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Diversity and Degradative Potency of Extant Autochthonous Crude Oil-Metabolizing Species in a Chronically Polluted River

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ABSTRACT

Persistent pollution of surface waters by hydrocarbon compounds is one of the foremost threats to limited global freshwater resources. This study analyzed the abundance, diversity and degradative capacities of hydrocarbon-utilizing bacteria in chronically polluted Kono River in the Nigerian Niger Delta in order to establish the bacterial drivers of ecological regeneration of the river after an oil spill. The study further aimed to develop a specialized bacterial consortium for application in bioremediation interventions. *Bacillus*, *Pseudomonas* and *Enterobacter* spp. were predominant out of the 82 isolates obtained. *Klebsiella pneumoniae* and two species of *Enterobacter cloacae* were identified as the most efficient hydrocarbon utilizers. The isolates were also confirmed as biosurfactant producers and possessed the *alkB1* and *nahAc* genes for degradation of aliphatics and aromatics. *E. cloacae*-K11, *K. pneumoniae*-K05, *E. cloacae*-K12 and their consortium were able to degrade the total petroleum hydrocarbons and polycyclic aromatic hydrocarbons in batch systems by 59.37% – 96.06% and 68.40% – 92.46% respectively. *K. pneumoniae*-K05 showed the greatest petroleum degradation capacity of the three isolates but hydrocarbon degradation was most efficient with the bacterial consortium. The results obtained showed no significant differences at $p \leq 0.05$ between the degradation capacities of *K. pneumoniae*-K05 and the consortium for PAHs but a significant difference ($p \leq 0.05$) was seen with TPH degradation. A viable hydrocarbon degrading bacterial consortium was developed at the end of the study and it was concluded that the polluted river water displayed inherent potential for effective natural attenuation.

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INTRODUCTION

Environmental pollution is a major and somewhat inescapable consequence of anthropogenic endeavor especially those linked to development and industrialization. Pollution by petroleum and its derivatives has been classed as one of the foremost global environmental challenges (ITOPF, 2021). The growing demand for energy, however, continues to drive crude oil exploration with consequent pollution of both aquatic and terrestrial ecosystems. Statistics from 2015 highlighted repeated pollution as the sixth most significant global challenge with sub-Saharan Africa placed 3rd amongst regions most affected by the growing crisis (World Economic Forum, 2015). Several spill incidents with volumes exceeding 3 million barrels have been reported in

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Nigeria over recent decades (Onwurah *et al.*, 2007). The Nigerian Niger Delta is known to bear the brunt of this assault. Records from the National Oil Spill Detection and Response Agency (NOSDRA) in Nigeria estimated that over a 20–year period, about 2.4 million barrels of crude oil were spilled into aquatic and semi-aquatic environments in the Niger Delta and only 23% of the spilled oil was recovered (Kadafa, 2012; Macaulay, 2014). Spillage in terrestrial systems is typically delimited and more readily controlled than those on water as water presents the challenge of unfettered oil dispersion. Incidents of petroleum spillage in aquatic ecosystems are therefore more large-scale and devastating and marine spills ultimately ease into brackish and freshwater environments with time (Macaulay, 2014).

Crude oil spills in the aquatic environment are of great concern as they impact on surface waters, ground water and aquatic organisms. Water is a critical resource; one that is gradually becoming scarce according to current reports (UNECA, 2007). Water is essential for both rudimentary needs like drinking, domestic and sanitary activities and recreation; and more advanced enterprises like transportation, electricity generation, and infrastructure development. Water security is of paramount global concern; it is a challenge that cuts across several spheres including human health, environment and even human rights. African countries carry the greatest burden of limited water resources (UNECA, 2007). Rivers are one of the most ancient waterbodies in existence and their quality is fundamental to the survival of humans and the biome (Higler, 2012). It is estimated that in South Africa, more than 15% of individuals in rural areas live off polluted riverwater as their sole source of water. Alarmingly greater numbers of 40% and 70% are reported for Nigeria and Sudan respectively (Shirani *et al.*, 2018). These statistics highlight the urgent need for studies into water remediation technology.

The commonly employed physical and chemical remediation techniques are normally environmentally invasive, deleterious to ecosystem players, expensive and do not ensure the total elimination of the organic pollutants. Biological remediation techniques are distinctive in this regard as they utilize biological systems and processes to ensure the complete removal of the hazardous forms of organic pollutants. Microorganisms play a crucial role here as bioremediation very commonly entails the use of microorganisms or their enzymes to restore an environment impaired by pollutants to conditions as close as possible to its pristine state. Microbial bioremediation is particularly unique because it exploits the metabolic potential of microbial groups to completely mineralize hydrocarbons or transform them into less noxious forms – the system of elimination employed in nature (Shahsavari *et al.*, 2018; Ramdass & Rampersad, 2021). There are several different genera of bacteria and fungi with the inherent capacity to actively degrade hydrocarbons and they are found in almost any soil or aquatic environment (Lima *et al.*, 2020). Studies targeted at optimizing the biodegradation process are pivotal to the advancement of bioremediation technology. The identification of petroleum utilizing species is a crucial part of bioremediation in any system as it provides insight into specific growth requirements, metabolic pathways and enzymatic activity.

With the unfolding environmental and water security crises across the globe, the need for effective, minimally intrusive and sustainable pollution management techniques has become apparent. The importance of biodegradation by the autochthonous aquatic microbial community cannot be over-emphasized. It is fundamental to ecosystem regeneration after a disturbance. The presence of this specialized group of microorganisms, able to biodegrade petroleum hydrocarbons, is an indication of a system's inherent capacity for natural attenuation and would normally provide a measure of the system's ability to effectively and quickly recover from hydrocarbon stressors. This study aimed to establish the microbiological players involved in ecological regeneration following spillage in a riverine environment and then develop a specialized bacterial consortium for application in bioremediation of petroleum spills in aquatic ecosystems. The objectives were to analyze the growth properties, abundance, diversity and degradative capacities of hydrocarbon utilizing bacteria isolated from chronically polluted

freshwater from Kono River in Rivers state, Nigeria as well as to ascertain the presence of relevant hydrocarbon catabolic genes in the isolates and determine their ability to produce biosurfactants.

MATERIALS AND METHODS

Kono River is situated in Gokana Local Government area of Rivers state, Nigeria, an area with several reported cases of spillage dating back up to five decades. Contaminated water samples were collected from 12 equidistant points along the length of Kono River using sterile plastic bottles. Sampling was along the water column. The samples were appropriately labelled and immediately transported to the laboratory for analysis.

The pH values of the water samples were determined using a combined glass calomel electrode and a pH meter (Jenway, UK) while a benchtop combination meter was used to determine electrical conductivity. The chemical oxygen demand (COD) and the five-day biochemical oxygen demand (BOD_5) of the samples were determined using the methods outlined by ASTM (2012) and USEPA (2012) respectively. COD was determined by the dichromate closed reflux method. For BOD_5, the sample was incubated at 20°C for 5 days and the oxygen consumed was measured. The TDS and TSS were determined according to the respective methods of evaporation and filtration recommended by APHA (2005).

The study was designed to identify the three top hydrocarbon degrading bacteria from the river water samples in order to use them in development of a viable consortium. All experiments were done in triplicates unless otherwise stated. The experimental design consisted of, first, the enumeration of total cultivable heterotrophic bacteria (TCHB) and then the isolation, enumeration and identification of cultivable hydrocarbon utilizing bacteria (CHUB) from the composite river water samples collected (n=10). The top fast-proliferating isolates (n=12) on crude oil-tainted medium were subjected to tests to determine their growth rate and whether they produce biosurfactants. The five fast-growing isolates were screened for their crude oil utilization capacity using growth monitoring and turbidimetry techniques. Finally, the top three utilizers were tested for their ability to effectively degrade crude oil. These isolates (n=3) were then identified based on their genomic characteristics.

Total cultivable heterotrophic bacteria (TCHB) were enumerated as follows: ten sets of 500 ml volumes of composite water samples were filtered through a 0.22 µm membrane filter. The membranes from each individual composite sample (10 in total) were then aseptically introduced into sterile normal saline. After rigorous shaking, the samples were then serially diluted and 0.1 mL aliquots of suitable dilutions inoculated unto nutrient agar plates in triplicates making a total of thirty plates. Incubation was at room temperature for 48 hours. Plate counts were done at the end of the incubation period using an automated digital colony counter (Balance Instrument Co., China). Plates with counts in excess of 300 colonies were discarded.

Isolation and enumeration of cultivable hydrocarbon utilizing bacteria (CHUB) were done using nystatin–amended mineral salts medium (MSM) via the vapor phase method (Azuwike *et al.*, 2020) with 3 replicates per samples totaling 30 plates in all. Approximately 0.1 mL aliquots of dilutions of the broth culture were plated out on the MSM. Sterile Whatmann No.1 filter papers were then saturated with crude oil and aseptically placed into the lids of individual inverted agar plates. The plates were incubated in the inverted position at 30 °C for 5 – 7 days. Plates with visible colonies ranging from 30 – 300 were enumerated using an automated digital colony counter and expressed as colony forming units per milliliter of river water sample. Discrete colonies were purified by sub-culturing twice via the streaking plate technique. The pure isolates were then transferred to slants and stored for further analysis and testing.

The percentage content of hydrocarbon utilizing bacteria in the river water was determined using the formula:

$$\% \text{ CHUB} = \frac{\text{CHUBC}}{\text{TCHBC}} \times 100$$

Where: CHUBC – cultivable hydrocarbon utilizing bacterial counts; TCHBC – total cultivable heterotrophic bacterial counts

The preliminary identification of the isolates obtained (n=82) was on the basis of their cell morphology and cultural and biochemical characteristics (Holt *et al.*, 1994; Cheesborough, 2006). Apart from the microscopic observation of the cells and observation of colony characteristics, several standard biochemical tests were employed in the characterization of the isolates. Some of the tests used in the preliminary identification were Gram's staining, spore staining, urease production, lysine utilization, nitrate reduction, hydrogen sulfide production, citrate utilization, motility, Methyl Red, Voges Proskauer reaction, ornithine utilization, gelatin liquefaction, triple sugar iron test, phenylamine deamination, indole production, starch utilization, catalase reaction, oxidase production and fermentation of multiple simple and complex sugars. Enzymatic reactions – phenylalanine deaminase, acetate utilisation, arginine dehydrolase, lipase and ornithine decarboxylase – were also tested

The modified technique of Gerhardt *et al.* (1994) was adopted in the preparation of standard cultures. Each purified isolate was inoculated from a stock culture into 100 mL of mineral salts broth containing crude oil as the sole carbon source in a conical flask and incubated at room temperature for 24 h with agitation. The isolate from the 24 h standard culture was serially diluted and the extent of growth for the individual isolates was determined using 0.1 ml aliquots of the dilutions via the vapor phase method described above. The isolates that grew within 48 h were considered fast-growing and were analyzed for the production of biosurfactant. The five best growers were screened for hydrocarbon utilization.

The oil spreading technique was employed to test for biosurfactant production in the isolates. The 48 h broth culture of the test isolate was centrifuged and about 10 µL of the culture supernatant dropped carefully on the oil-laden surface of a petri dish containing 20 µL of Bonny light crude oil layered unto 20 mL sterile distilled water. Set-ups with an emulsified halo of up to 10 mm in diameter after 30 s were considered positive for biosurfactant production. Duplicate assays were carried out per isolate (Walter *et al.*, 2013).

The ability of each isolate to utilize crude oil was determined *in vitro* by growth monitoring using viable plate count and by turbidimetry using a UV-visible Spectrophotometer. The modified method of Okpokwasili and Okorie (1988) using Bonny light crude was employed for the screening analysis. The crude oil was sterilized by filtration using a 0.22 µm membrane filter. Sterile mineral salts broth (MSB) containing 5% sterilized crude oil in testtubes was inoculated with 0.1 mL of the bacterial suspension from the enrichment broth. This was done in three replicates for each pure test isolate. The control tubes consisted of MSM supplemented with crude oil without inoculation. The tubes were incubated at room temperature on a rotary shaker for 15 days. The pH, optical density (OD) of each set-up at 540 nm and the total viable bacterial counts (TVBC) (using pour plate method) were determined at regular intervals. The isolates with the greatest utilization potential based on specific growth rate and increase in optical density were subjected to the degradation assay. The formula used to determine the specific growth rate is as below.

$$\text{Specific Growth Rate } (\mu) = \frac{2.303(\log Nt - \log No)}{\text{Tt} - \text{To}}$$

Where: No – bacterial count at the onset of the exponential growth phase; Nt – bacterial count at time t during the exponential growth phase; To – time (h) at the start of the exponential growth phase; Tt – time (h) at selected point during the exponential growth phase

The techniques outlined by Okpokwasili and Okorie (1988) and Odokuma and Okpokwasili

(1993) were adapted for the hydrocarbon degradation assay using crude oil supplemented mineral salts broth (MSB). Approximately 1 ml of each of the three selected test isolates from the enrichment broth was inoculated into an Erlenmeyer flask containing about 100 ml MSM with 10 % v/v of Bonny light crude oil as the sole carbon source. The set up for each isolate was done in with replicates such that there were 15 set-ups in total. Incubation was at room temperature on a rotary shaker for 21 days with monitoring of total petroleum hydrocarbon and polycyclic aromatic hydrocarbon contents at 7-day intervals. The pH was maintained at 7.0. All set-ups were replicated. The total petroleum hydrocarbon and polycyclic hydrocarbon contents of samples were determined using a gas chromatograph fitted with a flame ionization detector (Agilent 6890N, USA) via liquid-liquid extraction method as per Protocol 3560 (USEPA, 1996). The samples were extracted with dichloromethane and eluted using pentane.

The most efficient hydrocarbon degrading bacterial isolates were sequenced to determine their specific identities. The technique of Nasser *et al.* (2017) was modified for the study. The cells of pure isolates cultivated in Luria-Bertani medium were harvested by centrifugation and the bacterial DNA extracted using ZR fungal/bacterial DNA Miniprep (Zymo research, USA) as outlined by the manufacturer. The Nanodrop 2000 spectrophotometer was used to determine the concentration of the DNA obtained from the extraction process and establish its purity while the integrity of the DNA sample was established by quantifying and visualizing the DNA using a UV transilluminator on 1% w/v agarose gel. The 16S region of the rRNA genes of the isolates were amplified using the 27F and 1492R forward and reverse universal primers on GeneAmp® PCR System 9700 (Applied Biosystems, USA) at a final volume of 50 µl. The PCR parameters were: initial denaturation at 94 °C for 5 minutes, 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 then final elongation at 72°C for 7 minutes. Hold temperature was 10 °C. The amplified PCR products were resolved on a 1.5 % agarose gel at 120V for 15 minutes then visualized using a UV transilluminator. Sequencing was done using the BigDye® Terminator 117 v3.1 kit on 3510 ABI sequencer (Inqaba Biotechnological, South Africa).

Sequence identification entailed checking the 16S rRNA sequences obtained for each test isolate against the National Centre for Biotechnology Information (NCBI) database using the 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. Screening for the alkane-1-monooxygenase (*alkB1*) and naphthalene-1,2-dioxygenase (*nahAc*) degradative genes was done by visualizing on a 1.5 % agarose gel using a UV transilluminator. This compared the nucleotide sequences in PCR-amplified DNA extracted from the test bacterial isolates to selected gene-targeting primers for the *alkB1* and *nahAc* degradative genes. The forward and reverse primers used were *nahAc* F 5'-TGGCGATGAAGAACTTTTCC-'3 and R 5'-AACGTACGCTGAACCGAGTC-'3 and *alkB1* F 5'-TACGGGCACTTCGCGATTGA-'3 and *alkB1* R 5'-CGCCCAGTTCGAMACGATGTG-'3, for the *nahAc* and *alkB1* degradative genes respectively.

Basic statistical distribution analysis of the data obtained was conducted using Microsoft Excel® 2016 and SPSS 23.0. The degradative capacities, based on residual concentrations, among the bacterial isolates and their consortium were compared using one way ANOVA at 95% confidence interval. The null hypothesis, H_0, was that there was no significant difference in TPH and PAHs removal amongst the three isolates and their consortium.

RESULTS AND DISCUSSION

The baseline physicochemical characteristics of the Kono river water samples were indicative of organic pollution. The mean pH of the river water samples was 8.82 ± 0.02 while the electrical conductivity was 1375.60 ± 0.05 µS/cm. The mean total petroleum hydrocarbon (TPH) and

polycyclic aromatic hydrocarbons (PAHs) levels in the water samples were 23.55 ± 1.17 mg/L and 1.79 ± 0.19 mg/L respectively with five-day biochemical oxygen demand (BOD_5) and chemical oxygen demand (COD) levels of 18.0 ± 0.91 mg/L and 53.48 ± 2.03 mg/L respectively. These values are higher than the permissible limits stipulated by regulatory agencies (FMoE, 1995; DPR, 2002). The mean values obtained for total dissolved solids (TDS) and total suspended solids (TSS) in the polluted Kono river water samples were 307.59 ± 1.33 mg/L and 2.70 ± 0.72 mg/L respectively.

The mean abundance of total cultivable heterotrophic bacteria (TCHB) and cultivable hydrocarbon utilizing bacteria (CHUB) were 6.505 log CFU/ ml and 3.895 log CFU/ mL respectively with the CHUB being only a miniscule 0.245% of the total cultivable heterotrophic bacterial community in the river water. Out of the 82 bacterial isolates obtained from the polluted river water samples, 16 genera were identified (Fig. 1). The predominant isolates were *Bacillus*, *Pseudomonas* and *Enterobacter*. *Vibrio* sp. had the lowest occurrence.

The percentage of hydrocarbon utilizers obtained in the present study was unexpected. A higher fraction of hydrocarbon (HC) utilizers was expected because the river and the region as a whole are linked to long-term crude oil pollution. High abundance of HC utilizers are expected in environments that suffer from repeated spillage over a long period of time. One researcher suggested that after a pollution event, the abundance of HC utilizers will often rise significantly in the environment; these numbers drop over time as the pollutant levels decrease but will scarcely return to the pre-pollution levels. This is especially true of environments that experience chronic pollution (Neethu *et al*., 2019; Galitskaya *et al*., 2021). The impacted system will often revert to a new equilibrium that would see greater abundance of HC utilizing bacteria

A total of 12 isolates were classed as fast growing utilizers based on their ability to grow extensively on crude oil-amended MSM within 48 hours of incubation. Their observed extent of growth and their ability of produce biosurfactants are outlined in Table 1. Biosurfactant production by a bacterium provides a further advantage during biodegradation of organic compounds as the presence of the biosurfactant increases the bioavailability of the substrate which, in turn, aids attack by microbial enzymes (Iyobosa *et al*., 2020). It is therefore foreseeable that the effective fast-growing hydrocarbon utilizers would be biosurfactant producers.

Bacillus sp. (K02), *Pseudomonas* sp. (K11), *Enterobacter* sp. (K12), *Klebsiella pneumoniae* (K05) and *Arthrobacter* sp. (K64) displayed the most extensive growth properties on crude oil

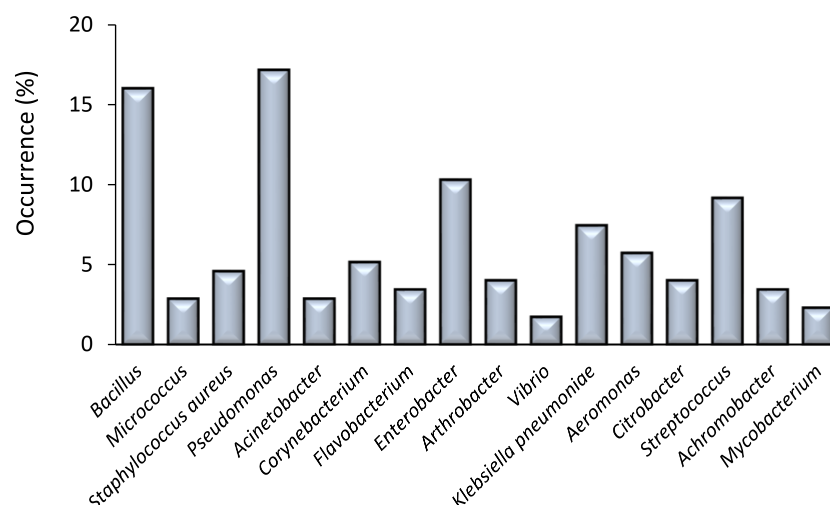

Fig. 1. Occurrence of the hydrocarbon utilizing bacterial isolates in the petroleum-polluted river water.

Table 1. Biosurfactant production and observed growth patterns of selected isolates on mineral salts agar with crude oil as the sole carbon source after 48 h incubation

Isolate code	Tentative Identity	Growth Pattern	Biosurfactant production
K01	*Klebsiella pneumoniae*	+++	+
K02	*Bacillus* sp.	+++	+
K05	*Klebsiella pneumoniae*	+++	+
K08	*Bacillus* sp.	++	–
K11	*Pseudomonas* sp.	+++	+
K12	*Enterobacter* sp.	+++	+
K33	*Pseudomonas* sp.	++	+
K46	*Acinetobacter* sp.	++	+
K47	*Bacillus* sp.	+++	+
K59	*Flavobacterium* sp.	+	+
K64	*Arthrobacter* sp.	+++	+
K71	*Staphylococcus aureus*	++	–

Growth: +++ High growth (> 50 CFU); ++ Medium growth (10 – 50 CFU); + Low growth (< 10 CFU)
Biosurfactant: + Confirmed; – Not confirmed

amended mineral salts medium after 48 hours. The specific growth rates for the isolates ranged from 0.3/h – 1.06/h. The results from the hydrocarbon utilization assay of these isolates based on turbidimetry and viable plate counts and the variation in the pH of the growth medium are illustrated in Figure 2. The greatest optical densities of about 2.5 and 2.4 were seen with *Pseudomonas* and *K. pneumonia* respectively. Most of the isolates reached maximum abundance on day 9 of the study. *Bacillus* showed the greatest abundance during the study of about 4.398 log CFU/ml while *Arthrobacter* reached its maximum count of about 3.699 log CFU/ml at the end of the study. Only *Arthrobacter* demonstrated a distinct lag phase during the HC utilization study (Fig. 2). With the exception of the *Bacillus* and *Arthrobacter* isolates, HC utilization in all the other isolates seemed to produce alkaline by-products as evidenced by the rise in pH of the medium. With *Bacillus*, the pH of the MSM dropped quite sharply suggesting the generation of more acidic metabolites during the process. This could allude to the utilization of the fermentative metabolic pathway by *Bacillus* and the oxidative pathway by the other isolates as the production of acidic end-products is a major difference between the fermentative metabolic pathway and the oxidative one. Different groups of organisms produce varying metabolic by-products during biodegradation; the metabolites produced will often depend on the precise degradation pathway employed by the organism (Joutey *et al.*, 2013; Truskewycz *et al.*, 2019). The initial reduction in pH exhibited by *Pseudomonas* and *K. pneumoniae* indicate the possible production of acidic intermediates.

Genomic analysis verified the identities of the top three HC degrading isolates as *Enterobacter cloacae* (K11), *Klebsiella pneumoniae* (K05) and *Enterobacter cloacae* (K12) (Fig. 3). The nucleotide sequences obtained for the isolates have been registered in the NCBI GenBank® under accession numbers OK077562 – OK077564. All three isolates carried the *alkB1* and *nahAc* hydrocarbon degradative genes (Fig. 4) and were able to effectively degrade TPH and PAHs in batch microcosms as depicted in Figure 5.

The *alkB1* gene codes for the alkane monooxygenase enzyme that catalyzes initial hydroxylation during aerobic degradation of alkanes while the *nahAc* gene codes for the naphthalene-1,2-dioxygenase reductase component responsible for the oxidation of aromatic hydrocarbons compounds to cis-arene diols. The presence of these indicator genes in the test isolates is significant as they are indicative of hydrocarbon degradation capacity in the bacteria and their

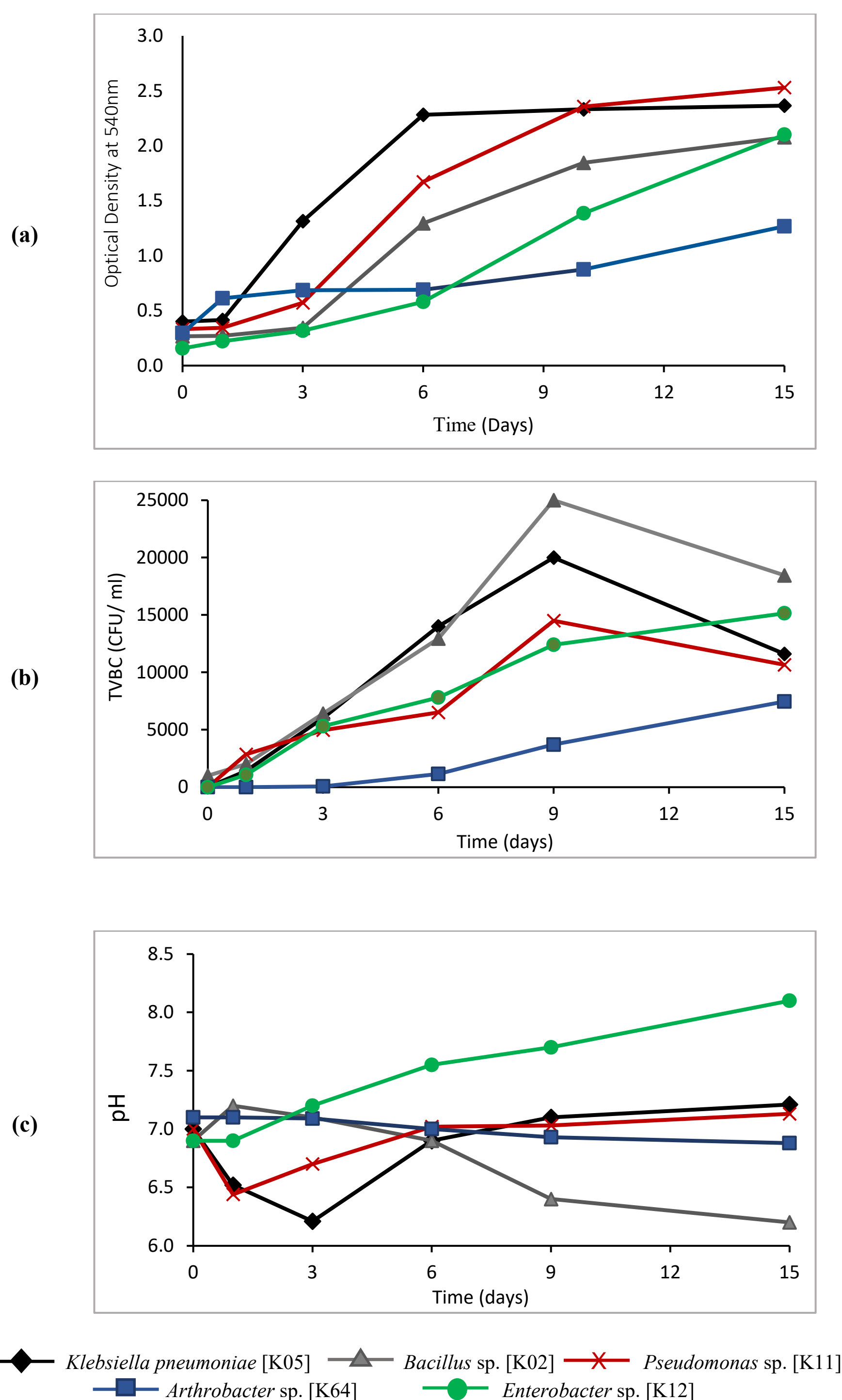

Fig. 2. Hydrocarbon Utilization Assay: Variations in mean optical density (a), total viable bacterial counts (TVBC) (b) and pH (c) of the mineral salts broth containing crude oil as sole carbon source

copy numbers may be used to predict degradation potential and rates in environmental media.

The individual test isolates and their consortium were able to degrade the total petroleum hydrocarbon and polycyclic aromatic hydrocarbon contents in the batch systems by 59.37% – 96.06% and 68.40% – 92.46% respectively. *K. pneuminiae*-K05 was the most efficient crude oil degrader matching the removal levels attained by the consortium of the three isolates (Fig. 5). *E.*

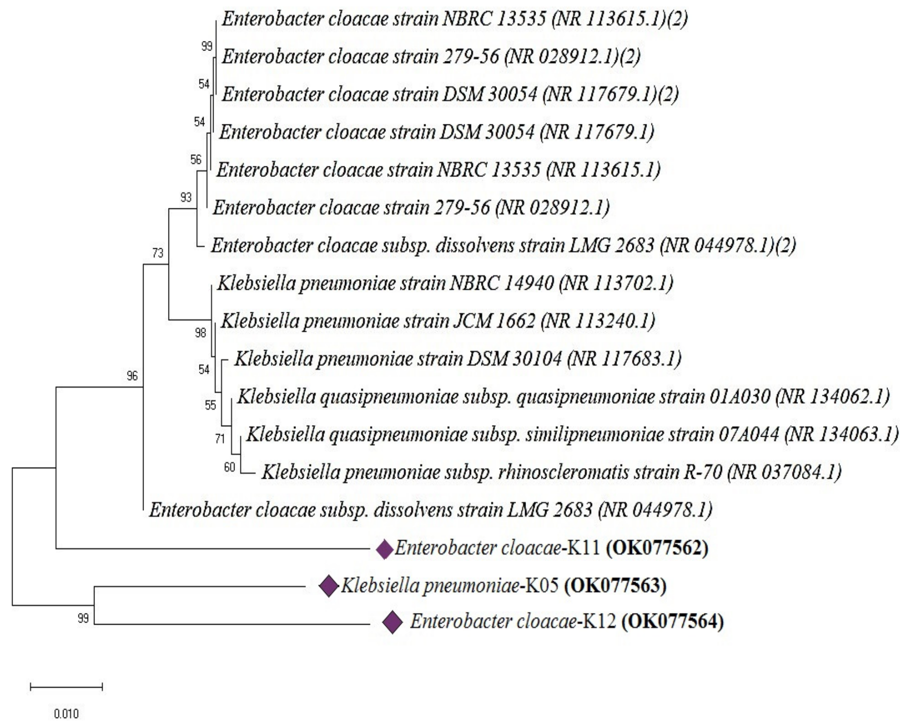

Fig. 3. Phylogenetic tree based on partial sequences of 16S rRNA indicating the evolutionary distances among the most efficient hydrocarbon degrading isolates

The purple diamond ◆ indicates isolates from this study with the accession numbers in parenthesis. The identities of the top three isolates were confirmed via Sanger sequencing as: *Enterobacter cloacae* (K11), *Klebsiella pneumoniae* (K05) and *Enterobacter cloacae* (K12)

cloacae-K11 was the least efficient degrader. Based on the F-statistic and p-values at $p < 0.05$ and total degrees of freedom, n=13, there were no significant differences between the degradation capacities of *K. pneumoniae*-K05 and the consortium for PAHs (p=0.1198) but a significant difference was seen with TPH degradation (p=0.000283). Similarly, PAHs degradation between *E. cloacae*-K11 and *E. cloacae*-K12 did not differ significantly (p = 0.1514) from each other but the two isolates differed significantly (p = 0.0005) in their ability to degrade TPH. The degradation levels for *K. pneumoniae*-K05 and the consortium differed significantly (p=0.0005; 0.00196) from those achieved by *E. cloacae*-K11 and *E. cloacae*-K12 for both TPH and PAHs.

The results for the consortium are somewhat unanticipated as it would be expected that the consortium of proven hydrocarbon degraders would totally eliminate the crude oil long before the end of the 21-day study. This reduced efficiency observed could be attributed to the high pollution level (10 % v/v) used in the study; several researchers have shown that microorganisms are more efficient degraders at lower substrate concentration (Jørgensen, 2008; Ławniczak *et al*., 2020). Chen *et al.* (2017) confirm a drop in degradation efficiency with increasing crude oil concentration. High concentrations of petroleum hydrocarbons may greatly inhibit growth of microorganisms, even microorganisms with the capacity for petroleum hydrocarbon degradation (Xu *et al*., 2018).

The analyses of the residual fractions of the total petroleum hydrocarbons and polycyclic aromatic hydrocarbons using GC-FID are presented in Figures 6 and 7 respectively. Predictably, the isolates and the consortium were able to utilize the C_8 – C_{14} aliphatic fractions more readily than other fractions. Shorter and straighter chained-hydrocarbons are well known to be more

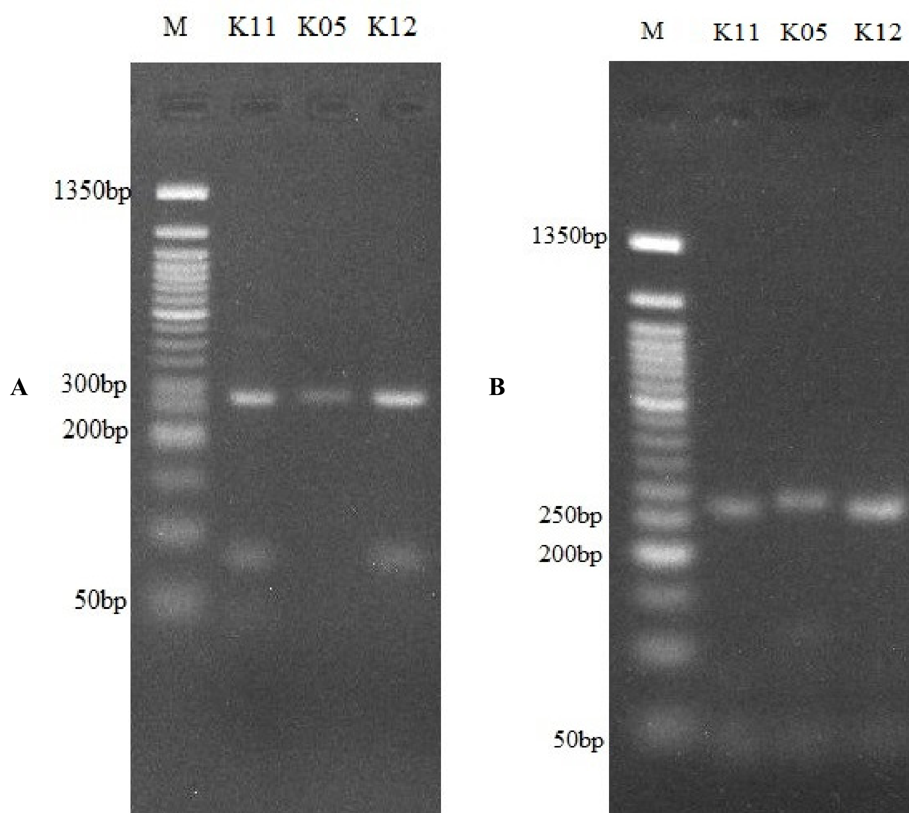

Fig. 4. Gel images of *nahAc* gene amplification at 300bp (A) and *alkB1* gene amplification at 250bp (B)
M is the DNA ladder

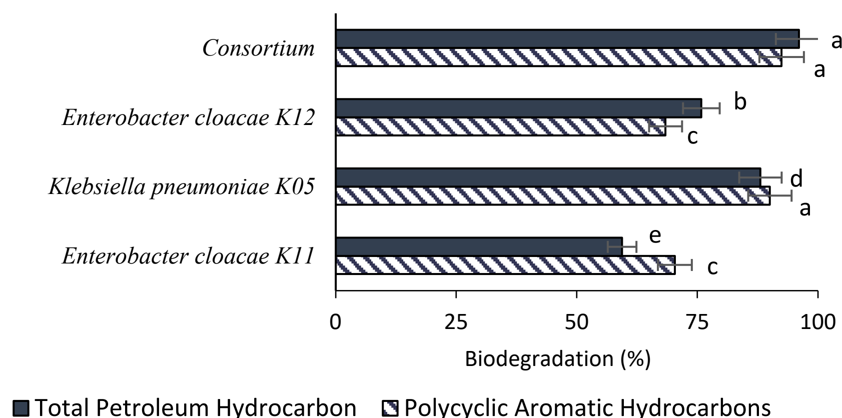

Fig. 5. Primary biodegradation of TPH and PAHs by the selected isolates and their consortium
Different letters indicate statistically significant differences between groups at p≤0.05.

biodegradable than their branched or longer chained counterparts (Jørgensen, 2008).

Amongst polycyclic aromatic hydrocarbons, pyrene, benzo(a)pyrene, phenanthrene, chrysene and naphthalene proved to be the most recalcitrant fractions (Fig. 7). *K. pneumoniae*-K05 proved even more efficient in the degradation of naphthalene, chrysene and benzo(a)pyrene than the consortium. Similar to the current study, reports by Ekanem & Ogunjobi (2017) and Chaudhary *et al.* (2020) found that C_8 – C_{16} carbon fractions in spent lubricating oil and diesel respectively

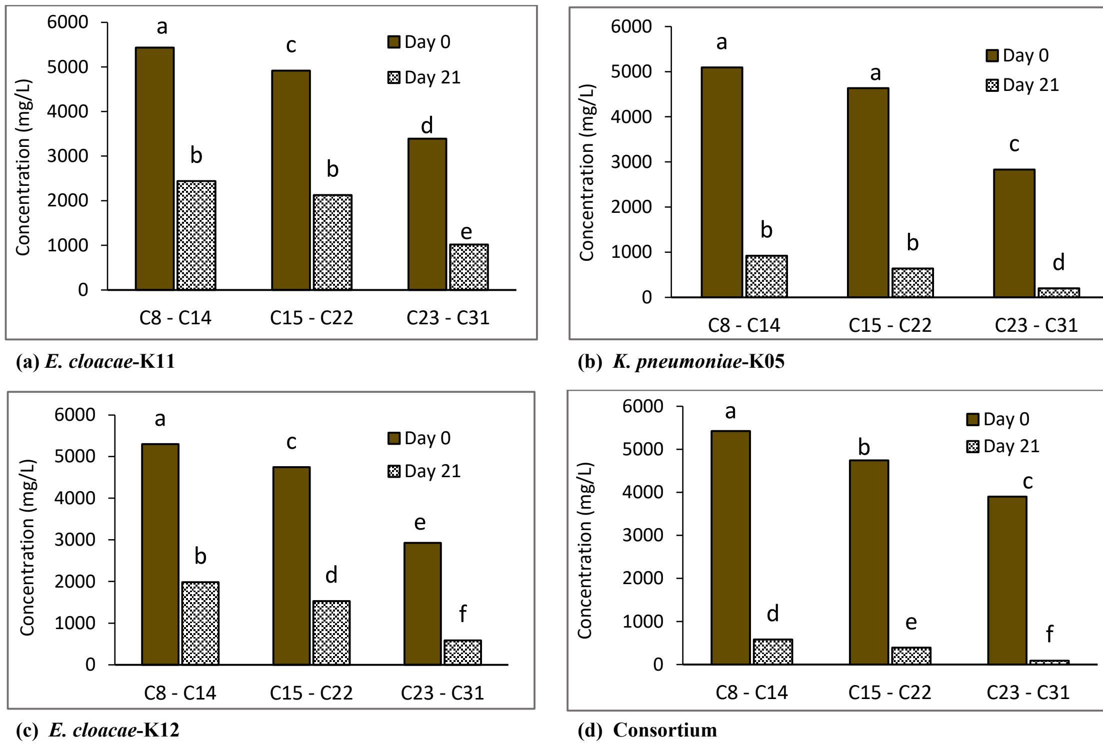

Fig. 6. Fractional analysis of residual total petroleum hydrocarbon content of petroleum contaminated medium augmented with the three selected isolates and the consortium during the 21-day study period. Different letters indicate statistically significant differences between groups at 95 % confidence interval.

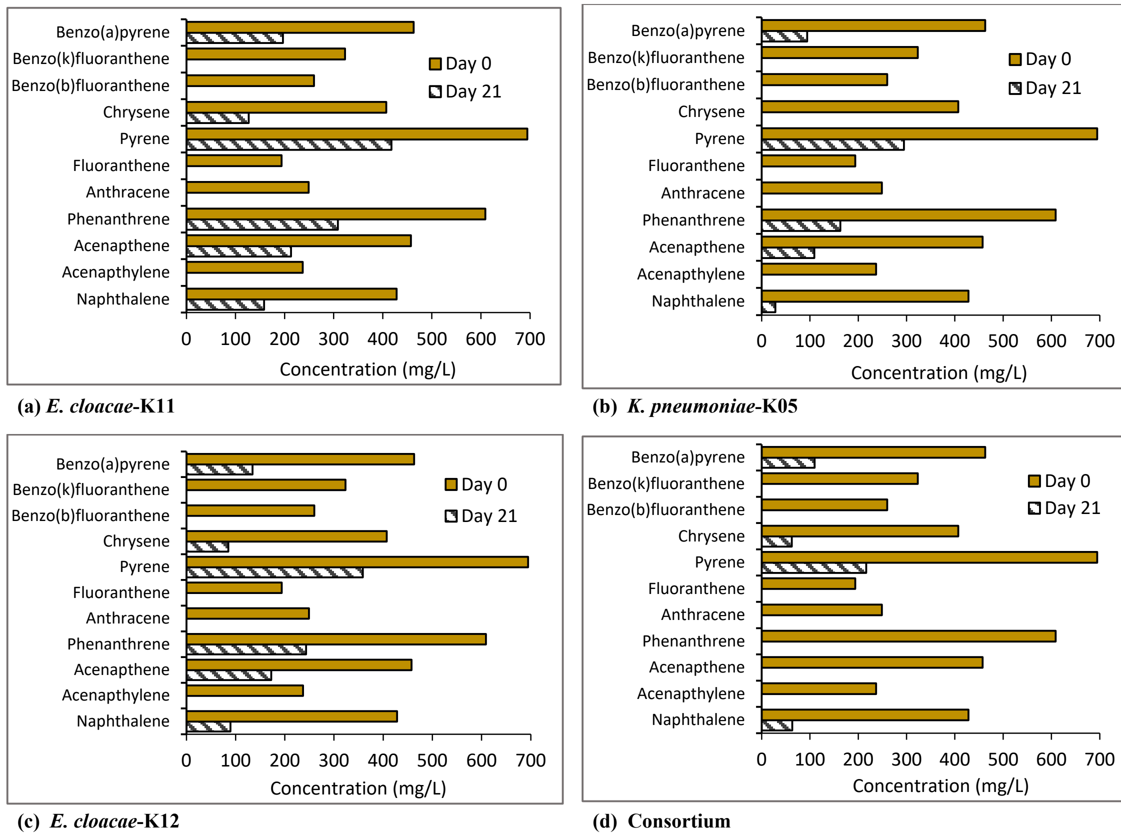

Fig. 7. Fractional analysis of residual polycyclic aromatic hydrocarbon content of petroleum contaminated medium augmented with the three selected isolates and the consortium during the 21-day study period

were the most readily degraded by bacterial isolates; and like the current study, the persistent PAHs fraction was chrysene. Another study identified phenanthrene, fluorene and anthracene as the recalcitrant PAHs fractions during the biodegradation of crude oil in a marine environment (Chen *et al.*, 2017). The condensed ring structure of these compounds plays an important role here as pyrene and benzo(a)pyrene consist of four and five fused benzene rings respectively and are categorized as high molecular weight PAHs (Al Farraj *et al.*, 2019). The persistence of naphthalene (which consists of two benzene rings) in the current study is, however, surprising as it was expected that it would be depleted before large 3 – 4-ringed compounds like chrysene, fluoranthene and anthracene.

A consortium of bacteria was confirmed more competent than axenic strains in the degradation of crude oil. Microbial consortia will always fare better than individual isolates in the degradation of organic compounds as no single organism has the full cocktail of enzymes or metabolic capability for breakdown of all organic compound (Chen *et al.*, 2017). Microorganisms tend to be genetically specific for a particular group of compounds and exposure to such compounds trigger the expression of sometimes even previously dormant genes that, in turn, facilitate the secretion of pertinent degradative enzymes (Frische & Hofrichter, 2005). A consortium provides the advantage of having a diversity of organisms capable of degrading different types of compounds. Effective environmental bioremediation therefore requires the combined effort of multiple functional species. Co-metabolism and/or simultaneous metabolism may be the prominent phenomena driving the degradation prowess of these consortia. Bacteria within a consortium are also known to display better stability and resilience than individual isolates (Xu & Yu, 2021).

The dominance of Gram negative rods in the petroleum contaminated water in the current study buttresses the findings of similar studies on biodegradation of hydrocarbon compounds (Joutey *et al.*, 2013; Xu *et al.*, 2018). This resilience by the Gram negative bacteria may be due to their cell wall structure; the lipopolysaccharide outer layer which surrounds the inner peptidoglycan membrane provides additional protection against environmental stressors leaving the bacterium with more time during the lag period to adapt and synthesize the required enzymes for biodegradation. Several comparable studies equally report the biodegradation of petroleum hydrocarbons by the bacterial species isolated in the current study (Adebusoye *et al.*, 2007; Kafilzadeh *et al.*, 2011; Joutey *et al.*, 2013; Xu *et al.*, 2018). *Pseudomonas, Klebsiella* and *Enterobacter* are particularly well-known for their capacity to degrade both aliphatic and aromatic HC compounds (You *et al.*, 2018; Rajkumari *et al.*, 2021) while *Pseudomonas*, *Bacillus*, *Micrococcus*, *Corynebacterium* and *Flavobacterium* have been highlighted as being somewhat specific to the utilization of PAHs (Macauley, 2014). The isolates noted as the most efficient hydrocarbon degraders in the degradation of spent lubricating oil were *Pseudomonas*, *Nocardia* and *Bacillus* spp. (Ekanem & Ogunjobi, 2017) while Nwinyi *et al.* (2013) highlighted *Enterobacter* sp. as an effective degrader of PAHs including chrysene, pyrene, fluoranthene and naphthalene in polluted media. These findings seem to be at odds with the observed persistence of chrysene, pyrene and naphthalene in the presence of *Klebsiella pneumoniae* and *Enterobacter* sp. obtained in the current study.

CONCLUSION

In this study, it was determined that the fraction of autochthonous bacteria from the river water that could utilize petroleum was only about 0.245%. The predominant hydrocarbon utilizing bacteria in the system were *Bacillus*, *Pseudomonas* and *Enterobacter* spp. but *Klebsiella pneumoniae* and two species of *Enterobacter cloacae* were identified as the most efficient hydrocarbon utilizers. These isolates were confirmed to possess the *alkB1* and *nahAc* genes for degradation of aliphatic and aromatic hydrocarbon compounds. The selected degraders and

their consortium proved proficient in the degradation of the total petroleum hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) in batch systems with contaminant elimination in the magnitude of 59.37 % – 96.06 % and 68.40 % – 92.46 % obtained respectively. The three top hydrocarbon degrading isolates and their consortium were able to utilize the C_8 – C_{14} aliphatic fractions more readily than other fractions. Amongst the PAHs, pyrene, benzo(a)pyrene, phenanathrene and naphthalene proved to be the most recalcitrant fractions. *K. pneumoniae* showed the greatest petroleum degradation capacity of the selected isolates but hydrocarbon degradation was most efficient with the bacterial consortium of *K. pneumoniae* and two species of *E. cloacae*. The consortium of the three selected isolates will, thus, be researched further to establish its stability and the practicability of its application in remediation of aquatic ecosystems distressed by petroleum hydrocarbons. It was concluded that the river had satisfactory potential for natural attenuation.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/ or falsification, double publication and/ or submission, and redundancy has been completely observed by the authors.

LIFE SCIENCE REPORTING

No life science threat was practiced in this research.

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