocarbons endow the microorganisms with superior competence and versatility for the utilisation of similar hydrocarbon compounds as they already have the necessary catabolic genes and are consequently better able to promptly synthesise the requisite enzymes for degradation. Quite a few studies have highlighted this superior readiness by microorganisms to tackle xenobiotics to which they have been previously subjected; a trait that is noted to be transferrable to ensuing generations by genetic adaptation. Experienced microbial degraders have presented biodegradation rates surpassing 10 to 400 times those seen in their inexperienced and unexposed counterparts (Chaillan et al. 2006). It was found that bacterial cells grown in media enriched with pyrene were able to degrade pyrene in laboratory studies by 55% in contrast to the mere 1% seen with previously unexposed bacteria (Laleh et al. 2006). This is further buttressed by another study showing greater degradation levels following prior exposure of selected species (Chaîneau et al. 2005). In yet another study, species of Pseudomonas and Actinomyces obtained from diesel-impacted soil more readily utilised crude oil, diesel, kerosene, engine oil, cyclohexane and dodecanol than isolates from uncontaminated soils (Agwu et al. 2013).

There are very limited studies on organism-specific half-lives for crude oil and its derivatives. At a more elevated (compared to the current study) crude oil concentration of 100 mg L^{-1}, degradation half-lives of 6.39 days, 17.33 days and 79.67 days for crude oil during remediation studies using palm bunch biostimulant, oil dispersant and natural attenuation, respectively, were obtained (Abonyi et al. 2022). These values are much higher than those obtained in the 1% spike in the present study but lower than the values seen with the 10% spike. The half-life for diesel in the present study was similar to that obtained in a study using the bioventing technique (Agarry, Latinwo 2015). They reported degradation half-lives for diesel of 43.3 days and 16.1 days using natural attenuation and bioventing respectively. When bioventing was combined with brewery waste amendments, the half-life value further declined to 6.4 days. In contrast, much higher half-lives of 33.53 to 35.09 days for diesel using different amendments and 103.43 days with natural attenuation was obtained (Uba et al. 2019). Similarly, the natural attenuation half-life for diesel was estimated at 42.26 days at 1% spike in a phytoremediation study (Dadrasnia, Agamuthu 2013).

Conclusions

This study has added to the library of knowledge on hydrocarbon degrading species by investigating the ability of Rhizobium petrolearium to utilise hydrocarbon compounds as an energy source. The bacterium was able to effectively utilise crude oil, petrol, diesel and kerosene at single and tenfold concentrations. The study further identified the most recalcitrant n-alkane fractions across the four compounds as C_{13} to C_{21} for single fold contaminant concentrations and C_{22} to C_{30} for tenfold concentrations. Biodegradation half-lives ranged from 0.601 to 0.682 days at single fold spike and 14.204 to 39.608 days at tenfold concentrations. Further studies that explore how the isolate would fare in the field as a bioaugmentation inoculant are recommended. The application of nutrient amendment alongside bioventing or biosparging techniques in soil or water could improve the outcome of biodegradation by the isolate in field studies.

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