ISSN: 2350-0328

International Journal of AdvancedResearch in Science, Engineering and Technology

Vol. 5, Issue 11, November 2018

Response of Fungi to Diesel Oil Contamination of a Soil in Nigeria

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ABSTRACT: Extensive petroleum hydrocarbon exploration and production activities have resulted in pollution of natural environments by crude oil and its products; exposure of microorganisms to the petroleum hydrocarbon contamination could lead to disastrous consequences for the soil microbial community and in turn alter soil fertility. Frequent tanker accidents along the highways have released large quantities of petroleum products into nearby agricultural lands; the magnitude of damage to the ecosystem could only be imagined. This study therefore focused on the response of fungi to diesel oil contamination in a soil. Three setups of soil samples (3kg weight), designated B, C, and D soil options were contaminated with 90ml, 180ml, and 270ml volumes of diesel oil respectively; one setup of soil (A soil option) was left uncontaminated (0ml volume) to serve as control. Microbiological analysis of the soil samples was carryout on sabuoraud dextrose agar and mineral salts oil ager at days 1, 7, 14 and 21 intervals after addition of diesel oil to the soils. Mean counts of heterotrophic fungi ($X10^3$CFU G^{-1} soil) in the soil options were 0ml: 7.0, 90ml: 4.5, 180ml: 4.5, and 270ml: 4.0. Mean densities of hydrocarbon-utilizing fungi ($X10^2$CFU G^{-1} soil) for 0ml, 90ml, 180ml, and 270ml soil options were: 5.0, 3.5, 6.8 and 3.0 respectively. Fungal organisms isolated include *Aspergillus niger*, *Aspergillus species*, *Fusarium species*, *Mucor species*, *Rhizopus species* and *Saccharomyces species*, which occurred in control soil and diesel-polluted soils but *Mucor species* did not occur in 90ml soil option. Results showed that heterotrophic fungi responded negatively to addition of diesel oil to soil while hydrocarbon-utilizing fungi decreased in 90ml diesel-polluted soil, peaked in 180ml polluted soil, and decreased again in 270ml polluted soil compared to control soil. Fungal organisms survived the toxic effect of diesel oil in the soils which means that the study site harbour fungi with the potential to utilize diesel oil which could be isolated and used in cleanup of crude oil spilled sites. In conclusion, pollution of soil with diesel oil reduced population of heterotrophic fungi and at the same time stimulated growth of indigenous fungi with the potentials to participate in natural remediation process of diesel fuel spilled sites; also 180ml volume of diesel stimulated optimum growth of fungal degraders in the study soil.

KEY WORDS: Response**,** diesel oil, contamination, fungi, soil

I. INTRODUCTION

Petroleum production began in 1958 and since then, cases of petroleum and refined petroleum products' spills onto agricultural lands through petroleum production operations have been reported (Odu, 1977; Awobanjo, 1981; De *et al*., 2000; Obire and Anyanwu, 2009; Al-Nasrawi, 2012; El Hanafy *et al*., 2015). The incidences of recorded environmental pollution, due to high rate of petroleum related activities have been associated with frequent oil spills especially through oil well blowouts, tanker accidents, rupture of pipelines, and sabotage. These mishaps result in the release of crude oil and refined petroleum products into the terrestrial and aquatic environment (Okpokwasili and Amanchukwu, 1988; Al-Nasrawi, 2012).

Diesel fuel is a hydrocarbon product boiling between approximately 150^{o}C and 400^{o}C with carbon chain length of C_{15}–C_{22}. Classification of diesel differs from country to country; some classes containing selected cracked distillates such as light cycle oils (Campos *et al*; 1974). A variety of additives maybe used to improve the stability of the fuel; these include aliphatic amines, chelating agents, detergents, and corrosion inhibitors (Mukhopadhyay and Chakraborty, 2015; Ali *et al*., 2015). Some of which can act as nutrient source for microorganisms. Petroleum derived diesel is composed of about 15% saturated hydrocarbons (primarily paraffins such as n-, iso- and cyclo-paraffins) and 25% aromatic hydrocarbons including naphthalenes and alkylbenzenes. The average chemical formula for common diesel fuel is $C_{12}H_{23}$ (range $C_{10}H_{20}$ to $C_{15}H_{28}$) (Anyanwu, 2014).

ISSN: 2350-0328

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The soil is the most dynamic site of interactions in nature and it is also the region in which many biochemical reactions concerned in the decomposition of organic matter and nutrition of plants particularly agricultural crops occur (Marquez–Rocha *et al*., 2001). Soil pollution with petroleum and its derivatives is one of the causes of degradation of natural environment (Riis *et al*., 1995; Ojumu *et al*., 2004). The use of microorganisms and their activities in tests for effects of a specific chemical substance in soil, as well as in studies of soil pollution, has often been recommended (Riis *et al*., 1995; Ojumu *et al*., 2004). Diesel oil pollution could affect soil microbial flora such as the fungal community also known as "mycoflora of the soil" in a way that could hamper soil productivity. This could reduce numbers of microbes in polluted soil below those of non-polluted soil. Soil microorganisms are responsible for the breakdown of organic matter including hydrocarbons, conversion of inorganic components from one form to another (Ojumu *et al*., 2004).

Many fungal species inhabit the soil, and at high level of water activity can cause spoilage. Some members of the genera are however, known to have relevance in man's daily life. *Aspergillus niger* is responsible for the spoilage of many foods (Ikediugwu and Ejale, 1980; Kuku, 1985). Also, the metabolic process of *A. niger* has been harnessed into very useful products (Nishio *et al*., 1981; Kuku, 1985; Dinchev, 1981; Sauer and Borroughts, 1980). *Penicillium* species has the ability to produce antibiotics (Okafor, 1987).

The effect of oil pollution in the soil environment is largely determined by both biotic and abiotic factors of the soil. These biotic and abiotic properties of soil determine the persistence of oil pollutants in the environment, although this can be dependent to some extent on the quality and mixture of the hydrocarbon. In fact, there are measurable effects of oil on ecologically important microbial populations caused by exposure to crude and refined oils (La-Rue, 1977). Once crude oil is discharged into the soil, it rapidly sinks into soil and volatile fraction escapes leaving the less volatile fraction for microbial degradation (Davis and Huges, 1968; Ojumu *et al*., 2004). This is due to a major complex mixture of hydrocarbons containing paraffins, olefins, kerosene and octane (Atlas, 1981). The sensitivity of soil microflora to petroleum hydrocarbons is a factor of the quantity and quality of oil spilled and previous exposure of natural soil microbes to oil (Bossert and Bartha, 1984).

The development of petroleum industry into new frontiers, the apparent inevitable spillages that occur during routine operations and records of acute accidents during transportation of petroleum products require more attention into oil pollution problems (Okoh, 2006). Also, the extensive use of petroleum products leads to the contamination of almost all components of the environment, and biodegradation of the hydrocarbons by natural populations of microorganisms has been reported to be the main process acting in the depuration of hydrocarbon-polluted environments (Challaina *et al*., 2004). In Nigeria, incessant tanker accidents that results in spillages of large quantity of petroleum products, including diesel fuel, into surrounding agricultural lands along traffic highways (Nwilo and Badejo, 2005; Anyanwu, 2014;

Okoye and Okunrobo, 2014) called for this study into the pollution problems. The implication of such acute pollution of the environment could be alteration of the ecosystem microbial community structure in a way that the soil productivity is hampered leading to food scarcity.

II. SIGNIFICANCE OF THE STUDY

This study therefore, was undertaken to assess the impact of diesel oil on the fungal community structure of soil, and to evaluate the potential of soil fungi to withstand toxic effect of diesel oil in tropical rainforest vegetation. Furthermore, to isolate fungi in the soils as to know the type of fungi associated with the diesel-polluted experimental soil capable of utilizing hydrocarbon as sole source of carbon and energy. Also, the study focused on the response of fungi to different concentrations of diesel fuel in the soil.

III. MATERIALS AND METHODS

A) Study Area

The study site was a fallow agricultural garden around Nkpolu-Oroworukwo community in Diobu area of Port Harcourt metropolis. The area is a plan land within the tropical rainforest vegetation of Niger Delta in Southern Nigeria. The study site has no presence of crude oil production activity and no history of petroleum hydrocarbon spillage.

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B) Collection of Soil and Diesel Oil Samples

Soil samples were collected from the study area in four replicates which were measured in 3kg weight into fresh unused black polythene bags each. Surface soil (0 - 15cm depth) was collected using a clean auger borer and bulked into the fresh polythene bags. The bulked soil samples were taken to the green house for treatment application. Diesel oil was obtained from Conoil filling station located along Ikwerre Road in Mile 3 Diobu Area of Port Harcourt. This was used for polluting the soil.

C) Preparation and Treatment of Soil Samples

Treatment application involved addition of diesel oil to the soil samples to simulate pollution in the soils. The soil samples were packaged in 3kg weight into fresh unused polythene bags perforated at the bottom to allow excess leachate drain off during the period of the experiment. Four sets of samples were labeled A, B, C and D and left for 2 days before being watered/moistened with 600ml sterile tap water. Thereafter, pollution of the soil samples was done by adding 90ml, 180ml and 270ml volumes of diesel oil to the 3kg weight (w/v) of soils designated: B, C, and D soil options respectively. Soil sample A was left unpolluted (0ml diesel oil) and served as control. Microbiological analysis of the soil samples was done at weekly intervals after treatment application.

D) Microbiological Analysis of Soil Samples

Microbiological analysis of soil samples was done at days 1, 7, 14 and 21 intervals after addition of diesel oil. For the purpose of enumeration and isolation of heterotrotrophic fungi and hydrocarbon-utilizing fungi, ten-fold serial dilution, as described by Obire and Wemedo (1996), was employed to obtain appropriate dilutions used to inoculate agar plates by spread-plating: sabuoraud agar for heterotrotrophic fungi and mineral-oil agar for hydrocarbon utilizing fungi. The inoculated plates were incubated at 28 ± 2^{0}C for 2 – 5 days. After incubation, plates were examined and colonies that developed were counted and recorded; and taken as total heterotrophic fungal counts and hydrocarbon-utilizing fungal counts enumerated. The difference in counts between the control soil and diesel-contaminated soils was taken as the effect of diesel contamination on the fungal population of the study soil.
Identification of fungal isolates was done by macroscopy to observed colonial morphology: colour of colony, texture, shape and surface appearance; and microscopy by wet preparation and slide culture to reveal the nature of the filaments and reproductive structures such as conidiospores and sporangiospores. All identification of pure isolates was made on the basis of their cultural and morphological characteristics and by reference to Alexopoulos and Sun, 1962; Barnett and Hunter, 1972; Abbey, 1995; Winn *et al*., 2006).

IV. EXPERIMENTAL RESULTS

Results of this study was based on the enumeration of heterotrophic fungi and hydrocarbon-utilizing fungi to obtain their populations at the various concentrations of diesel-contaminated soils and control soil; and to know the types of fungi associated with the diesel-contaminated soils. Fungal populations for heterotrophic fungi and hydrocarbon-utilizers are shown in tables 1 and 2 respectively. Types of fungi isolated from the soils are shown in table 3.0

Table 1.0: Counts of heterotrophic fungi in control and diesel-polluted soils

Days of Analysis	Number of colony forming units per gram soil ($X10^{3}CFU\ G^{-1}$)			
	Control soil (0ml)	Concentration of diesel in polluted soils		
		90ml	180ml	270ml
1	6.9	5.0	3.0	5.0
7	7.0	3.0	6.2	3.8
14	8.2	4.9	4.0	3.0
21	6.0	5.2	4.8	4.2
Mean	7.0	4.5	4.5	4.0

ISSN: 2350-0328

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Ranges of counts of heterotrophic fungi in control and diesel-polluted soils are 0ml: 6.0 to 8.2X10^{3}CFU G^{-1}; 90ml: 3.0 to 5.0X10^{3}CFU G^{-1}; 180ml: 3.0 to 6.2X10^{3}CFU G^{-1} and 270ml: 3.0 to 5.0X10^{3}CFU G^{-1}. Ranges of hydrocarbon-utilizers in the soils are 0ml: 4.9 to 5.0X10^{2}CFU G^{-1}; 90ml: 3.0 to 4.0X10^{2}CFU G^{-1}; 180ml: 5.9 to 8.1X10^{2}CFU G^{-1} and 270ml: 2.2 to 4.0X10^{2}CFU G^{-1}. Fungal types isolated during this study include *Aspergillus niger, Aspergillus species, Fusaruim species, Mucor species, Penicillium species, Rhizopus species* and *Saccharomyces species.* Of the seven fungi isolated, all occurred in both uncontaminated and diesel-contaminated soils except *Mucor species* which did not occur in 180ml concentration.

V. DISCUSSION

Response of fungi (heterotrophic and hydrocarbon-utilizers) to diesel oil contamination of a soil was investigated in this study. Counts of heterotrophic fungi fluctuated between the control (uncontaminated) soil and diesel-contaminated soils; and within the days of the experiment. Densities of fungi were highest in control (0ml, unpolluted) soil throughout the period of the experiment when compared to the polluted soils. At day 1 of addition of diesel, fungal counts were highest in 0ml concentration, decreased at 90ml and 180ml concentrations and increased again at 270ml soil option. At day 7 after addition of diesel oil, counts of fungi were highest in 0ml, decreased in 90ml, increased in 180ml and decreased again in 270ml diesel-polluted soil option. At day 14 and day 21 of diesel treatment, fungal counts almost had similar pattern of fluctuations with control soil having highest counts, which decreased gradually with increasing concentrations of diesel oil from 90ml to 270ml diesel-polluted soil option.

Table 2.0: Counts of hydrocarbon-utilizing fungi in control and diesel-polluted soils

Days of Analysis	Number of colony forming units per gram soil (X10^{2}CFU G^{-1})			
	Control soil (0ml)	Concentration of diesel in polluted soils		
		90ml	180ml	270ml
1	5.1	4.0	7.0	3.3
7	4.9	3.8	6.3	3.0
14	5.3	3.0	5.9	4.0
21	5.0	3.2	8.1	2.2
Mean	5.1	3.5	6.8	3.1

Mean densities of fungi were highest in control soil, decreased and remained the same in 90ml and 180ml soil options but decreased slightly in 270ml diesel-polluted soil. Results showed that heterotrophic fungal populations decreased as the pollutant concentration increased; and that diesel oil generally depressed fungal densities of the study soil. Statistically there was significant difference ($P<0.005$) between fungal counts of control soil and counts of the diesel-polluted soils but no significant difference between fungal counts of polluted soils. The statistical difference observed in the fungal populations of control soil and diesel-polluted soils showed real pollutant effect which caused decrease in fungal densities of polluted soils far below the counts in control soils.

Table 3.0: Fungal types isolated from control and polluted soils

Fungal types	Concentration of diesel in polluted soils			
	Control(0ml)	0ml	180ml	270ml
Aspergillus niger	+	+	+	+
Aspergillus species	+	+	+	+
Fusarium species	+	+	+	+
Mucor species	+	-	+	+
Penicillium species	+	+	+	+
Rhizopus species	+	+	+	+
Saccharomyces species	+	+	+	+

ISSN: 2350-0328
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KEY: + = bacterial species isolated, - = bacterial species not isolated

Counts of hydrocarbon-utilizing fungi of control soil and diesel-polluted soils showed similar pattern of fluctuations throughout the period of the experiment. Control soil (unpolluted soil) had somewhat high counts which decreased in 90ml diesel-treated soil option became highest in 180ml soil option and decreased again in 270ml diesel-treated soil option. Mean counts of hydrocarbon-utilizing fungi were highest in 180ml diesel-polluted soil followed by control soil option with 90ml diesel-polluted soil next to control soil and 270ml having the lowest counts. High counts of hydrocarbon-utilizing fungi observed in unpolluted soil, without any history of oil spillage, showed that microorganisms occur in nature particularly in region where oil exploration activity takes place. In polluted soils, pollutant effect was expressed differently; diesel oil depressed microbial growth in 90ml diesel-polluted soil, which peaked in 180ml polluted soil option and decreased again in 270ml diesel-polluted soil. Counts of hydrocarbon utilizing fungi were highest in 180ml diesel-polluted soil, which could be taken as the optimum concentration of diesel oil for every 3kg of soil capable of stimulating optimum growth/activity of hydrocarbon-utilizing fungi in soil. Statistically there was significant difference ($P<0.005$) between counts of hydrocarbon-utilizing fungi of control soil and diesel-polluted soils and among counts of diesel-polluted soils.

Of the six fungal organisms isolated, all occurred in control soil and diesel-polluted soils except *Mucor species* which did not occur in 90ml concentration of diesel oil. The involvement of fungal organisms in degradation of petroleum hydrocarbon in nature had been extensively studied. Several species of fungi have been implicated in hydrocarbon degradation (Barth, 2003; Lliros *et al*., 2003; Chaillana *et al*., 2004; Brito *et al*., 2006; Stauffert *et al*., 2014). Molds belonging to the genera *Aspergillus, Fusarium* and *Penicillium* implicated in this study as petroleum hydrocarbon utilizers were similar to those implicated in previous research (Chaillan *et al*., 2006).

The ability of fungi to survive at the different concentrations of diesel oil showed that fungal organisms participate in the natural process of degradation of the diesel oil. How far fungi can withstand the devastitating effects of diesel oil spillage through tanker accidents in the tropical rainforest vegetation was the main purpose of this study. The implication was that pollution of the soil with diesel oil could alter ecosystem structure affecting microbial community activity and in turn destroy soil productivity leading to food scarcity. However, this study established the fact that soil with no history of oil spillage harbour fungi with the potential to degrade petroleum hydrocarbons which can be isolated and used for decontaminating diesel oil spillage in the environment. The study soil harbour significant numbers of fungi capable of degrading petroleum hydrocarbons.

Reports in literature showed that some filamentous fungi such as *Cladosporium* and *Aspergillus* participate in aliphatic hydrocarbon biodegradation whereas fungi belonging to the genera *Cullinghamella*, *Penicillium, Fusarium* and *Aspergillus* can take part in aromatic hydrocarbon decomposition (Husaini *et al*., 2008; Steliga, 2012). Similar fungi occurred in the soil of the study area. Also, numerous works devoted to research on biodegradation with the use of fungi stated that majority of filamentous fungi is unable to totally mineralize aromatic hydrocarbons; they only transform them into indirect products of decreased toxicity and increased susceptibility to decomposition with the use of bacteria (Husaini *et al*., 2008; Steliga, 2012). This research highlighted the fact that fungi isolated in the study soil could play similar role that will lead to complete mineralization of spilled diesel fuel in the soil ecosystem.

VI. CONCLUSION

Assessment of potential impact of diesel oil spillage on fungal community structure and evaluation of the potential of fungal organisms to withstand toxic effect of the diesel oil were the purpose of this research. Assessment of the problems of pollution by diesel fuel spillage into the environments due to incessant tanker accidents on Nigerian traffic highways formed the significance of the present study. Results showed significant reduction in the counts of soil heterotrophic fungi in the diesel-polluted soil compared to the control soil. The difference in heterotrophic fungal

populations of the control soil and those of the diesel-contaminated soils was taken as the effect of addition of diesel oil on the soil fungi. Heterotrophic fungi responded negatively to addition of diesel oil to the soil which means that diesel oil had depressive effect on the growth of soil fungi. In contrast, hydrocarbon-utilizing fungi responded differently to the various concentrations of diesel oil in the soil. Results showed initial reduction in counts of hydrocarbon-utilizing

ISSN: 2350-0328

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fungi in 90ml diesel-polluted soil compared to control soil, which peaked in 180ml polluted soil and decreased to lowest counts in 270ml polluted soil option. In this study it was established that fungi survived toxic effect of diesel fuel and that soil with no history of oil spillage harbour fungal organisms with the potential to utilize hydrocarbons in diesel
oil. Also, the study showed that hydrocarbon-utilizing fungi occur naturally in soil, and that 180ml volume of diesel oil to 3kg soil was the optimum concentration that stimulated optimum growth of fungal organisms with the potentials to participate in natural remediation process of the diesel fuel contaminated sites.

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