Microbial Communities found in a Diesel Contaminated Soil.

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Abstract

Bacterial and fungal populations in an uncontaminated soil, diesel contaminated soil; taken close to a diesel generator plant and artificially contaminated soil (an uncontaminated soil sample to which diesel was added). These soil samples were analysed to know their microbial population and diversity. Plate counts using plate count agar, mineral salt agar, and potato dextrose agar was used for the enumeration of total culturable heterotrophic bacteria(TCHB), hydrocarbon utilizing bacteria(HUB), hydrocarbon utilizing fungi(HUF) and total culturable heterotrophic fungi(TCHF) counts respectively. The microbial species identified includes members of the genera; *Flavobacterium, Bacillus, Pseudomonas, Citrobacter, Alcaligenes*, *Acinetobacter, Staphylococcus, Azotobacter, Micrococcus, Serratia, Corynebacterium, Aspergillus, Penicillium, Rhizopus, Fusarium, Candida, Mucor, Geotridum* and *Cladosporium.* The uncontaminated control had eleven bacterial and eight fungal genera isolated, while the contaminated and artificially contaminated had seven and four bacterial isolates respectively. The results revealed that the presence of the diesel in the soil enriches some specific microbial species; HUB and HUF, with *Bacillus* and *Pseudomonas* species of bacteria, also *Aspergillus* and *Penicillium* sp of fungi, respectively, having highest frequency of occurrence in the contaminated soils. Therefore, the present study demonstrated that the diesel contaminated soil harbours hydrocarbon utilizing bacteria and fungi populations which can be harnessed to achieve effective bioremediation of diesel oil-contaminated soil.

Keywords: Diesel, hydrocarbon utilizing fungi, hydrocarbon utilizing bacteria, microbial diversity, microbial load.

Introduction

Our environment is continually loaded with petroleum hydrocarbons as a result of oil spills arising from the development of petroleum and petrochemical industries. When oil is released into the soil environment, it undergoes a natural attenuation, the extent and speed which depend on the nature of the spilled products and prevailing local environmental conditions. Some of the processes that determine the removal of the huge amount of oil includes; leaching, volatilisation, photochemical oxidation and microbial degradation [Ladousse and Tramier, 1991].

The toxicity of crude oil or petroleum products varies greatly, depending on their composition, concentration, environmental factors and on the biological state of the organisms at the time of the spill. Different species and different stages of the life cycles of the organisms have been demonstrated to have different susceptibilities to pollution (Obire and Anyanwu, 2009). The scale of pollution depends on the quantity of oil and

the damage done to the environment. In areas where there's heavy oil pollution, there will be an immediate detrimental effect on the life of flora and fauna. Fungi and bacteria are very important agents in biogeochemical cycling and mineralization. Therefore, any substance whose presence in the environment affects these microbial activities, will also adversely affect plant growth, as well as detoxification of organic pollutants. Microbes play a very important role in predicting the impact of a particular stress on the environment by their ability to respond to these adverse conditions through a change in their numbers (Obire, 1988) and the elimination of some species types (Adeyemo *et al*; 2013).

The discovery of diesel brought a lot of relief to the world's energy requirement because of ease of sourcing and conversion. The increased need for fuel has resulted in increased production, refining, distribution and consumption, which has brought with it, its attendant problem of environmental pollution. Diesel is obtained in the fractional distillation of crude oil between 250^{0}C and 350^{0}C at atmospheric pressure. Pollution often diminishes the total microbial diversity in soil; moreover the community composition may be altered by environmental contaminants (Lynch *et al*., 2004). Several bacterial species are capable of oil hydrocarbon degradation because hydrocarbons are naturally produced by plants and microbes (Sylvia *et al*., 2005). Fungi have been shown to have great potential for degrading organic compounds, due to their spreading mode of growth and symbiotic association with plant roots that spreads to cover a large area (Adeyemo *et al*., 2013; Hosokawa *et al*., 2009). Although, bacteria are considered as more important colonizers in oil - contaminated soil than fungi. Fungi and bacteria can have a mutualistic relationship, so the presence of fungi can support bacterial growth by the structures of hyphae and mycorrhizae, and some bacteria can improve mycelial growth and so improve the growth conditions of the fungal community (Sarand *et al*., 2000). Various pathways are known for the bacterial degradation of different hydrocarbons (Sylvia *et al*., 2005).

Biodegradation is the major weathering process for middle distillates, such as the diesel oil (Mariano *et al*., 2008). However, the continuous low-level inputs of oil into the environment are rarely noticed, and may pose a serious threat to the environment as the contaminant accumulates over time. Hence, diesel hydrocarbons released into the environment create a world-wide problem of contaminated water and soil that require decontamination. Contamination along with other physical and chemical changes in soil can cause adaptive changes in the composition of the microbial community found in that soil environment (Saul *et al*., 2005; Grant *et al*., 2007). Competition within and between species influences the bacterial community composition and hence degradation of contaminants. Soil pore size also influences degradation by providing surface for colonization and because of predation that reduces bacterial biomass especially in pores that are larger than 2µm (Johnsen *et al*., 2005; Mariano *et al*., 2008). Therefore in soils where the pore size is large, there are fewer microbes available for degradation than in soils with smaller pore sizes. Bacteria and fungi have developed the ability to degrade petroleum hydrocarbons, the objectives of this study were therefore,

to isolate and identity some of the indigenous bacterial and fungal flora found in diesel oil contaminated soils, to determine if the oil encourages the growth of specific microbial population and also the microbial diversity of a chronically diesel contaminated soil, which is a soil that is continuously receiving low doses of diesel as the engine is been fuelled regularly.

Materials and Methods

Study Area and Sample Collection

Contaminated soil samples were collected using soil auger close to the generator house where diesel is use as a source of fuel at the Department of Estate, Works and Transport. Three samples were collected from the soil surface (5 – 10 cm depth) at an interval of about 1.0m apart and bulked for homogeneity. An uncontaminated soil sample was collected from the school farm (this site does not have any history of previous oil contamination) all in the River State University of Science and Technology, Port Harcourt under aseptic conditions. The samples were put into sterile polyethene bags and labelled appropriately, transported to the laboratory immediately. The diesel used was bought from Oando Filling station along Ikwerre road, close to mile 3 park, Port Harcourt.

In the laboratory, according to Chikere *et al*., (2012) 1kg of the uncontaminated soil sample was weighed and put into a sterile pot, which was contaminated by adding 40ml of diesel oil, mixed thoroughly with a sterile forceps. This was labelled as artificially contaminated soil, and was allowed to stand for 24 hours before plating out. The soil characteristics were also carried out.

Microbiological Analysis

Enumeration of total culturable heterotrophic and hydrocarbon utilizing bacteria

This was done using the spread plate count method on plate count agar after serial dilution of the soil sample using physiological saline(Chikere and Ekwuabu, 2014). While the culturable hydrocarbon utilizing bacteria (HUB) were enumerated by vapour phase transfer method using mineral salt medium Mills *et al* .,1978, as modified by Odokuma and Dickson, 2003. Colonies were counted and further purified by sub-culturing. Pure cultures were analysed biochemically and identified based on the identification in the Bergey's Manual for Determinative Bacteriology (Holt *et al*., 1994)

Enumeration of total heterotrophic and hydrocarbon utilizing fungi

Potato dextrose agar (PDA) with tetracycline added was used for isolation and enumeration of total heterotrophic fungi. The Tetracycline is added to prevent bacterial growth while selectively permitting the isolation of yeasts and moulds (Harrigan and McCance, 1990; Obire and Anyanwu, 2009). For the HUF, vapour phase transfer using mineral salts medium (MSM) agar composition of Mills *et al*., (1978) as modified by Okpokwasili and Okorie (1988) was used. With Tetracycline added to prevent bacterial growth. The microscopic examination of the isolates was carried out by needle mount method by Cheesebrough, (1991). Isolates were identified on the basis of their cultural

characteristics, spores and vegetative mycelium according to Barnett and Hunter(1972, Larone, 1995; Doggett, 2000).

Physicochemical and Particle Size Analysis

Conductivities were determined using a Jenway 4010 conductivity meter. The pH was determined using a pH meter (Jenway pH meter 3015 model).The following physicochemical parameters were carried out: percentage nitrogen, percentage potassium, percentage phosphorous (APHA, 1989) and total organic carbon was done based on the method used by Craze (1990). The soil samples were also characterized into sand, silt and clay, as determined by the method of Ketler *el al*., 2001.

Results

The presence of these microorganisms was observed from the enumeration of THB, HUB, THF and HUF showed in Figure 1. From the uncontaminated soil samples, the following bacteria genera were isolated*; Bacillus, Alcaligenes, Serratia, Pseudomonas, Corybacterium, Micrococcus, Citrobacter, Flavobacterium, Azotobacter, Staphylococcus* and *Acinetobacter*. This uncontaminated soil sample also served as the control. The chronically contaminated soil contained the following genera*; Flavobacterium 16.0%, Bacillus 28.0%, Pseudomonas 20.0%, Citrobacter 12.0%, Alcaligenes 12.0%*, *Staphylococcus* 4% and *Acinetobacter 8.0%*. While the artificially contaminated soil had the following genera; *Pseudomonas* 29.42%*, Flavobacterium* 23.53%*, Acinetobacter* 5.88% and *Bacillus* 41.17%. The fungi genera isolated from the uncontaminated soil includes: *Aspergillus, Penicillium, Fusarium, Rhizopus, Cladosporilum, Candida, Mucor* and *Geotridum*. For contaminated soil the following genera were observed: *Aspergillus, Penicillium, Rhizopus, Fusarium, Geotridum* and *Cladosporium,* while in the artificially contaminated the following genera were also isolated: *Penicillium, Aspergillus, Rhizopus* and *Fusarium*. Most of these isolates have been reported to have the metabolic capacity to use diesel, as a substrate and degrade hydrocarbons in the process (Adeyemo *et al*., 2013). The total culturable heterotrophic Bacteria counts, ranged between 2.0 X 10^5 to 7.2 X 10^5 cfu/g, HUB ranged between; 2.4 X 10^3 to 5.6 X 10^3 cfu/g, TCHFC(total culturable heterotrophic fungal counts) ranged between; 1.4 X 10^3 to 4.4 X 10^3 SFU/g, HUF; 1.6 X 10^2 to 3.2 X 10^2 sfu/g for contaminated soil. For uncontaminated soil, the TCHBC ranged between 3.3 X 10^6 to 8.3 X 10^6 cfu/g, TCHFC; 1.6 X 10^4 to 6.4 X 10^4, HUB; 1.1 X 10^1 to 3.0 X 10^1 cfu/g, HUF, 0 to 1.5 X 10^1 sfu/g, while the artificially contaminated soil samples have values that ranged between 2.3 X 10^4 to 6.4 X 10^4 cfu/g of TCHBC, HUB 1.2 X 10^3 to 4.4 X 10^3 cfu/g, TCHFC; 1.8 X 10^2 to 2.6 X 10^2 sfu/g and HUF 1.0 X 10^2 to 3.0 X 10^2 sfu/g(spore forming unit per gram). The pH of the soil samples ranged between 5.7 to 6.6, 2,140 – 2, 500µs/g. The values of the physicochemical parameters of the soil samples used are displayed on Table 1.

Table 1: Physicochemical characteristics of the soil samples used in the study

Parameters	Uncontaminated	Contaminated	Artificially Contaminated
pH	6.6	5.7	5.85
Electrical Conductivity(µs/g)	2,140	2,500	2,541
% Nitrogen(mg/kg)	1	0.2	0.1
% Potassium(mg/kg)	0.51	0.45	0.4
% Phosphorous(mg/kg)	8.05	7.1	7.13
Total organic carbon(%)	0.23	0.35	0.32
Particle size distribution			
% Coarse sand	75	74.4	73.2
% Slit	15	19.6	14
% Clay	10	6	12.8

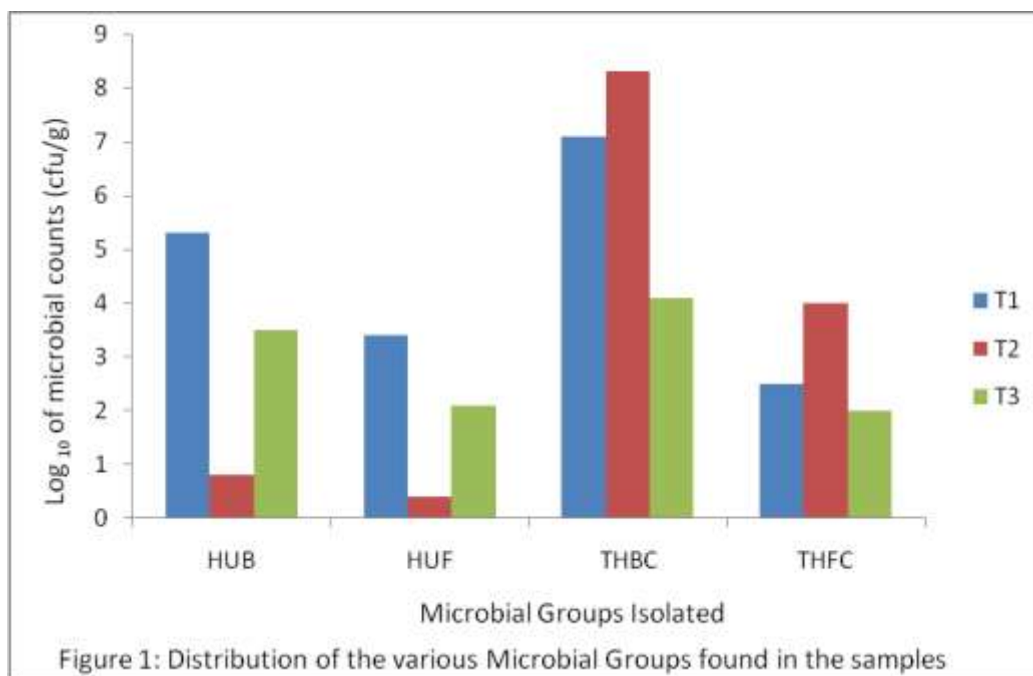

Figure 1: Distribution of the various Microbial Groups found in the samples

Table 2: Fungi Genera Isolated from the Soil Samples

Tentative Isolates	T1	T2	T3
Aspergillus	*	*	*
Mucor	Ab	*	Ab
Rhizopus	*	*	*
Penicillin	*	*	*
Geotridum	Ab	*	Ab
Fusarium	*	*	*
Cladosporium	*	*	Ab
Candida	Ab	*	Ab

Key: T1 - contaminated soil * = Present
Ab = not isolated
T2 - uncontaminated soil
T3 - artificially contaminated soil

Table 3: Bacterial Genera isolated from the Soil Samples

Isolates	Uncontaminated soil	Contaminated soil	Artificially Contaminated soil
Bacillus sp	+	+	+
Pseudomonas sp	+	+	+
Citrobacter sp	+	+	-
Alcaligenes sp	+	+	-
Flavobacterium sp	+	+	+
Micrococcus sp	+	-	-
Corybacterium sp	+	-	-
Serratia sp	+	-	-
Azotobacter sp	+	-	-
Acinetobacter sp	+	+	+
Staphylococcus sp	+	+	-

Key: + = isolated - = not isolated

Discussion

From the results the soil samples have a sandy- clay texture; uncontaminated soil had higher bacterial and fungal population and diversity when compared with chronically and artificially contaminated soil. A total of eleven bacterial and eight fungal genera were isolated which includes; *Bacillus, Alcaligenes, Serratia, Pseudomonas, Corybacterium, Micrococcus, Citrobacter, Flavobacterium, Azotobacter, Staphylococcus* and *Escherichia*. The following for fungi; *Aspergillus, Penicillium, Fusarium, Rhizopus, Cladosporilum, Candida, Mucor* and *Geotridum.* While the artificially contaminated soil had the least bacteria and fungi counts and diversity, four genera each respectively. In all contaminated soil samples *Bacillus* sp had the highest frequency of isolation in all soils, with 33.17% in contaminated, while the artificial contamination had 41.17%. Adeyemo *et al*., 2013, in their work also observed high frequency of occurrence for *Bacillus* sp. They also isolated some genera like; *Pseudomonas*, *Micrococcus, Aspergillus*. With the exception of the following genera which were not isolated in this work; *Klebsiella, Lactobacillus, Neisseria* and the following fungal genera; *Beauveria, Gibellula, Humicola* and *Cumminghamilla*. Obire and Anyanwu (2009), also made a similar observation, that there is a decrease in microbial load and diversity in crude oil contaminated soil. Okerentugba and Ezeronye (2003), in their work also shown that *Penicillium* sp and *Aspergillus* sp were able to degrade hydrocarbons especially when used in single cultures. In the Niger Delta regions of Nigeria, Obire (1988) found several species of oil-degrading aquatic fungi in the genera of *Aspergillus niger*, *Aspergillus terreus, Fusarium* sp., *Penicillium* sp which were also observed in this work. Mariano *et al*., (2008), have reported *Bacillus cereus, Pseudomonas aeruginosa*, and *Staphylococcus* sp as capable of using diesel as carbon source. The ability of *Bacillus* sp to withstand high concentrations of diesel oil may be due to the possession of the capacity to mineralize diesel. The isolation of increased number of hydrocarbon utilizing microorganisms from petroleum-polluted environment is taken as evidence that those organisms are the active hydrocarbon degraders in that environment. The microbial group capable of survival in such environment are those that have developed enzymatic and physiological responses that enable them to use the hydrocarbon present as substrates (Chikere and Ekwuabu, 2014). When these organisms use the hydrocarbon as substrate for growth, they release extra cellular enzymes and acids which are capable of breaking down the hydrocarbon molecule, by reducing the long chains of hydrogen and carbon, hence converting the hydrocarbon into simpler forms or products that can be absorbed by the organisms for nutrition and growth. These organisms that are capable of growth on this substrate, use the energy produced to synthesize cellular components, releasing carbon (iv) oxide, water and energy used for the production of biomass(Adekunle and Adebambo, 2007). Batelle (2000), in his study observed that fungi were better degraders than the other bioremediation techniques including bacteria. And unlike other groups of microorganisms, filamentous fungi do not show preferential degradation for any particular chain lengths of alkanes. Fungal mycelia are able to penetrate oil and increase the surface area available for degradation by other

microbes. These organisms (Fungi) are known to be aerobic and can also grow under environmentally stressed conditions such as low pH and poor nutrient status, where bacterial growth might be limited. The findings from this study have shown that there's a reasonably number of active indigenous hydrocarbon utilizing organisms in the oil contaminated sites.

Conclusion

Since bacteria and fungi are ubiquitously distributed in the environment, they are readily isolated from oil-contaminated environments. This indicates that they are able to use the hydrocarbon as a source of carbon and energy, hence play an important role in the degradation of oil spill in the environment. The presence of the diesel in the soil resulted in a decrease in microbial population and diversity, resulting in the selective increase in population of HUB and HUF and reduction of species diversity by completely eliminating certain species. However, since these organisms play an important role in nutrient cycling, the presence of the oil may interfere with some metabolic activities carried out by these organisms, which may in turn affect performance of the ecosystem or the ecological balance.

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