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Effects of Long-Term Kerosene Spillage on Heterotrophic Microorganisms in Soil from Niger Delta, Southern Nigeria

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ABSTRACT: Kerosene contaminated soil was obtained from four different locations in Calabar while pristine soil served as control. Bacterial species isolated from kerosene contaminated soil samples included species of *Bacillus, Pseudomonas, Micrococcus* and *Serratia* while bacteria isolated from pristine soil samples comprised of species of *Streptococcus, Salmonella, Escherichia coli, Staphylococcus aureus, Bacillus, Serratia, Micrococcus* and *Pseudomonas.* Similar fungal species which included species of *Aspergillus, Penicillium, Mucor, Rhizopus,* and *Fusarium* were isolated from both chronic kerosene contaminated and pristine soil samples. There was no significant difference ($p \geq 0.05$) in heterotrophic bacteria (HTB), fungal (HTF) and kerosene utilizing fungal counts (KUF) between chronic kerosene contaminated and pristine soil samples. However, there was significant difference ($p \leq 0.05$) between kerosene utilizing bacteria (KUB) between chronic kerosene contaminated and pristine samples. This revealed that long-term kerosene slippage had a selecting effect on soil bacteria as opposed to soil fungal. @JASEM

Keywords: Chronic kerosene contamination; pristine soil; slippage; isolated; bacterial fungal.

The Niger Delta area of Southern Nigeria is vulnerable to hydrocarbon pollution because increasing crude oil exploration and exploitation activities in this area which has led to widespread contamination of most creeks, rivers, swamps, soils, underground water and costal regions. (Okpokwasili and Odokuma, 1990; Nnubia and Okpokwasili, 1992; Odokuma and Okpokwasili, 1992; Okpokwasili and Nnubia, 1995).

The oil industry is the main stay of Nigerian economy thus, the vigorous extraction, transportation and storage of crude oil, subjects the Niger Delta region to frequently contamination by hydrocarbon. Kerosene as a refined hydrocarbon product is extensively utilized as a source of energy for both domestic and sub-industrial activities due to its relative availability, affordability and applicability.

Thus, it's vigorous commercialization and usage (utility) results in constant spillage in minute to appreciable quantities. This contamination present serious environmental effect on physiological processes, population and behavioural profiles of microorganisms (Teh, 1974; Teh and Lee, 1974; Sander *et al.,* 1985; Okpokwasili and Odokuma, 1994).

Exposure of soil ecosystem to hydrocarbon contamination (such as kerosene spillage) may cause a selective inhibition on certain members of the microbial biocenosis, while increasing relative population of those microorganisms able to use hydrocarbon for growth (Bossert and Bartha, 1984; Rosenberg, 1991). Limited in formation exists on the pollution status of kerosene on the environment and its effects on the microbial communities in the soil. The objectives of this study therefore, were to investigate the effects of kerosene on soil bacteria and fungi and to enumerate, isolate and identify bacteria and fungi associated with kerosene-contaminated soil and compare them with pristine soil samples.

MATERIAL AND METHOD

*Sample Collection***:** Kerosene contaminated soil samples(A-D) were collected from four kerosene surface tank location within Calabar, Cross River State, Southern Nigeria while pristine soil (sample PC) from fallow patch of land around the Department of Microbiology, University of Calabar, Calabar. Surface soil samples (0-30cm depth) were collected using auger borer.

*Preparation and incubation of soil samples***:** The soil samples obtained for analysis were bulked, air dried and passed through a 2mm sieve to remove the coarse fragments. The soil samples were identified as sandy-loam. Microbiological analyses were carried out immediately.

*Enumeration of total heterotrophic bacterial (HTB) count and hydrocarbon utilizing bacterial (KUB) counts***:** *Total heterotrophic bacterial counts:* The total heterotrophic bacterial plate counts were performed using the pour plate method on nutrient agar (LAB 8). The plates which were in triplicates were incubated at 28^{o}C. Bacterial colonies were counted after 24 hours of incubation and reported as heterotrophic bacterial (HTB) count.

*Kerosene utilizing bacterial counts***:** Kerosene utilizing bacteria (KUB) were enumerated using pour

plate technique on mineral salt medium (Zajic and Supplision, 1972) pH 7.0 incoporated with 50ug/ml of nystatin to inhibit fungal growth. Vapour phase technique was employed by placing sterile Whatman No. 1 filter paper saturated with 0.5mls of kerosene and aseptically unto the lid of the plate. The plates which were in triplicates were inverted and incubated at 28^{o}C for 4-7 days. After incubation, counts of average colonies from the triplicate were recorded as kerosene utilizing bacterial (KUB) counts.

Enumeration of total heterotrophic fungi (HTF) counts and kerosene utilizing fungi (KUF) counts:
Total heterotrophic fungal count: Total heterotrophic fungal (HTF) counts were performed using pour plate technique on sabouraud dextrose agar (ANTEC). The plates which were in triplicates were incubated at 28^{o}C. Fungal colonies were counted after 72 hours of incubation and reported as heterotrophic fungal (HTF) count.

*Kerosene utilizing fungal counts***:** Kerosene utilizing fungi (KUF) were enumerated using pour plate technique on mineral salt medium (Zajic and Supplision, 1972) pH 5.8 incoporated with 50ug/ml of streptomycin and 30ug/ml of penicillin to inhibit bacterial growth. Vapour phase technique was employed by placing sterile Whatman No. 1 filter paper saturated with 0.5mls of kerosene and aseptically unto the lid of the plate. The plates which were in triplicates were inverted and incubated at 28^{o}C for 4-7 days. After incubation, counts of average colonies from the triplicate were recorded as kerosene utilizing fungi (KUF) count.

Biochemical identification and characterization of bacterial isolates: Pure bacterial isolates obtained by streaking were stored at 4^{o}C on agar slants which were re-inoculated for growth of individual colonies which would be identified using morphological and biochemical techniques using the taxonomic scheme of Bergey's manual of determinative bacteriology (Holt *et al.,* 1994).

*Identification of fungal isolates***:** Macroscopic and microscopic examinations was carried out for observation of colonial morphology, colour, texture, shape and surface appearance, cultural characteristic, asexual and sexual reproduction structure like sporangia, conidial head, arthrosphores, the vegetative mycelia, septate or non-septate (Alexopoulous and Sun, 1962; Banette and Hunter, 1972). Microscopic examination of the mould was carried out using needle mount method. A small portion of each colony was picked with sterile needle and teased out in a drop of clean distilled water on a microscopic slide. Slides were prepared using methylene blue as the primary stain. This was covered with clean cover slips and examined under microscopic starting with a lower power objective (X10), then higher power (X40), for magnification (Wemedo *et al.,* 2002).

Statistical analysis: Collected data was subjected to analysis of variance (ANOVA) using completely randomized design (CRD). Means were separated using least significant difference (LSD) test.

RESULTS AND DISCUSSION

Bacterial Counts: Heterotrophic bacterial counts (HTB) in pristine soil samples was 1,97±0.4x10^{5} (CFU/g) colony forming unit per gram of soil sample while kerosene utilizing bacteria (KUB) was 3.77 ± 0.15 x 10^{2} CFU/g. In chronic kerosene contaminated soil samples the heterotrophic bacterial (HTB) count ranged from 1.74 ± 0.3x10^{4} CFU/g to 2.13 ± 0.6x10^{5} CFU/g while kerosene utilizing bacteria (KUB) range from 6.37 + 0.7 x 10^{3} CFU/g to 7.00 ± 0.12 x 10^{2} CFU/g (Table 1).

Table 1 Heterotrophic bacterial and kerosene utilizing bacterial counts in chronic kerosene contaminated and pristine soil samples.

Samples	HTB (CFU/g)	KUB (CFU/g)
PC	1.97 ± 0.4 x 10^{5}	3.77 ± 0.15 x 10^{2}
A	2.00 ± 0.3 x 10^{5}	6.37 ± 0.7 x 10^{3}
B	2.13 ± 0.6x 10^{5}	6.93 ± 0.13 x 10^{2}
C	1.74 ± 0.3 x 10^{4}	6.60 ± 0.1 x 10^{2}
D	2.08 ± 0.4 x 10^{5}	7.00 ± 0.12 x 10^{2}

KEY: A-D = Kerosene contaminated soil samples, PC = Pristine soil sample, HTB = Heterotrophic bacterial count, KUB = Kerosene utilizing bacterial count, (CFU/g) = Colony forming unit per gram.

Fungal Count: Heterotrophic fungal count (HTF) in the pristine soil samples was 5.67 ± 0.87x10^{3} CFU/g while kerosene utilizing fungal count (KUF) was 3.63 ± 0.89x10^{3} CFU/g. In chronic kerosene contaminated soil samples, the heterotrophic fungi (HTF) count ranged from 4.63 ± 0.9x10^{4} CFU/g to 5.13 ± 0.35x10^{3} CFU/g while kerosene utilizing fungi (KUF) ranged from 3.70 ± 0.58x10^{3} CFU/g to 4.10 ± 0.21x10^{2} CFU/g (Table 2).

Table 2 Heterotrophic fungal and hydrocarbon utilizing fungal count in chronic kerosene contaminated and pristine soil sample.

Samples	HTF (CFU/g)	KUF (CFU/g)
PC	$5.67 \pm 0.87 \times 10^3$	$3.63 \pm 0.89 \times 10^3$
A	$5.10 \pm 0.32 \times 10^3$	$4.10 \pm 0.21 \times 10^3$
B	$4.63 \pm 0.9 \times 10^4$	$3.97 \pm 0.88 \times 10^3$
C	$5.13 \pm 0.35 \times 10^3$	$4.07 \pm 0.15 \times 10^2$
D	$5.10 \pm 0.32 \times 10^3$	$3.70 \pm 0.58 \times 10^3$

KEY: A-D = Kerosene contaminated soil samples, PC = Pristine soil sample, HTF = Heterotrophic fungal count, KUF = Kerosene utilizing fungal count, (CFU/g) = Colony forming unit per gram.

The microbial community structure plays a critical role in the biogeochemical cycling in the soil where they are responsible for biodegradation and other metabolic activities. The microbial community structure is altered in various ways when petroleum products such as kerosene are spilled on the soil. In this study, it was observed that bacterial abundance was not altered in the presence of the kerosene contaminant.

Table 3 Bacteria species isolated from chronic kerosene contaminated samples A-D and pristine soil sample PC

Isolates	Bacterial Species
ABI	*Pseudomonas spp*
AB2	*Bacillus spp*
AB3	*Micrococcus spp*
AB4	*Serratia spp*
BB1	*Serratia spp*
BB2	*Pseudomonas spp*
BB3	*Bacillus spp*
BB4	*Micrococcus spp*
CB1	*Pseudomonas spp*
CB2	*Bacillus spp*
CB3	*Micrococcus spp*
DB1	*Bacillus spp*
DB2	*Pseudomonas spp*
PCB1	*Escherichia coli*
PCB2	*Salmonella spp*
PCB3	*Pseudomonas spp*
PCB4	*Bacillus spp*
PCB5	*Micrococcus spp*
PCB6	*Streptococcus spp*
PCB7	*Staphylococcus aureus*

KEY: AB = Bacterial isolates from sample A, BB = Bacterial isolates from sample B, CB = Bacterial isolates from sample C, DB = Bacterial isolates from sample D, PCB=Bacterial isolates from sample PC

Statistically analysis revealed that the difference in heterotrophic bacterial count between kerosene contaminated and pristine soil samples was not significant ($p \geq 0.05$). However, it was observed that there was a reduction in bacterial diversity in kerosene contaminated soil samples. A total of eight different bacterial species which included *Staphylococcus aureus, Escherichia coli, Micrococcus spp, Salmonella spp, Serratia spp, Streptococcus spp, Pseudomonas spp* and *Bacillius spp* were isolated from pristine soil samples while only four of these bacteria mostly hydrocarbon utilizing bacterial which included *Pseudomonas spp, Serratia spp, Bacillus spp* and *Micrococcus spp* were isolated from kerosene contaminated soil samples (Table 3). Song *et al.,* (1990) reported similar results that introduction of crude oil into the soil resulted in the selection of hydrocarbonclastic bacteria reaching approximately the 100% of total micro-organism.

They suggested that these hydro-carbon utilizers can make up the whole community. It was also observed that there was no significant difference ($p \geq 0.05$) in the fungal abundance fungal communities between kerosene contaminated and pristine soil samples. There was also no alteration in fungal diversity present in both kerosene contaminated and pristine soil samples as similar fungal species such as *Penicillium, Aspergillus, Fusarium, Rhizopus and Mucor* were isolated from both sample types (Table 4).

Table 4: Fungal species isolated from chronic kerosene contaminated soil sample A-D and pristine soil sample PC

Isolates	Fungal Species
AFI	*Aspergillus spp*
AF2	*Penicillium spp*
AF3	*Mucor spp*
BF1	*Aspergillus spp*
BF2	*Penicillium spp*
CF1	*Penicillium spp*
CF2	*Rhizopus spp*
CF3	*Mucor spp*
CF4	*Aspergillus spp*
CF5	*Fusarium spp*
DF1	*Rhizopus spp*
DF2	*Mucor spp*
PCF1	*Penicillium spp*
PCF2	*Mucor spp*
PCF3	*Rhizopus spp*
PCF4	*Fusarium spp*
PCF5	*Aspergillus spp*

KEY: AF = Fungal isolates from sample A, BF = Fungal isolates from sample B, CF = Fungal isolates from sample C, DF = Fungal isolates from sample D, PCF=Fungal isolates from sample PC

Statistical analysis using least significant difference test showed that there was significant difference ($p \leq 0.05$) in kerosene utilizing bacterial (KUB) counts between kerosene contaminated and pristine soil samples. The reason for higher counts in kerosene contaminated soil may be due to the presence of residual kerosene which enhances the growth of kerosene utilizing bacteria as against comparison to pristine soil (Ijah and Antai, 2003). However, there was no significant difference ($p \geq 0.05$) in kerosene utilizing fungal (KUF) counts between kerosene contaminated and pristine soil samples.The

percentage occurrence of bacterial species from both kerosene contaminated and pristine soil samples. *Pseudomonas* spp, had a percentage occurrence of 25.42%, *Staphylococcus aureus* 5.09%, *Streptococcus spp* 3.39%, *Bacillus spp* 25.42%, *Escherichia coli* 5.09% *Micrococcus spp* 22.03%, *Serratia spp* 8.48% and *Salmonella spp* 5.09% (Table 5).While, the percentage occurrence of fungal species from both kerosene contaminated and pristine soil samples. *Aspergillus spp* 28.95%, *Fusarium spp* 7.89%, *Penicillium spp* 26.32%, *Rhizopus spp* 7.89%, and *Mucor spp* 28.95% (Table 6).

Table 5 Percentage of occurrence of bacterial isolates from chronic kerosene contaminated and pristine soil samples

ORGANISMS	SAMPLE PC	SAMPLE A	SAMPLE B	SAMPLE C	SAMPLE D	T	%
Pseudomonas spp	3 (20%)	3 (20%)	3 (20%)	3 (20%)	3 (20%)	15	25.42%
Staphylococcus aureus	3 (100%)	-	-	-	-	3	5.09%
Streptococcus spp	2 (100%)	-	-	-	-	2	3.39%
Bacillus spp	3 (20%)	3 (20%)	3 (20%)	3 (20%)	3 (20%)	15	25.42%
Escherichia coli	3 (100%)	-	-	-	-	3	5.09%
Micrococcus spp	2 (15.39%)	3 (23.08%)	3 (23.08%)	2 (15.39%)	3 (23.08%)	13	22.03%
Serratia spp	1 (20%)	2 (40%)	2 (40%)	-	-	5	8.48%
Salmonella spp	3 (100%)	-	-	-	-	3	5.09%
						59	100%

KEY: A-D = Chronic kerosene contaminated soil sample, PC = Pristine soil sample, T = Total

TABLE 6 Percentage occurrence of fungal isolates from chronic kerosene contaminated and pristine soil samples

ORGANISMS	SAMPLE PC	SAMPLE B	SAMPLE C	SAMPLE D	T	%
Aspergillus spp	3 (27.27%)	2 (18.18%)	3 (27.27%)	-	11	28.95%
Fusarium spp	2 (66.67%)	-	1 (33.33%)	-	3	7.89%
Penicillium spp	3 (30%)	3 (30%)	2 (20%)	-	10	26.32%
Rhizpous spp	1 (33.33%)	-	1 (33.33%)	1 (33.33%)	3	7.89%
Mucor spp	3 (27.27%)	-	2 (18.18%)	3 (27.27%)	11	28.95%
					38	100%

KEY *A-D = Chronic kerosene contaminated soil sample, PC = Pristine soil sample, T = Total*

In conclusion, this study shows that kerosene can serve as a selective agent against certain members of the bacterial community in the soil. The response of the microbial community as indicated by differences in diversity between chronic kerosene contaminated and the pristine soil samples is an indicator of intrinsic bioremediation potential i.e. microorganisms present in chronic kerosene contaminated soil samples possess the ability to metabolize on the hydrocarbon waste and hence, can facilitate bioremediation.

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