Journal of Advances in Microbiology

19(2): 1-12, 2019; Article no.JAMB.53321
ISSN: 2456-7116

Fungal Population Dynamics Associated with Active-phase of Hydrocarbon Degradation in Oil-polluted Soil

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Authors' contributions

This work was carried out in collaboration among all authors. Author AUO designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors CBC and GCO managed the analyses of the study. Author AUO managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMB/2019/v19i230190
Editor(s):
(1) Dr. Veysi Okumus, Associate Professor, Department of Biology, Faculty of Arts and Science, Siirt University, 56100, Siirt, Turkey.
Reviewers:
(1) Chadetrik Rout, Maharishi Markandeshwar University, Mullana, India.
(2) Sainudeen Pattazhy, Kerala University, India.
Complete Peer review History: http://www.sdiarticle4.com/review-history/53321

Original Research Article

Received 15 October 2019
Accepted 18 December 2019
Published 25 December 2019

ABSTRACT

Fungal population dynamics was monitored in an oil-polluted soil undergoing remediation by enhanced natural attenuation (RENA) at Ibaa, Emohua L. G. A. Rivers State. Total petroleum hydrocarbons (TPH) and polycyclic aromatic hydrocarbons (PAHs) were determined from baseline [pre-RENA (POL B)] and sampling days 0 (POL 0), 9 (POL 9), 18 (POL 18), 36 (POL 36) and day 56 (POL 56)] using gas chromatographic-mass spectrometry. The baseline TPH and PAHs were 9,146.65 ppm and 3,454.10 ppm in the polluted soil (pre-RENA) and 479.67 ppm and 279.72 ppm for unpolluted soil (pristine control) respectively. By day 56, percentages of contaminants degradation were 97% and 89% for TPH and PAHs respectively. Higher counts for both heterotrophic fungal (THF) counts and culturable hydrocarbon utilizing fungal (HUF) counts were obtained on day 36 with values of 5.6 x 10^5 cfu/g and 4.77 x 10^6 cfu/g respectively. Out of the 47 HUF isolated and characterized, 34 hydrocarbon utilizing fungi were isolated in the active phase between day 9 (POL 9) to day 36 (POL 36). These recovered hydrocarbon degrading fungal

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isolates associated with the active phase of hydrocarbon degradation *(Mucor* sp., *Malbranchia* sp., *Prototheca* sp., *Cladosporium* spp., *Trichosporon* sp., *Acremonium* spp., *Rhizomucor* spp.). RENA treatment effectively reduced the pollutant levels in the impacted soil.

Keywords: Fungal population dynamics; active phase; oil-polluted; enhanced natural attenuation (RENA).

1. INTRODUCTION

Fungi are known to participate in many degradation processes including, the breakdown of petroleum hydrocarbon by producing numerous extracellular enzymes. Increase in industrialization, exploration, extraction, refining, transport of crude oil, use of petroleum and derivative products in the Niger-Delta region has exposed the soil to continuous release of crude oil. This has negatively affected the well endowed ecosystem and food chain. These activities subsequently lead to widespread of pollutants in its rivers, swamps, farmlands with petroleum hydrocarbon [1,2]. Oil spillage is of life-threatening environmental concern in the Niger-Delta region and has result in huge damage to ecology [3]. Its brings about harm to public health, contaminate drinking water, impede natural resources such as the mangrove swamp, and disturb land farming and other agricultural system [4].

Petroleum pollution has antagonistically influenced the biological system, food chain and is of significant ecological concern in the Niger Delta area [5,6]. These results to loss of biodiversity mostly observed as destruction to ecosystem, flora and fauna performance and therefore have been a crucial issue for environmental scientists throughout the last decades [7-12]. Both the environment and humans are exposed to danger by this hydrocarbon pollution [13]. There is increasing need to remedy the adverse effects of anthropogenic activities in this area. This has prompted the advancement of viable bioremediation methodologies [13-15].

In this study, we investigated the effectiveness of remediation by enhanced natural attenuation, (RENA) technique (also called land farming) in the recovery of oil-polluted site. This method involved the treatment of an oil-polluted site in Ibaa, Emohua Local Government Area, Rivers State the eastern Niger Delta of Nigeria by spreading top soil on the contaminated soil, addition of inorganic fertilizer to bio stimulate microbial growth and aerating the soil through tilling to enhance mixing with unpolluted soil and increasing available surface area for microbial activity. The essence of the tilling and homogenization was to evenly distribute the petroleum contaminants and fragment the soil lumps to fine particles thereby increasing the surface area [16-19].

The indigenous fungal flora of the oil-polluted soil in Ibaa, Emohua Local Government Area, Niger Delta,Nigeria was isolated and characterized. We characterized remediation site using physicochemical indices, petroleum hydrocarbon quantification by gas chromatography, identified, determined and evaluated fungal population dynamics during remediation. We evaluated the effect of nutrient addition to the remediation process. This study aims at providing a good platform for effective bioremediation strategies using the hydrocarbon utilizing fungi in oil-polluted Soil, effectiveness of remediation by enhanced natural attenuation (RENA).

2. MATERIALS AND METHODS

2.1 Sampling Site/ Collection

The study was performed on a crude oil-spill polluted site located in Ibaa, Emohua Local Government Area, Rivers State the eastern Niger Delta of Nigeria. Composite soil samples were collected on each sample with soil auger into polyethylene bags sanitized with 70% ethanol from the crude oil polluted site undergoing bioremediation at the depth (0-0.5M) and unpolluted soil (US) served as control. Sampling was done prior commencement of bioremediation pre-RENA [baseline sample designated as POL (B)] and on commencement of bioremediation on days 0, 9, 18, 36 and 56 designated as [POL (0), POL (9), POL (18), POL (36) and POL (56)]. All samples were transported to the laboratory within 6h at 4ºC for analysis.

2.2 Baseline Characterization

Physicochemical parameters such as moisture content, nitrate, phosphate, total organic carbon

(TOC), turbidity, temperature, pH and conductivity will be determined using methods from [20] and heavy metals Cadmium (Cd), Lead (Pb) and Nickel (Ni). Gas chromatography tracing of residual crude oil was done to determine extent of hydrocarbon attenuation during and at termination of site remediation. Total culturable hydrocarbon utilizing and heterotrophic bacteria and fungi counts was determined using methods of [21,22].

2.3 Isolation and Identification of Hydrocarbon Utilizing Fungi (HUF)

2.3.1 Crude oil preliminary enrichment

One gram (1 g) of each soil sample was inoculated into 250 mL Erlenmeyer flask containing 100 ml of sterilized Bushnell-Haas broth supplemented with 1% (v/v) sterilized crude oil as the sole carbon source, The setup was incubated in rotary shaker at 30ºC at 130 rpm for 7 days. Using methods of [23,24].

2.3.2 Isolation of hydrocarbon utilizing fungi (HUF)

Hydrocarbon utilizing fungi (HUF) counts, 10-fold serial dilution of crude oil enrichment was done using sterile distilled water. Appropriate aliquots were plated out in triplicates on spread plate method using Petri dishes containing sterilized Bushnell-Haas agar (Sigma-Aldrich, USA) modified with crude oil and chloramphenicol (to inhibit bacterial growth). Plates were incubated at 30ºC for 7-14 days.

2.3.3 Purification and characterization of hydrocarbon utilizing fungi

Discreet colonies of different HUF was randomly picked using a sterile inoculating wire loop and sub cultured for purification by streaking on nutrient agar plates and incubated at 30ºC for 48 hrs. The identification of petroleum hydrocarbon utilizing fungi was done microscopically using the tease mount method made by using an inoculating needle, pick a small portion of each fungus growth containing a drop of Lacto-phenol cotton blue (LPCB) placed on separate slides and a cover slip to examine individual isolate using under low and high power objectives lens (microscope) [25].

2.4 Total Heterotrophic Fungal (THF) Count

The total heterotrophic fungal count was recovered using spread plate method in Dichloran Rose bengal (DRBC) Agar (Sigma-Aldrich, USA) and Chloramphenicol was added (to inhibit bacterial growth). For heterotrophic fungal counts, 10-fold serial dilution from the crude oil enrichment broth was done using sterile distilled water and appropriate aliquots were plated out in triplicates on Petri dish plates containing sterilized Dichloran Rose bengal (DBRC) Agar (Sigma-Aldrich, USA) with Chloramphenicol (to inhibit bacterial growth) using spread plate method. Plates were incubated at 30ºC for 7-14 days. Discreet colonies of different THF was randomly picked using a sterile inoculating wire loop and sub cultured for purification by streaking on nutrient agar plates and incubated at 30ºC for 48 h.

3. RESULTS AND DISCUSSION

This study involved remediation by enhanced natural attenuation (RENA). RENA is a land farming treatment technology [26,27] involving a form of natural bioremediation [25,27] suggested RENA as a possible choice in remediating crude oil polluted soil as it supports an aerobic microbial process which is essential for remediation of petroleum hydrocarbons to occur.

3.1 Gas Chromatographic Analysis

The study revealed that TPH and PAHs values (baseline POL (B) analysis) prior to commencement of bioremediation for the polluted soil was 9,146.65 ppm and 3,451.88 ppm and unpolluted control [US] was 479.67 ppm and 279.72 ppm respectively. The concentrations for the polluted soil indicate that the site is chronically polluted. The residual TPH and PAHs was analyzed each sampling days 9, 18, 36 and 56 designated as [POL (0), POL (9), POL (18), POL (36) and POL (56)]. A baseline characteristic of soil sample is shown in Table 1. POL (0) (polluted soil as RENA started on day zero) was 8,538.80 ppm and 2,248.75ppm for TPH and PAHs respectively.

Fig. 1 illustrates the residual concentrations of total petroleum hydrocarbons (TPH) and polycyclic aromatic hydrocarbons (PAHs) during the 56-day bioremediation. The depletion in TPH and PAHs was observed at varying rates on all sampling days during the remediation period and by day 56 when remediation ended. Percentages of contaminants degradation were 97% and 89% for TPH and PAHs respectively as illustrated in Fig. 2.

Table 1. Baseline characteristics of soil samples

Parameters	Polluted soil pre-RENA baseline (POLB)	Polluted soil as RENA started on day zero (POL 0)	Unpolluted Control (US)
Total petroleum hydrocarbon (TPH)	9,200 ppm	8, 600 ppm	480 ppm
Polycyclic aromatic hydrocarbon (PAH)	3,500 ppm	2,300 ppm	280 ppm
pH	5.03	4.82	6.12
Nitrate (NO^{3-})	8.33mg/l	6.67 mg/l	3.84 mg/l
Phosphate (PO^{4+})	8.11mg/l	10.834 mg/l	20.52mg/l
Temperature	27.6 ^{o}C	29.3 ^{o}C	30 ^{o}C
lectrical Conductivity	141.1 µs/cm	140.73 µs/cm	185.46 µs/cm
Total organic carbon (TOC)	5.64 %	5.068 %	1.97 %
Culturable heterotrophic fungal count (CHF)	2.77 x 10^{6}	4.77x 10^{6}	4.14 x 10^{5}
Hydrocarbon utilizing fungal count (HUF)	3.4 x 10^{5}	4.6 x 10^{5}	4.0 x 10^{3}
Moisture Content	7.93 %	6.76 %	4.88%
Nickel (Ni)	0.38 mg/l	0.36 mg/l	0.68 mg/l
Lead (Pb)	0.54mg/l	0.41mg/l	0.0065 mg/l
Cadmium (Cd)	1.2mg/l	1.26 mg/l	0.013 mg/l

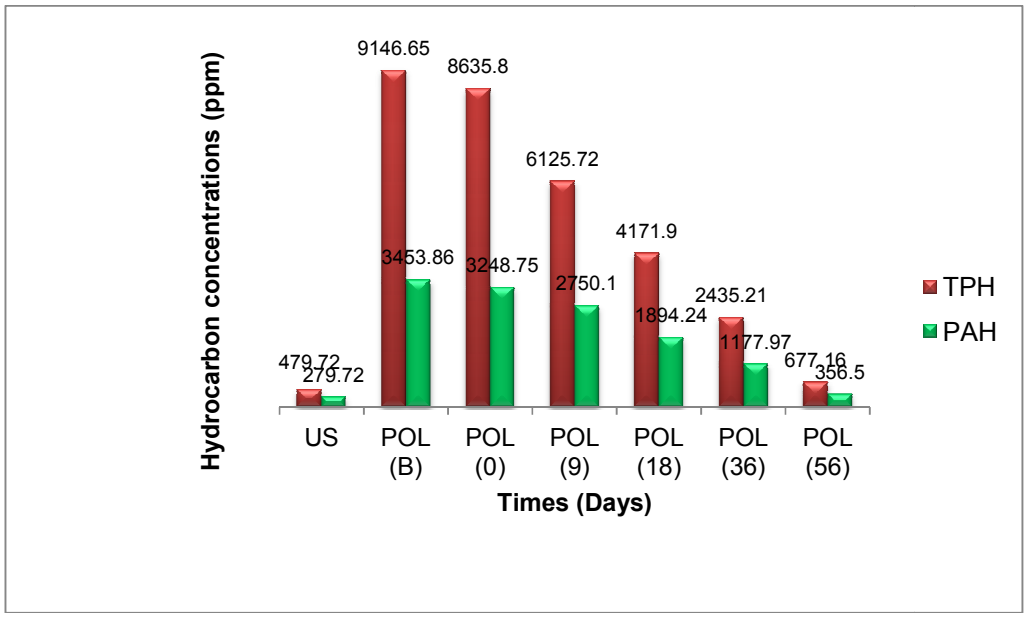

Fig. 1. Residual concentrations of TPH and PAHs during the 56-day bioremediation n

The result of Gas Chromatography-Mass Spectrophotometer on total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAHs) degradation showed that these fungal isolates exhibited biodegradation capabilities. Several findings suggest that fungal isolates are potential degraders for bioremediation of crude oil-contaminated soil. Dai et al. [28] reported that in oil spill cleanup, polluted soil act as reservoirs for these groups of beneficial microorganisms [29].

The TPH and PAHs analyzed by GC-MS during the 56-day remediation gave TPH consisting of hydrocarbon fractions (carbon number chain lengths range C_8-C_{34}) as illustrated in Fig. 3a.

Heavier hydrocarbons fractions of carbon chain lengths C_{21}, C_{23}, and C_{28}-C_{34} were completely depleted by day 56 as illustrated in Fig. 3a-b

PAHs consisting of Benzo(b)fluoranthene, Acenaphthylene, Acenaphthene, Benzo(k)

fluoranthene, Fluorene, Fluoranthene, Benzo (a) anthracene, Indeno (1,2,3-cd) pyrene, Dibenz (a, h) anthracene, Pyrene, Phenanthrene, Chrysene, Naphthalene, Benzo (a) pyrene, Benzo (g, h, perylene,Anthracene. For the PAHs, Acenaphthylene, Benzo(k) fluoranthene, Fluorene, Fluoranthene, Benzo (a) anthracene, Indeno (1,2,3-cd) pyrene, Pyrene, Phenanthrene, Naphthalene, Benzo (g, h, i) perylene, Benzo (g, h, i) perylene were completely degraded below detectable limit (BDL) as shown in Fig. 4. [30,31] determined the following: benzo[a]anthracene, benzo[a]pyrene, benzo[b] fluoranthene, indeno[1,2,3-c,d]pyrene, anthracene, benzo[g,h,i]perylene, benzo[e]pyrene, chrysene, fluoranthene, fluorene, phenanthrene, and pyrene to be classifiable as human carcinogens.

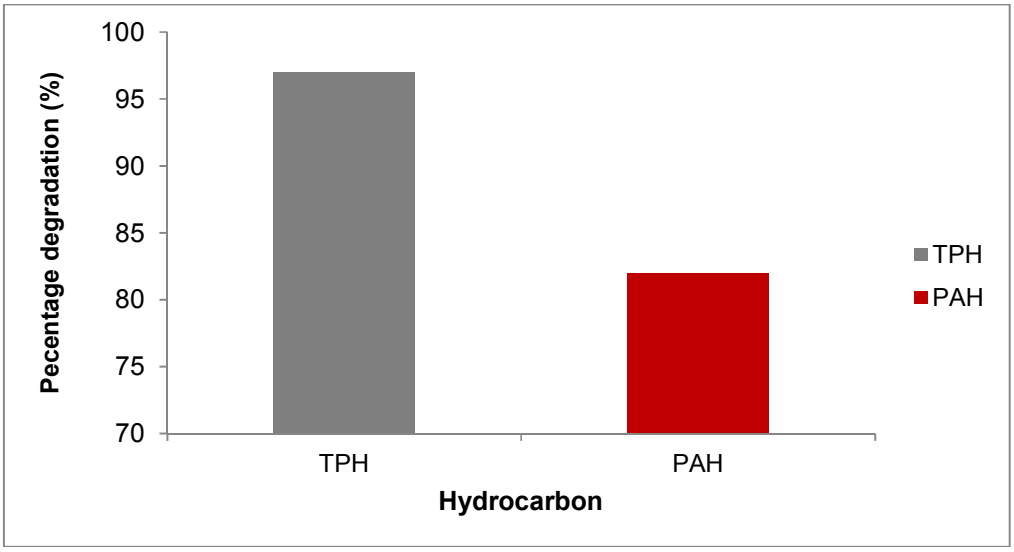

Fig. 2. Percentage depletion of (TPH) and (PAH) during the 56-day bioremediation

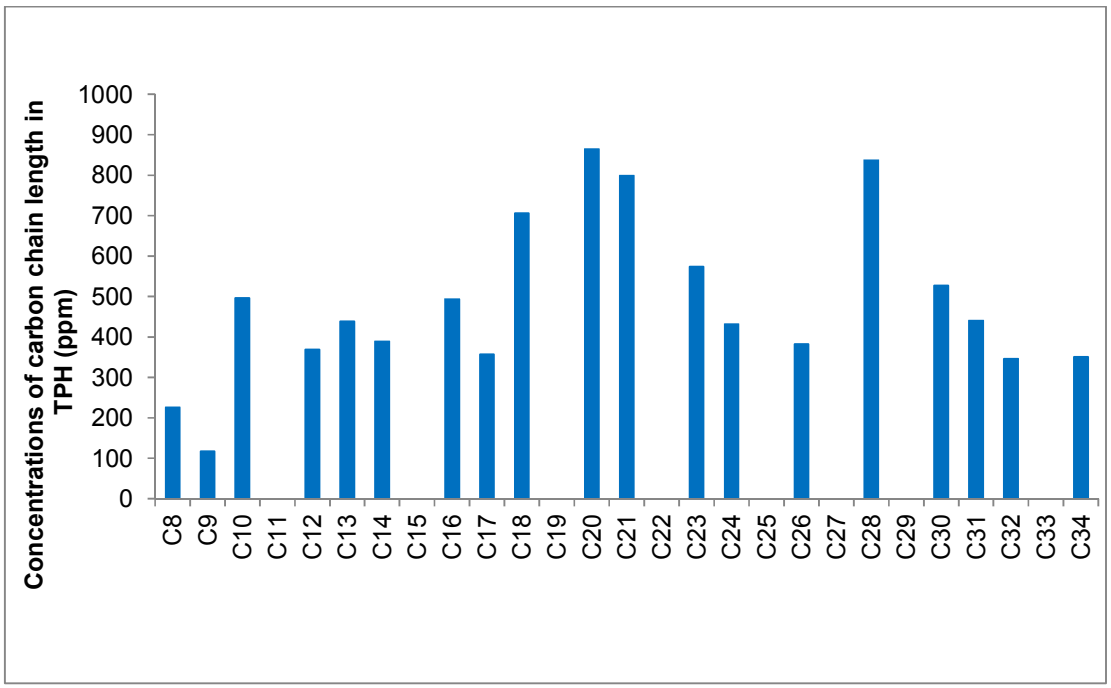

Fig. 3a. Baseline residual TPH concentration prior to start of bioremediation

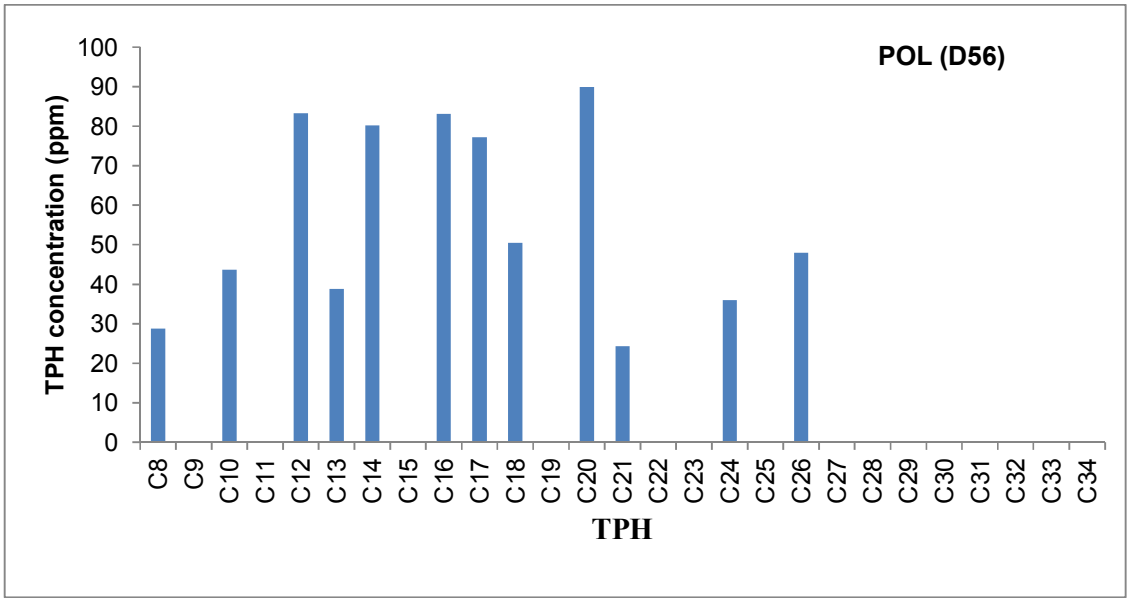

Fig. 3b. Residual TPH concentration on day 56

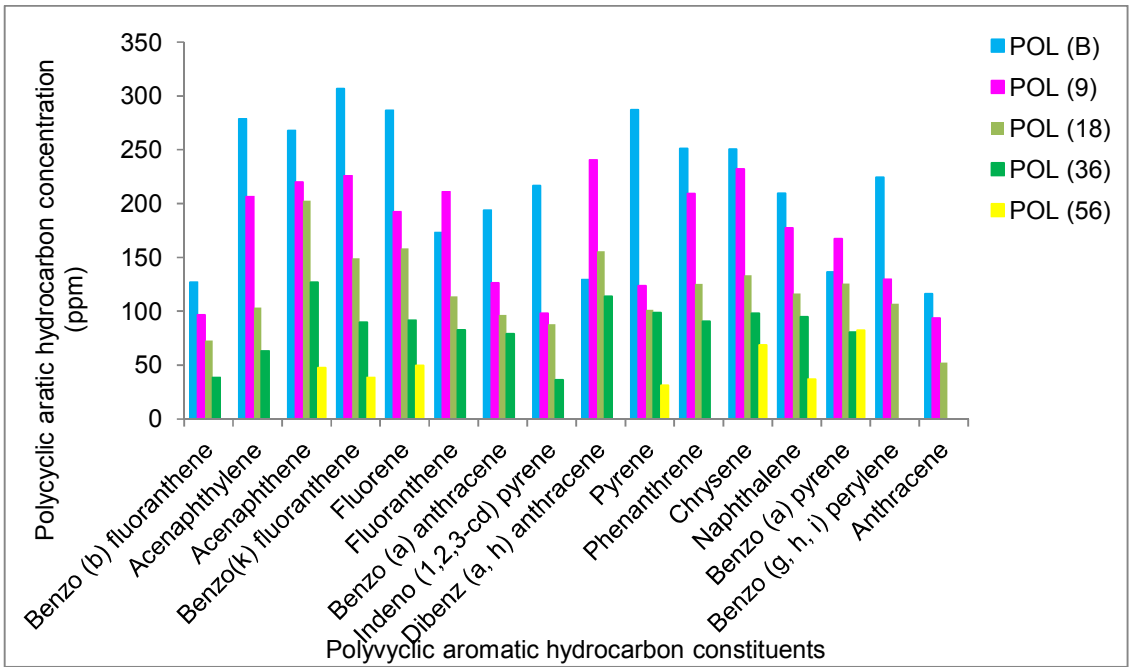

Fig. 4. Residual PAHs concentrations during the 56-day bioremediation

3.2 Enumeration of Heterotrophic and Hydrocarbon Utilizing Fungi in Soil Samples

The culturable hydrocarbon utilizing (CHUF) and heterotrophic (CHF) fungal counts were values 4.2 x 10^6 cfu/g and 5.3 x 10^6 cfu/g respectively on day 0 (POL 0). By day 9 (POL 9), the fungal counts increased in both CHUF and TCHF with the highest number of counts for total culturable heterotrophic fungal (THF) and total culturable heterotrophic fungal (THF) count of 5.6 x 10^5 cfu/g and 4.77 x 10^6 cfu/g on day-36 (POL 36) respectively as shown in Fig. 5a and b.

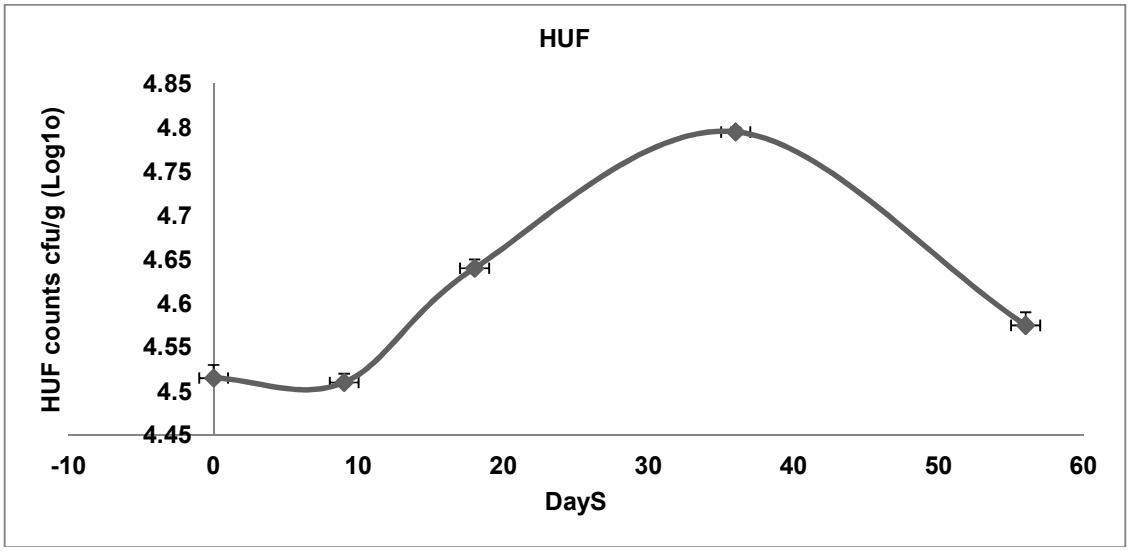

Fig. 5a. Culturable Hydrocarbon utilization fungal (THUF) counts during the 56-day bioremediation

Error bars are standard deviation of replicate counts

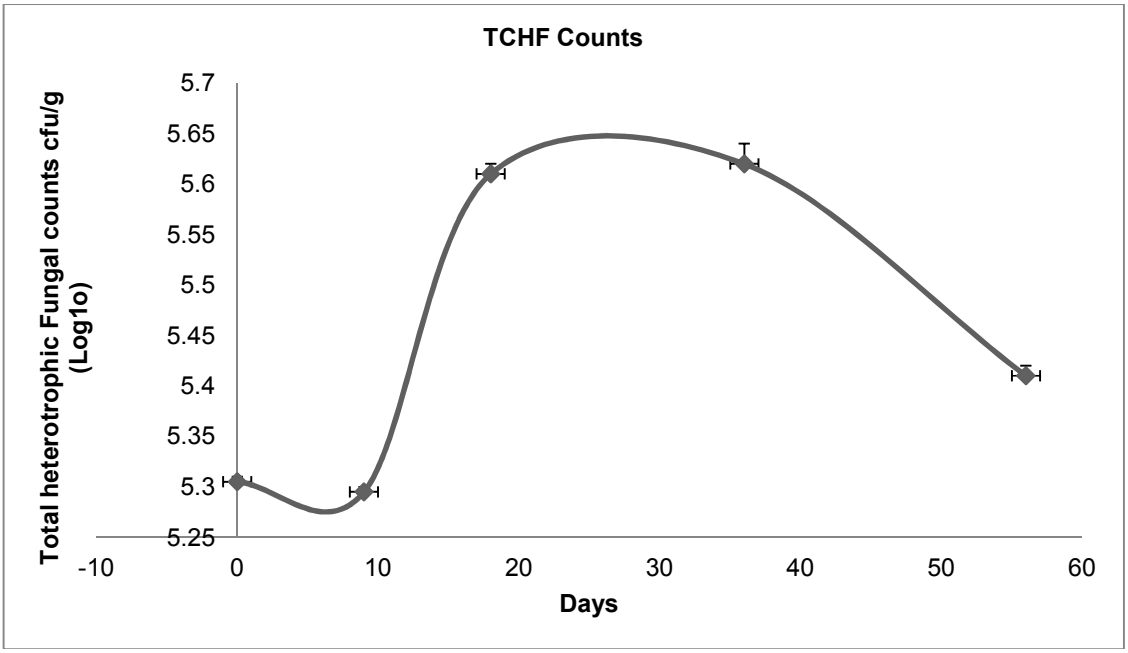

Fig. 5b. Total culturable heterotrophic fungal counts

Error bars are standard deviation of replicate counts

The growth profiles of the fungal cultures revealed a continuous growth and drop on day-36.The highest number of hydrocarbon utilizing fungi (34 hydrocarbon utilizing fungi) was isolated in the active phase between day 9 (POL 9) to day 36 (POL 36). It was remarkably noticed that counts of hydrocarbon degraders in the unpolluted was low. A total number of two unidentified and 47 identified heterotrophic fungal counts were obtained from the samples during the 56-day bioremediation.

3.2.1 Fungal characterisation

Based on morphological and biochemical characteristic, forty seven (47) hydrocarbon

utilizing fungal isolates were revealed as *Mucor* sp., *Malbranchia* sp., sp., *Cladosporium* spp.(2), *Trichosporon* sp., *Acremonium* spp (3)., *Rhizomucor* spp (3). They were identified to belong to either of these Yeast: *Candida, Cryptococcos, Sporothrix schenckii, Rhodotorula* and. Algae (*Prototheca* sp) is an achlorophyllous alga which degrades oil. Although *Prototheca* is generally considered to be a colorless member of the *Chlorococcales*, there have been a number of attempts to relate it to the fungi. Colonies of *Prototheca* appeared yeast-like on media. However, our observations support the conclusion that it is indeed a non photosynthetic alga and not a fungus. One such structural feature that is of special interest is the presence of membrane-bounded starch granules which are considered to be storage plastids. Fungi are not known to produce plastids and *Cladophialophora boppii* a black yeast-like fungus. The highest numbers of isolates (16 fungal isolates) were recovered on POL (36).

However, majority of the fungi isolated in this study was filamentous fungi which are *Cladosporium carrionii, Phialophora verrucosa, Madurella, Exophiala jeanselmei, Mucor spp., Aspergillus fumigatus, Fusarium* sp *Fusarium* sp., *Penicillium* spp., some attributes that enable them as good potential agents of degradation. They possess some attributes that enable them as potential agents for the degradation of hydrocarbon. Once they come in contact with hydrocarbon, they quickly digesting it through the secretion of extracellular enzymes [32]. Previous study reported by George-Okafor et al. [33] reported that filamentous fungi have good potential for bioremediation processes and are capable of removing and degrading recalcitrant hydrocarbons.

Some of these organisms have earlier been reported as hydrocarbon bio-degraders by [34,35] are *Aspergillus*, *Cephalosporium,* and *Pencillium* which are found to be the potential degrader of hydrocarbons. The yeast species, namely, *Candida , Rhodotorula , Prototheca* sp., *Geotrichum* sp*, and Trichosporon* isolated from contaminated water were noted to degrade petroleum compounds [36,37]. In this study we also reported that *Aspergillus*, *Candida*, *Cephalosporium*, *Cladosporium*, *Fusarium, Geotrichum*, *Mucor, Penicillium*, *Rhodotorula* and *Trichoderma* genera to be be capable of growth in oil-contaminated soil. Fig. 6 represents the percentage occurrence of individual fungal isolate during the 56-day bioremediation.

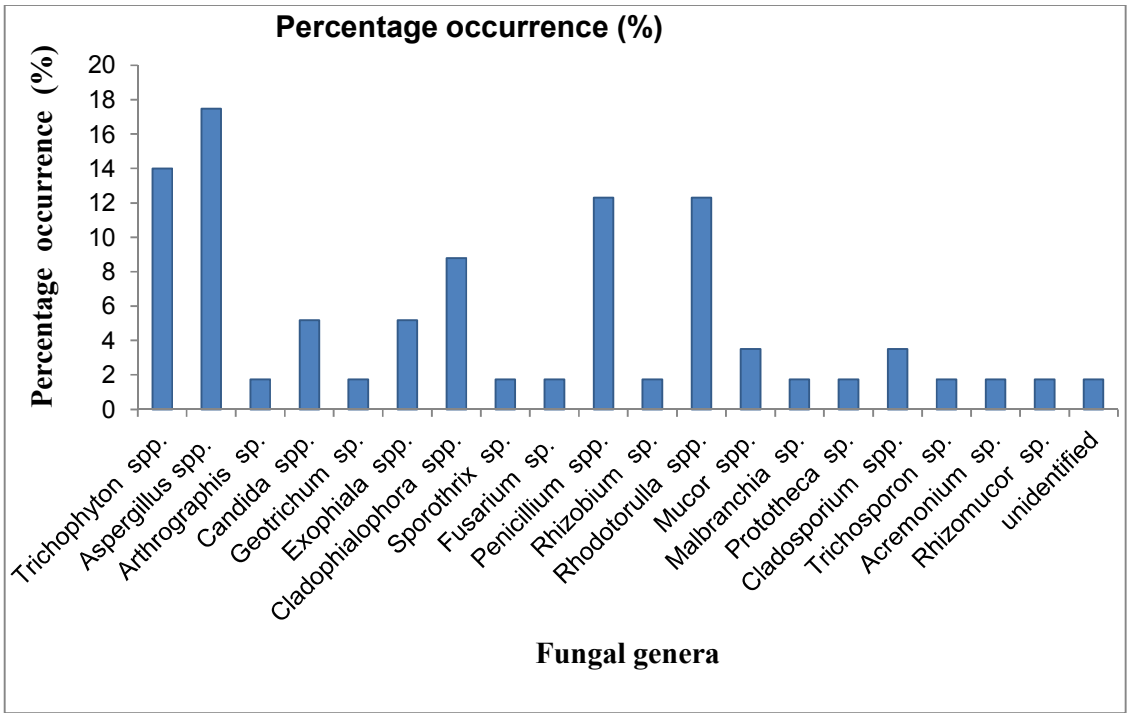

Fig. 6. Percentage occurrence of individual fungal isolate during the 56-day bioremediation

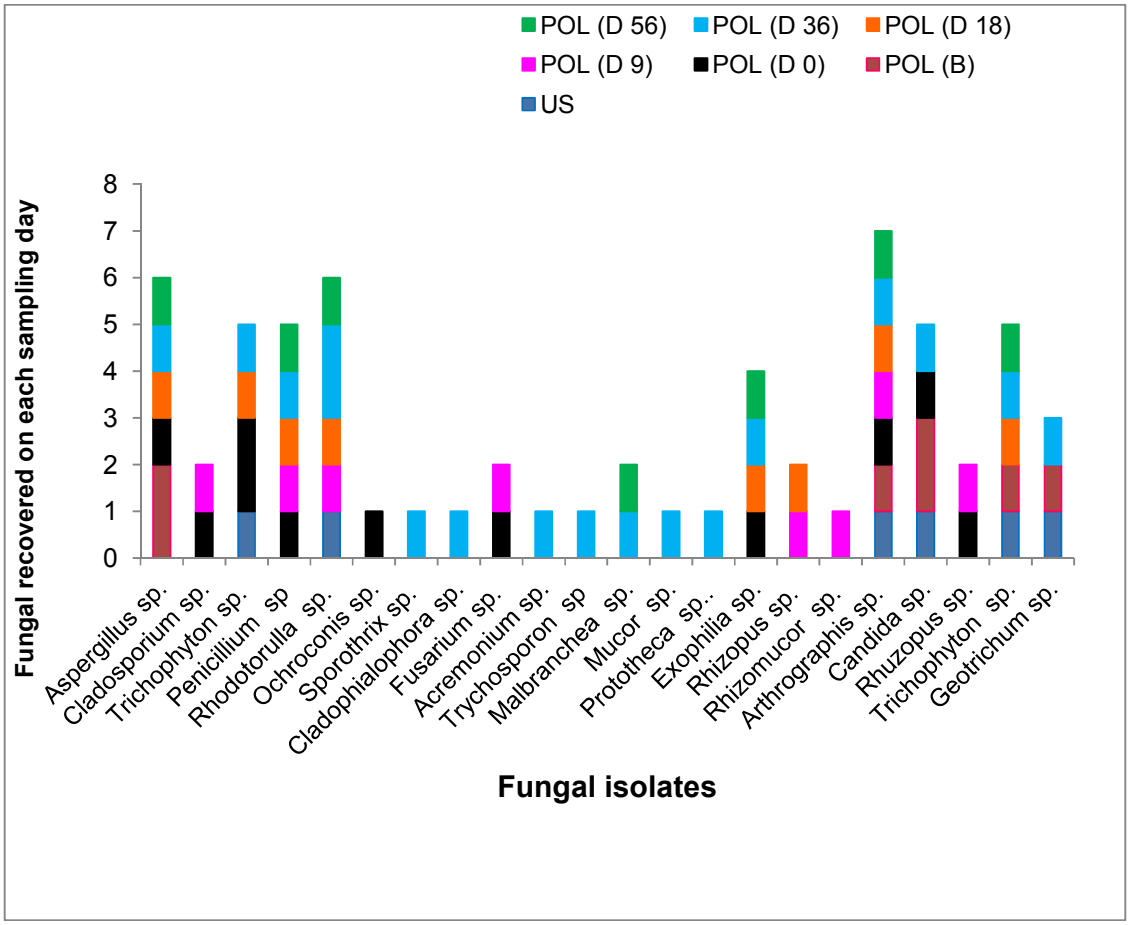

Fig. 7. Diversity of fungi recovered on each sampling day (POL (B), POL (0), PO; (9), POL (18), POL (36) POL (56) and unpolluted (US)

The highest occurring fungal genera as observed in this study are *Aspergillus* (17.5%), *Trichophyton* (14%), *Penicillium* (12.3%) and *Rhodotorula* (12.3%). *Aspergillus* and *Penicillum* have also been observed by Dawoodi et al. [35] to be dominant genera in the characterization of hydrocarbon utilizing fungi from hydrocarbon polluted sediments and water from four different hydrocarbon polluted sites in Ogala-Bonny, Rivers State. Fig. 7 shows the diversity of fungi recovered on each sampling day (POL (B), POL (0), PO; (9), POL (18), POL (36) POL (56) and unpolluted (US).

Gargouri et al. [37] reported that yeasts (*Candida tropicalis* and *Trichosporon asahii*) could efficiently degrade total petroleum hydrocarbon to about 97% and 95%, respectively**.** Okerekeet al. [38] reported that fungal isolates are involve hydrocarbon reduction. These fungal isolates have also been implicated in bioremediation by [39-43] isolated a strain identified as *Geotrichum* sp. capable of degrading benzo[a]pyrene in a PAH-contaminated soil.

4. CONCLUSION

In conclusion, novel hydrocarbon degrading fungal isolates were recovered which are *Arthrographis kalrae*, *Sporothrix schenckii, Malbranchia* sp., *Prototheca* sp., *Acremonium* spp. and *Rhizomucor* spp. (associated with the active phase of hydrocarbon degradation) were recovered. Data obtained from this research also shows that the extant, indigenous fungal populations in the crude oil-polluted soil have the potential to degrade hydrocarbons and biostimulation enhanced their biodegradation abilities and remediation by enhanced natural attenuation (RENA) is an effective method of bioremediation technique. It is evident from this investigation that oil degrading fungi could readily

be isolated from the oil-polluted soil from Ibaa, Emohua Local Government Area, Rivers State the eastern Niger Delta of Nigeria. These indigenous fungal isolates are promising candidates for further investigations regarding their ability to remove petroleum hydrocarbon from hydrocarbon contaminated environments.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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