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Levels of Some Polyaromatic Hydrocarbons, Heavy Metals and Capacity of MicrobesIsolated from Soil Contaminated with Diesel toUtilizeHexadecane

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Abstract

Soil samples contaminated with diesel were analysed to determine the bacterial and fungal diversity together with the concentration of polyaromatic hydrocarbons (PAHs) and some selected metals which are often targets in environmental checks. Bacteria and fungi were isolated and identified using standard microbiologicalmethods. Identification and quantification of PAH, metal was accomplished using standard methods. A total of fifteen fungal species were isolated and identified. *Enterobacter xiangfangensis*isolated was identified using molecular tools. Degradation test using *Rhizopus stolonifer*and *Enterobacter xiangfangensis* separately was done. The fungal growth pattern revealed that there was an increase in viable cell number from 0.05 to 0.25 (80%) at 84ppm of nickel nitrate (highest). The highest bacterial count was 18.0×10^6 cfu/ml. The results showed that six of the USEPA target PAH assayed were detected. The concentrations of the priority pollutants in the soil samples varied from 2.08 mg/kg to 1.28 mg/kg for site 1 and 1.99 mg/kg to 1.03 mg/kg in site 2. Cd was found below detection limit. These results are valuable in addition to other reports on decontamination of sites co-contaminated. Future efforts will be aimed at determining the extent that microorganisms can utilise both heavy metals and hydrocarbons.

Keywords: Microbes, Heavy Metals, Polyaromatic Hydrocarbons, Pollution.

1. Introduction

Environments polluted with both heavy metals and hydrocarbons are difficult to clean up. This is because of the toxic nature of the heavy metals to microorganisms that can degrade hydrocarbons [1]. [2] reported that heavy metals pollution is a universal problem. Hexadecane is difficult to degrade [3]. Hexadecane, a constituent of gasoline, jet fuel, kerosene and diesel, is often found as the pollutant in most soils and water [4]. Pollution of soils and water resources with crude oil hydrocarbons is one of the most important ecological issues globally [5]. Majority of these hydrocarbons can cause cancer and they are persistent in the environment with the high capability to bioaccumulate due to the persistence of the pollutants [6]. Fungi play a central role in the biodegradation of organic compounds and are producers of an array of extracellular enzymes [7]. The microbial utilization of hydrocarbons depends on the chemical nature of the components within the petroleum mixture and on environment determinants [8]. Many microorganisms have the ability to utilize hydrocarbons as sole sources of carbon and energy for metabolic activities and these microorganisms are ubiquitous and widely distributed in the nature [9]. Polycyclic aromatic hydrocarbons (PAHs) are important group of different organic compounds. These compounds are present and they are widely detected in different environmental matrixes [10]. PAHs are hydrophobic in nature and they have low water solubility, these features, made them to be persistent in the environment over time.The hydrophobicity nature of PAHs has made them to accumulate higher in bottom sediments than in water of contaminated aquatic environments [11]. Other properties of PAHs apart from solubility are; high sensitivity, heat resistant, conductivity, emitability and resistant to corrosion [12]. There are two main sources of PAHs in the environment: these are natural (biogenic and geochemical) and artificial sources (petrogenic and pyrolytic)[13]. The purpose of this study is to determine themicroorganisms present in diesel polluted soil, the concentration levels of total metals contents and PAHs in the soil and to test the utilization of hexadecane by select microorganisms.

2. Methodology

2.1 Sampling site and collection

The samples used in this study were obtained from two diesel contaminated soil. Site A was the generator house located opposite Industrial Chemistry Department while Site B was the generator house located at the Auditorium area of the University of Ilorin. Polluted soil samples were collected from three different points in addition to one control point. The three points were one mitre apart. All samples in each point were collected in triplicates. Samples were collected five times between February and May, 2018. The soil sample was collected with the aid of sterile hand trowel. The samples were carefully collected to avoid contamination into previously labelled sterile bags.

2.2 Chemicals and Reagents

All chemicals and reagents used for the study were of analytical grade of highest purity. N-Hexane-, acetone, Dichloromethane (DCM) were purchased from Sigma Aldrich, PAH standards; naphthalene, anthracene, and pyrene were purchased from Sigma Aldrich, South Africa.

2.3 Experimental procedures for the analysis of PAHs in the samples

Contaminated soil samples (0-15 cm depth) were collected from different locations near generator house in University of Ilorin campus. Three sampling points were randomly chosen at each of the two sampling point's locations at each location. A total of six sampling points was chosen from the study locations and two sampling points from non-contaminated location and these serves as control points. Samples were transported to the laboratory and air-dried at

room temperature after the removal of stones, residual roots and debris that might present in the samples during collection. The samples were sieved with a 1 mm sieve to remove the coarse soil fraction. Soil samples were later stored at 4^{o}C before further analysis. A 10-20 g soil samples were weighed into the soxhlet thimble. Appropriate amount of 1:1 DCM/acetone were added to the apparatus. Samples were extracted for 16 hours at 4 to 6 cycles per hour. After extraction, 1-2 ml of a low volatility keeper were added to the extracts prior to solvent reduction steps to prevent loss of volatile PAH components during evaporative concentrations. Extracts were concentrated to a known final volume using rotary evaporators. The final sample extract were analysed with the aid of Gas chromatography GC-MS instrument. (6890 series) equipped with a Mass spectrometer (Agilent 5975).

2.31Determination of physicochemical parameters and metal analysis

Soil samples were sieved through a 2 mm sieve in order to eliminate stones and other materials extraneous to soil, and then stored in polyethylene bags for transport to the laboratory. The sieved soils were spread over a polyethylene sheet and air-dried at room temperature for one week. The residual moisture was removed by heating at 105±5^{o}C for 3 hours. Samples were ground to a fine powder in a tungsten-carbide swing mill for 3 min and sieved through a <1 µm sieve. Standard methods were used to analyse the physio-chemical parameters such as pH, silt, clay and sand and organic matters [14]. 5 ml of HF and 10 ml $HClO_4$ were added to 1.0 gram of the soil samples and the samples were digested. The digestate were filtered and the filtrate were analysed for metal concentrations.

2.32Instrumentation

A Perkin Elmer 300 Zeeman atomic absorption spectrometer equipped with an HGA-800 graphite furnace and an auto sampler was used to determine the elemental composition of the samples. The graphite furnace temperature program for working elements were set as recommended by the manufacturer. A Unicam model 929AA flame atomic absorption spectrometer with deuterium lamp and air acetylene burner was used during analyses. Gas chromatography GC-MS instrument. (6890 series) equipped with a Mass spectrometer (Agilent 5975).

2.4Microbiological investigation

Immediately after sample collection, samples were transferred to the laboratory for analysis. Exactly 1g of each soil sample was carefully weighed and dispensed into 24 test tubes containing 9ml of sterile distilled water. The solutions were vigorously shaken and allowed to settle. Exactly 900µl of sterile distilled water was transferred into 72 Eppendorf tubes. Exactly 3 of the Eppendorf tubes with sterile distilled water represented each solution containing the samples. Using a sterile pipette 100µl of each sample solution were transferred into the 3 set of Eppendorf tubes and serial dilution was carried out at 10^{-1}, 10^{-2} and 10^{-3}. The appropriate dilutions (10^{-2} and 10^{-3}) with the aid of a sterile pipette under a strictly controlled condition were used and 200µl of each solution suspension were dispensed in parallel onto correctly labelled Petri dishes. Prepared nutrient agar and potato dextrose agar containing streptomycin were poured over the inoculated Petri dishes, gently swirled and allowed to cool. The platings were done in duplicates. The plates were incubated at 37 °C for 1 day for bacteria and 25 °C for 3-5 days for fungi. The numbers of the colonies on the plates were counted and their characteristics were observed. The different colonies obtained from the mixed cultures were subcultured to achieve purity of the selected cultures with the aid of an inoculating loop for bacteria and needle for fungi into fresh sterile NA and PDA plates. The plates were incubated at standard temperatures and days. For preservation of the isolates, the pure cultures were aseptically transferred into labelled sterile NA and PDA slants in McCartney bottles and were incubated 37^{o}C for 1 day for bacteria and at 25°C for 3-5 days for fungi before they were kept in the refrigerator for storage at -4°C.

2.5Characterization and identification of the fungal isolates

Characterization of the fungal isolates was done both macroscopically and microscopically. The growth of the colonies was observed and monitored. Macroscopic features of each plate were examined and recorded with the naked eye. Characteristics such as colour, mycelia, shape, size and presence of pigment were observed for both the base and front of the plates. Microscopically, the colonies were observed under a light microscope according to the method of [15]. Identification was done by referring to [16, 17].

2.6Characterization and identification of bacterial isolates

Characterization of the bacterial isolates was by determining their colonial morphology, cellular morphology and biochemical characteristics. The isolates' colonial morphology determination was carried out based on colony surface, edge, shape, size, elevation and other optical characterizations. The cellular morphology was based on microscopic examination and to determine characteristics such as shape of cells, Grams reaction and motility. Various biochemical tests were carried out to determine the features of the isolates [15]. *Enterobacter xiangfangensis* had the highest frequency of occurrence and hence was further identified using molecular tools. Genomic DNA was isolated using a commercially available kit (FastDNA spin kit for soil, MP Biomedicals, Santa Ana, CA). 16S rRNA-genes were amplified with 27 Forward and 1492 Reverse primers as previously reported [18]. Amplified 16S rRNA-gene sequence were cleaned and analysed at the International Institute for Tropical Agriculture (IITA) biotechnology facility. National Centre for Biotechnology Information (NCBI) database was used to compare and identify the organism using BLASTn.

2.7Fungal biodegradation test in the presence and absence of nickel nitrate

Hexadecane was used to challenge the isolate in the presence of nickel nitrate. Precisely 65µl of hexadecane was added into each reaction tube together with 1µl of the metal. Exactly 100µl of the *Rhizopus stolonifer* broth was inoculated into each of the reaction tubes except the controls. In the absence of nickel nitrate, 65µl of hexadecane was transferred into the reaction tubes containing mineral salts medium and then *Rhizopus stolonifer* was inoculated into the bottles except the control tubes. The tests were duplicated with one control in each case. Optical densities were read at 600nm with the aid of a spectrophotometer. The optical densities were read at day 0, 3, 6, 11 and 15 in the presence of the metal and day 0, 4, 6, 8, 11 and 15 in the absence of the metal.

2.8 Test for Hexadecane Utilizing Potential of Enterobacter xiangfangensis

Utilization of hexadecane was performed using mineral salts medium described by [19]. Growth of *Enterobacter xiangfangensis* on hexadecane substrate was carried out by the inoculation of *Enterobacter xiangfangensis* culture into a 50ml falcon reaction tube containing 50ml of mineral salts medium (MSM) as previously reported by [19, 20]. The composition of the MSM (g/L): 0.5 KH2PO4; 0.3 KNO3; 0.2 MgSO4.7H2O; 1.4 Na2HPO4; 1.0 (NH4)2SO4. The pH of the medium was adjusted to 7.0. Each reaction tube was supplemented with 65µL of hexadecane added to the medium as the only carbon and energy source. This set up was further supplemented with 42ppm, 84ppm, 126ppm and 168ppm of nickel nitrate and sodium dichromate. The different concentrations of the heavy metals were added in separate reaction tubes. The reaction mixtures were incubated for 15 days in stationary position. The optical density of the culture fluids was monitored every 72 hours at wavelength 600nm as indicators of utilization. Reaction tubes analysed were sacrificed every three days after analysis to prevent contamination.

3 Results and Discussion

The range of the pH was found to be mildly acidic between (6.45-6.90). The conductivity of the soil sample collected was found between the ranges of (0.02-0.05) Siemens/metre. The total dissolved solid of the soil samples were found to be between the ranges of (19.50-28.50). The percentage moisture content was found to be moderate considering the samples were taken during the rainy season of the year with a value ranging from (4.76-6.47)(table 1).

Table 1. Physicochemical parameters recorded across all locations

Location	pH	Conductivity (mS)	Total Dissolved Solid (ppm)	Moisture Content (%)
M	**6.90**	**0.02**	**28.50**	**6.47**
2	**6.60**	**0.03**	**23.25**	**6.23**
3	**6.45**	**0.02**	**19.50**	**6.32**
4	**6.70**	**0.04**	**24.25**	**5.05**
5	**6.80**	**0.04**	**22.50**	**4.76**
6	**6.68**	**0.05**	**24.63**	**5.33**

Table 2. Average metal concentrations across all locations

Location	Cu(g/kg)	Cr(g/Kg)	Cd(g/kg)	Pb(mg/ml)
1	**0.037 ± 0.025**	**0.007 ± 0.005**	**ND**	**0.027 ± 0.029**
2	**0.025 ± 0.032**	**0.026 ± 0.016**	**ND**	**0.027 ± 0.055**
3	**0.063 ± 0.011**	**0.042 ± 0.003**	**ND**	**0.058 ± 0.041**
4	**0.166 ± 0.208**	**0.027 ± 0.003**	**ND**	**0.018 ± 0.011**
5	**0.066 ± 0.015**	**0.036 ± 0.004**	**ND**	**0.043 ± 0.020**
6	**0.067 ± 0.016**	**0.051 ± 0.020**	**ND**	**0.043 ± 0.008**
Total mean & std. dev. for all locations	**0.071 ± 0.090**	**0.031 ± 0.017**	**ND**	**0.036 ± 0.031**
Ranges	**0.025 – 0.166**	**0.007 – 0.051**	**ND**	**0.018 – 0.058**
WHO limits (2006)	**1.00**		**0.005**	**0.01-0.05**

Concentration of metals (table 2) recorded across all locations ranged between 0.025- 0.166 for Cu; Cr (0.007- 0.051); Pb (0.018-0.058) while Cd was found below detection limit. Copper is a transition metals which belong to period 4 and group 1B of the periodic table with atomic number 29, atomic weight 63.5, and density 8.96 $kgcm^{-3}$, copper is the third most used metal in the world. Copper is indeed essential, but in high doses it can cause anaemia, liver and kidney damage, and stomach and intestinal irritation. Higher amount of copper was found in location 4.All soils examined had high total organic carbon contents. Higher percentages of metal concentration were found in sites 3, 5 and 6 respectively. These sites were severely affected with diesel oil pollution. The pH values increased with depth while the

organic carbon and organic matter contents decreased with increasing depth. No noticeable trend was observed for the CEC. The total concentrations of heavy metals reported in this study were found below the Department of Petroleum Resources (DPR) maximum allowable limits of metals in soils except for Cd in some sites. Like CEC, there is no noticeable trend in the concentration of heavy metals with depth. However, the topsoil had higher mean concentration of the heavy metals compared to the subsoil. All sites showed appreciably higher levels of metal concentration compared to background levels recorded prior to diesel handling in the generator house. In the present study, it was observed that the concentration of naphthalene ranges from 0.10-2.08 mg/kg which is above the WHO limits with the range value of 0.01-0.05 mg/kg. The concentration of acenaphthalene ranges from 0.05-1.85 mg/kg which is higher than the WHO limits of 0.1 mg/kg. Concentration recorded for fluorine ranges from 0.03-0.99 mg/kg which is above the WHO limits value of 0.01-0.05 mg/kg while concentrations of phenanthrene range from 0.02-1.09 mg/kg. The above results show that the concentration for anthracene ranges from 0.04-1.28 mg/kg was found to be above the WHO limit concentration value of 0.01 mg/kg and this shows toxicity in the soil sample. The total concentration for pyrene ranges from 0.02-1.22 mg/kg. The dominance of naphthalene, anthracene and pyrene in almost all the samples was observed. There is an indication that the treatment facility has some sites that are contaminated which have PAH levels of more than 0.01 mg/kg (table 3). The levels of PAHs detected in most of the sample areas were observed to be higher than the control point. This is an indication that the plant is adding contaminants to the surroundings where lecture theatres and mosques are close by. From the table above, it can be seen that site 1 has the highest concentration of priority pollutants to that of site 2. According to the European classification system of soil contamination [21],< 0.20 mg/kg show no contamination, 0.20–0.60 mg/kg corresponds to weak contamination, 0.60–1.0 mg/kg represents moderate contamination, and >1.0 mg/kg indicates heavy contamination, so all the sampling sites in this study were heavily contaminated, where by the PAHs concentrations are 12.6, 65.5 and 22.3 times higher, respectively, than the heavily contaminated level (1.0 mg/kg). It is indicated that the soil around the generator house stored great amounts of PAHs and regulatory measures are needed to prevent the generator site from turning into pollution sources, which would transfer PAHs into the air or groundwater in the region.

Table 3: Concentration of various PAHs from the two sampling sites

	Site 1				Site 2			
Compound	A	B	C	Ctrl	A	B	C	Ctrl
Naphthalene	2.08±0.09	1.99±0.07	0.95±0.05	Nd	1.99±0.07	1.97±0.10	0.99±0.07	Nd
Acenaphthene	1.85±0.09	1.57±0.06	0.95±0.05	Nd	1.33±0.05	1.03±0.09	0.83±0.05	Nd
Fluorene	0.06±0.05	0.83±0.04	0.03±0.02	Nd	0.99±0.03	0.05±0.07	Nd	Nd
Phenanthrene	1.09±0.07	0.99±0.05	N/D	Nd	0.85±0.01	0.65±0.07	0.33±0.02	Nd
Anthracene	1.28±0.08	1.21±0.08	0.66±0.06	Nd	1.09±0.04	0.950.05	0.60±0.04	Nd
Pyrene	1.22±0.08	1.02±0.07	0.54±0.05	Nd	0.95±0.05	0.14±0.02	0.12±0.01	Nd
Mean value±SD	4.26±0.71	4.16±0.04	0.72±0.08	Nd	3.75±0.02	2.85±0.01	0.95±0.35	Nd
Ranges	0.10-2.08	0.05-1.85	0.03-0.99	Nd	0.02-1.09	0.04-1.28	0.02-1.22	Nd

3.1 Statistical analysis - Pearson's correlation coefficient

The Pearson correlation coefficient (table 4) represents the correlation coefficient between two or more variables measured in the same interval. The correlation coefficient ranges from -1 (negative variables) to +1 (positive variables). A positive value between two continuous variables shows a positive relationship between the two variables while a negative value shows a negative relationship between the two variables. When the correlation coefficient of zero exists between two variables, it shows that there is no relationship between the two variables. A variable between 0.5 shows a weak correlation while values above 0.5 show a strong correlation between the two variables.

Table 4. Pearson's correlation coefficient

	Napthalene	Acenaphthene	Flourene	Phenanthrene	Anthracene	Pyrene
Napthalene	1.00	0.14	0.83	-0.02	0.75	0.90
Acenaphthene		1.00	-0.08	0.29	0.50	0.85
Flourene			1.00	0.25	0.65	0.80
Phenanthrene				1.00	0.25	0.78
Anthracene					1.00	0.05
Pyrene						1.00

The correlation matrix above shows that, throughout the study area, there is a strong positive correlation between the compound naphthalene with anthracene (0.75). The data was further treated to observe PAH pollutant compounds, which have similar pollution signatures with respect to the study area.

3.2 Description of fungal isolates

Fifteen fungal isolates were isolated, characterised and identified. They are *Aspergillus niger, Rhizopus stolonifer, Aspergillus fumigatus, Aspergillus tamarii, Trichophyton terrestre, Aspergillus nidulans, Trichoderma viride, Aspergillus flavus, Aspergillus versicolor, Syncephalastrumracemosum, Penicillium crystallinum, Geotrichumcandidum, Botrytis cinerea, Penicillium chrysogenum*and *Alternaria alternata*. *Rhizopus stolonifer* was most predominant. The least in terms of occurrence include *Trichophyton terrestre, Trichoderma viride, Aspergillus versicolor, Geotrichumcandidum*and *Penicillium chrysogenum.* Table 5 shows number of fungal isolates present in each plate at each sampling week at sampling site A and B including the control site. The most predominant was *Rhizopus stolonifer*. Table 6 shows the frequency of isolates occurrence over the sampling weeks.

Table 5: Total fungal count of isolates from the two sampling sites.

Week	**A1**	**A2**	**A3**	**AC**	**B1**	**B2**	**B3**	**BC**
1	1	42	50	11	12	11	42	4
2	1	3	15	2	0	6	1	6
3	2	4	1	2	2	31	4	8
4	4	3	14	14	19	1	5	6
5	14	4	14	38	9	9	14	10

Key: 1=immediate polluted site; 2=one metre away from 1; 3=two metre away from 1; C=Unpolluted Control; A= Sampling site A; B= Sampling site B.

Figure 1 represents the data that shows the increase in fungus growth (*Rhizopus stolonifer*) in mineral salts medium containing hexadecane and nickel nitrate. The tables depict the mean variation of optical density at 600nm. At day 0 to

day 3, microbial load was different in all concentration of nickel nitrate. At day 3 to day 6, microbial load at all concentration increased gradually. At day 6 to day 13, the microbial load at concentration 48ppm and 168ppm increased sharply and at concentration 84ppm and 126ppm, there was reduction. At day 13 to day 15, microbial load at concentration 48ppm and 168ppm reached decline phase. At concentration 84ppm, *Rhizopus stolonifer*was at its optimum growth.

Table 6: Occurrence of fungal isolates in the sampling sites

	Fungal isolates	**Weeks and sampling sites**										**Percent(%)**
		A1	**B1**	**A2**	**B2**	**A3**	**B3**	**A4**	**B4**	**A5**	**B5**	
1	*Aspergillus niger*	+	+	+	-	-	-	+	+	+	-	60
2	*Rhizopus stolonifer*	+	+	+	+	+	+	-	+	+	-	80
3	*Aspergillus fumigatus*	+	-	-	-	-	-	-	-	+	-	20
4	*Aspergillus tamarii*	+	+	-	+	+	+	-	+	-	+	70
5	*Trichophyton terrestre*	+	-	-	-	-	-	-	-	-	-	10
6	*Aspergillus nidulans*	+	-	-	-	-	-	-	-	+	+	30
7	*Trichoderma viride*	+	-	-	-	-	-	-	-	-	-	10
8	*Aspergillus flavus*	+	-	-	-	+	-	-	-	+	-	30
9	*Aspergillus versicolor*	+	-	-	-	-	-	-	-	-	-	10
10	*Syncephalastrumracemosum*	+	-	-	+	+	-	-	-	-	-	30
11	*Penicillium crystallinum*	-	+	+	-	+	+	+	-	+	+	70
12	*Geotrichumcandidum*	-	+	-	-	-	-	-	-	-	-	10
13	*Botrytis cinerea*	-	+	-	-	-	-	-	+	-	-	20
14	*Penicillium chrysogenum*	-	+	-	-	-	-	-	-	-	-	10
15	*Alternaria alternata*	-	+	-	-	-	-	-	+	+	+	40

Key: 1,2,3,4,5 = Weeks; A= Sampling site A; B= Sampling site B; +=Present; -=Absent.

Figure 2 shows increase in *Rhizopus stolonifer*growth in mineral salts medium containing hexadecane only. At day 0 to day 4, there was a sharp decline in microbial load, perhaps because the isolate was introduced into a new environment. This explains why *Rhizopusstolonifer* had to adjust and adapt compared to figure 1 where there was a gradual increase at day 0 to day 3. At day 4 to day 6, the gradual increase in the microbial load revealed that the isolate has been able to adapt and start the utilization of hydrocarbon. At day 13 to day 15 a gradual decrease was observed which explains that the isolate has gotten to its decline/death phase [22] demonstrated that species of *Penicillium*, *Aspergillus* and *Rhizopus* were capable of degrading hydrocarbons. *Aspergillus niger* is commonly found as a saprophyte [23](Timothy *et al.*, 2018). *Aspergillus nidulans* have been used for the production of large quantities of industrially relevant enzymes [24]. *Aspergillus flavus* has been reported as a saprotrophic fungus [25]. *Rhizopus stolonifer* is the most predominant fungal isolate in this study. They are commonly found in soil, compost and other decaying plant matter[26]. *Geotrichumcandidum* is notably associated with skin, sputum and faeces [27]. *Alternaria alternata* is a member of the imperfect fungi and one of the most important among the allergenic fungi [28]. The saprophytic and habitat of these fungus may account for their presence in the sampled soil. Filamentous fungus has ability to grow on many substrates by secreting extracellular hydrolytic enzymes, even capable of growing under ambient environment [29]. According to [30]*Rhizopus stolonifer*was isolated from Adayar site in India and it was

recorded that *Rhizopus stolonifer* has the ability to secrete extracellular hydrolytic enzymes that allows it to utilize hydrocarbons. It was observed that compared to other fungal isolates used in the research, *Rhizopus stolonifer* was able to utilize the hydrocarbon at a very low rate. This may explain the reason for a sharp decline in microbial load of 2.0 to 0.01 at day 0 to day 4 and gradual increase from day 4 to day 15 in mineral salts medium in the absence of the metal (figure 2).

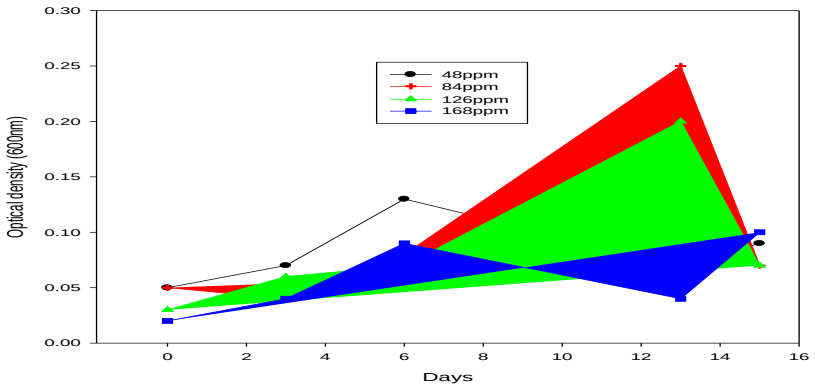

Figure 1: Growth of *Rhizopus stolonifer* in MSM with nickel nitrate and hexadecane.

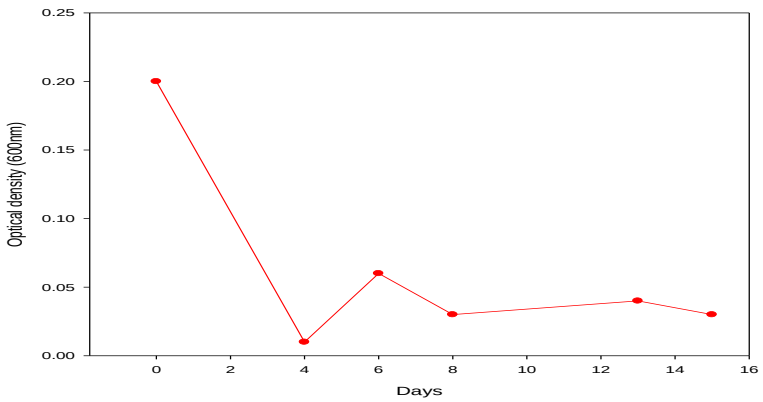

Figure 2: Growth of *Rhizopus stolonifer* in MSM with hexadecane.

Fungi are potential degraders under adverse conditions, such as in environments with low pH and poor in nutrients. Moreover, when compared with yeasts and bacteria, they have a higher ability to adapt to media with a low water activity. The results of heterogeneous fungi and diesel utilizing fungi in the soil samples suggested that the diesel utilizing fungi were adapted to the quantity of hydrocarbons in the environment. *Rhizopus stolonifer* degraded hexadecane better in the presence of nickel nitrate (figure 1) than in its absence (figure 2). Hexadecane degradation was best in 126 ppm (82%) of the metal used, this was followed by 84 ppm (80%) and then 48 ppm (58%). Degradation was least in 168 ppm (8%) of nickel nitrate. Hexadecane was mineralised by filamentous fungi in a study by [31]. In their study hexadecane could not degrade aromatic compounds. This study showed thatthe *Rhizopus stolonifer*found in the diesel contaminated soil degraded optimally in the presence of nickel nitrate. In a related study [32],*Pseudomonas aeruginosa* isolated from kerosene was challenged with chromium (III) solution and mercury(II) solution, it was observed that high concentration of some these trace heavy metals like Cr3+ and Hg2+ inhibit the growth of this bacterium. This is in variance with the current study where the fungus was able to grow well in the presence of nickel nitrate. The bacterial counts were done. Exactly 0.0x10^{6}cfu/ml and 1.0 x10^{6}cfu/ml was recorded for control of week 2 of the two sampling sites. Exactly 0.5 x10^{6}cfu/ml and 0.0 x10^{6}cfu/ml was recorded for control of

week 3 of the two sampling sites. These records and that for the others indicate that there is marked significance between the microbial load in the unpolluted soil and the polluted soil (Table 7). The controls generally had lower microbial loads. Bacteria with the potential of degrading diesel isolated from diesel polluted sites at the two diesel polluted sites were *Enterobacter xiangfangensis*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Micrococcus luteus*, *Streptococcus* sp. and *Bacillus subtilis* (Table 8). *Enterobacter xiangfangensis* had the highest frequency of occurrence (100%). It was detected throughout the five weeks of sampling. *Bacillus subtilis* occurred four times (80%) out of the five times that sampling was done. *Micrococcus luteus* and *Pseudomonas aeruginosa* had equal occurrences (60%) as they were seen in three separate weeks. *Streptococcus* sp.was next with two occurrences (40%) while *Proteus vulgaris* had the least with a single occurrence in the five weeks of sampling (Table 8). Molecular tools were used to confirm the identity of *Enterobacter xiangfangensis*after initial traditional means of identification was used. The growth pattern of *Enterobacter xiangfangensis* on hexadecane at different concentrations of nickel nitrate showed that it had the highest growth on the third day. After the third day it started decreasing on day 6, 9, 12 and 15 (Figure 3). The same pattern was recorded for sodium dichromate (Figure 4) except that 84ppm and 126ppm of sodium dichromate produced their highest growth on day 6 and day 9 respectively. [33] reported that *Bacillus subtilis* and *Pseudomonas aeruginosa* were able to degrade diesel, hexadecane and phenanthrene. These researchers also reported that resident bacteria are a promising technology to recover a polluted environment. In another study by [34], *Bacillus niabensis* ACSI85 was used in the degradation of hexadecane up to 90%. These are in agreement with the current study where resident bacteria in diesel polluted soil utilized hexadecane. The degradation of hexadecane by *Pseudomonas aeruginosa* NY3 in the presence of glutaric acid had been reported. It was stated that during the degradation of alkane, organic acids are produced which can make the pH to decrease [35]. [1] reported that the metabolic activities of bacteria were active in the presence of nickel and cobalt. In their report, hydrocarbon degradation was up to 90%. This implies that the presence of heavy metals can make microbial utilization of hydrocarbons to be more efficient. In the current report, there was a limited utilization of hexadecane in the presence of nickel nitrate and sodium dichromate. In a study by [32], the growth of *Pseudomonas aeruginosa* was inhibited in the presence of chromium (III) and mercury (II) ions. The inhibition became higher as the concentration of the heavy metals increased. [36] also reported that heavy metals inhibited biodegradation of hydrocarbons no matter the concentration that was used. These inhibitory effects of heavy metals may suggest why *Enterobacter xiangfangensis*could not grow for more than a few days before decline in hexadecane and the metals in this work. However, [37] used a consortium of bacteria to biodegrade heavy metals which normally were no degraded by single organisms. This shows that a consortium can be explored to degrade hydrocarbons in the presence of toxic metals.

Table 7: Mean Heterotrophic Bacterial Count in the Sampled Fields

Site	Number (cfu/ml)/Sampling time (week)				
	1	2	3	4	5
A1	5.5×10^6	1.5×10^6	8.0×10^6	5.5×10^6	5.0×10^6
A2	5.0×10^6	18.0×10^6	7.0×10^6	2.5×10^6	6.0×10^6
A3	12.5×10^6	12.5×10^6	4.0×10^6	4.0×10^6	3.5×10^6
AC	1.5×10^6	$0{,}0 \times 10^6$	0.50×10^6	11.5×10^6	4.5×10^6
B1	12.0×10^6	7.0×10^6	2.0×10^6	3.0×10^6	5.0×10^6
B2	4.0×10^6	14.0×10^6	4.5×10^6	2.5×10^6	7.0×10^6
B3	15.0×10^6	4.0×10^6	2.0×10^6	2.0×10^6	3.5×10^6
BC	10.0×10^6	1.0×10^6	0.0×10^6	1.5×10^6	2.0×10^6

Legend: A1=immediate polluted site in A; A2=one metre away from A1; A3=two metres away from A1; B1=immediate polluted site in B; B2=one metre away from B1; B3=two metres away from B1; AC=Unpolluted Control in A; BC= Unpolluted Control in B; Sampling site A; B= Sampling site B.

Table 8: Frequency of Bacterial Occurrence in the Sampled Fields

S/N	Isolates	Sampling time (week)					Percentage (%)
		1	2	3	4	5	
1	*Enterobacter xiangfangensis*	+	+	+	+	+	100
2	*Bacillus subtilis*	+	+	+	-	+	80
3	*Pseudomonas aeruginosa*	+	-	+	+	-	60
4	*Micrococcus luteus*	-	+	+	-	+	60
5	*Streptococcus* sp	-	+	+	-	-	40
6	*Proteus vulgaris*	-	-	+	-	-	20

Legend: + = Present; - = Absent

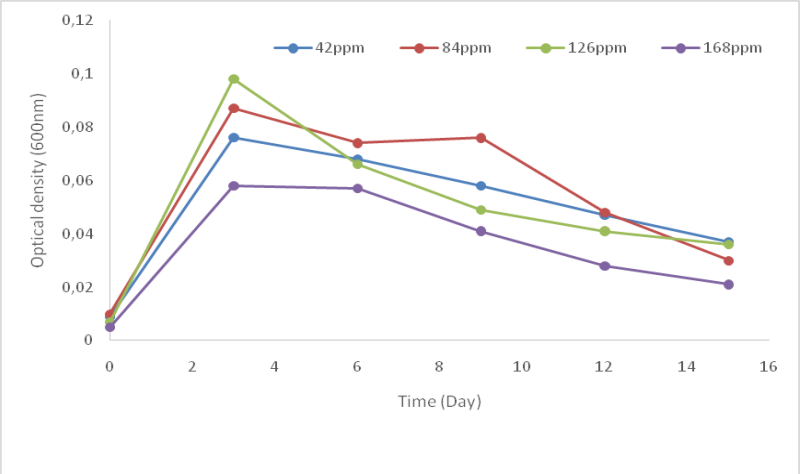

Figure 3: Growth Pattern of *Enterobacter xiangfangensis* on Hexadecane at Different Concentrations of Nickel Nitrate.

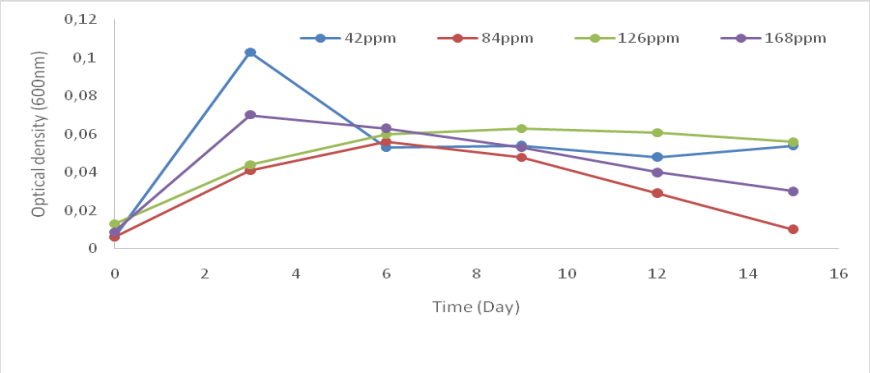

Figure 4: Growth Pattern of *Enterobacter xiangfangensis* on Hexadecane at Different Concentrations of Sodium Dichromate Solutions.

4 Conclusion

This study revealed that concentrations of the metals analysed in contaminated soils were greater than the level found at the control site. This suggests that exogenous metals are weakly bound to the soil particles. The results of this study would be helpful to understand the levels, distribution and sources of PAHs in each sampling location which can provide information for improving living environment and human health in the university of Ilorin main campus (PS). It is concluded that *Rhizopus stolonifer* has the capability to remove hexadecane and nickel nitrate contaminants from the environment. More studies need to be carried out to determine the extent that microorganisms can utilise both of heavy metals and hydrocarbons.

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