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VARIATION IN MICROBIAL DENSITY AND PHYSICO-CHEMICAL PROPERTIES OF KEROSENE-POLLUTED SOIL

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ABSTRACT

Microbial population density, pH, total nitrogen, organic carbon and heavy metal concentrations were investigated in kerosene polluted tropical garden soil. The soil was polluted with kerosene to achieve 1%, 5%, 10% and 20% pollution levels. Heterotrophic and kerosene utilizing bacteria, fungi and actinomycetes were enumerated in the unpolluted (control) and polluted soil at four weeks intervals. pH, total nitrogen, organic carbon and C:N ratio were also determined in the unpolluted and polluted soil at eight weeks intervals after pollution. Control and kerosene-polluted soil samples were analyzed for heavy metal after 24 weeks. Microbial population densities varied over the duration and with levels of pollution, indicating that kerosene is a source of carbon and energy for microbial growth. Species of *Pseudomonas, Flavobacterium, Serratia, Acinetobacter, Bacillus, Micrococcus, Aspergillus, Penicillium, Rhizopus, Fusarium, Mucor* and *Streptomyces* persisted over the duration of study and were isolated from polluted soil. pH and total nitrogen varied significantly ($P<0.05$) over the duration of pollution. C:N ratio increased steadily and varied significantly ($P<0.05$) over time and with pollution levels, suggesting the unsuitable nature of kerosene-polluted soil for agriculture except after bioremediation. Values of Zn in the polluted soils were greater than that of the control soil samples after 24 weeks of pollution, indicating metal pollution.

KEYWORD: Kerosene pollution of soil, Heavy metals, Microbial density variability, Carbon-nitrogen ratio, Tropical garden soil

INTRODUCTION

Environmental pollution is perceived generally as a problem of past and present century. Crude oil and widely used petroleum products such as kerosene, diesel and petrol are the common pollutants. It is estimated that each year more petroleum products spill on land than in the marine environment (Bartha, 1986). Also, large quantities of petroleum have been reported to enter the environment mostly from the land-base sources and toxicity have been related to their volatility, solubility and the presence of lower molecular weight components (Schmitt, 2000).

In carrying out their important role of soil transformation of organic compounds, soil microbes also break down pollutants and undertake recycling process that maintains the equilibrium of these elements in the food web Despite the ability of these microbes to breakdown pollutants released into the soil environment, a greater number of soil biota which cannot withstand the environmental stress posed by the pollutants are destroyed and this disrupts natural biogeochemical cycling carried out by them (Gadd, 1992; Deni and Penninckx, 1999). However, the ability of some of the microorganisms to survive and grow in the presence of pollutant provides the basis for the bioremediation of polluted sites.

The rate of the microbial degradation of petroleum hydrocarbon in soils is affected by physicochemical and biological parameters including the numbers and species of microorganisms present; the conditions for microbial degradation activity (e.g. presence of nutrient, oxygen, moisture content and temperature); the quality, quantity and bioavailability of the contaminants, the soil characteristics (Margesin and Schinner, 1997) and soil heavy metal concentrations (Kuo and Gethener, 1996; Said and Lewis, 1991). Various modification of bioremediation such as biostimulation (addition of agricultural fertilizer) and bioaugmentation (addition of hydrocarbon utilizing microbes) have been employed to enhance petroleum hydrocarbon degradation rates in soil (Lee *et al.*, 1993; Odokuma and Dickson, 2003). For biostimulation, it is the carbon: nitrogen ratio value of the soil that gives some idea on the quantity of fertilizer to be added.

Kerosene spillage on land has not often been reported in Nigeria but sparse spill occur on land around surface tank facilities, which serve as retail outlets for local consumers. The need to investigate the effect of kerosene on the soil pH, carbon, nitrogen, microbial population and possibility of added heavy metal arises as these lands in future may be used for agricultural purposes.

MATERIALS AND METHODS

Kerosene

Four litres of kerosene was purchased from Total filling station along Calabar road into sterile 2.5 litres glass bottles. Specific gravity (SG) of the kerosene was determined using hydrometer. This was done to ascertain that the SG was within the range of uncontaminated kerosene. The known SG was used to calculate the quantity of kerosene in milliliters that were equivalent to 50g, 250g, 500g and 1000g.

Soil samples

Surface and subsurface soil samples were collected from five different locations within the University of Calabar botanical garden by excavation with spade. Approximately 20g of soil sample was collected aseptically from the excavated soil. The sample was stored at 4°C and microbial analysis performed within 24 hours to avoid dramatic changes in the microbial population. The samples were mixed to obtain a composite sample. Five kilograms of this sample was weighed into perforated polythene bags. Pollution levels of 1%, 5%, 10%, and 20% were achieved by adding 50g, 250g, 500g and 1000g of kerosene respectively, to each 5kg of soil sample and mixed three times/day for two days to allow for even distribution of kerosene in the soil.

Microbial analysis

Soil (10g) was weighed out and added to 90 ml of sterile buffer solution (0.65g K_2HPO_4, 0.35g KH_2PO_4, 0.10g $MgSO_4 \cdot 7H_2O$, 1.0 mL Tween 80, 1.0 L distilled water)(Margesin and Schinner, 1997) contained in a stoppered 200ml volumetric flask with glass beads. This was gently agitated intermittently five times to extract adsorbed microbes from soil grains. From the initial dilution, aqueous solution representing ten-fold dilutions were prepared.

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Plating procedures

Dilutions (10^{-1}-10^{-6}) were plated in triplicates on nutrient agar, Martin's medium (Parkinson, 1994), sodium caesinate medium (ASIRC, 2002) and mineral salts medium (Zajic and Supplison, 1972). For actinomycetes count, dilutions to be plated were pretreated and decontaminated by heating for 6 minutes at 55°C (Pirouz *et al.*, 1999).

Enumeration of heterotrophic microorganisms

The total heterotrophic count was performed using pour plate technique (Tournas *et al.*, 2000). For bacterial count, nutrient agar was supplemented with antifungal agent (50µg/ml of nystatin), while for heterotrophic fungal counts martin's Medium supplemented with antibacterial agents (50µg/ml of streptomycin and 30µg/ml of penicillin) was used. Sodium caesinate medium supplemented with 50µg/ml of nystatin and 30µg/ml of tetracycline was used for the enumeration of heterotrophic actinomycetes. All plates were incubated at room temperature in the dark and heterotrophic bacteria, fungi and actinomycetes count recorded after 24 hour, 72 hours and 14 days incubation, respectively.

Enumeration of kerosene utilizing microorganisms

Kerosene utilizing microorganisms in the soil were enumerated using spread-plate technique (Tournas *et al.*, 2000). 0.1ml of soil dilutions (10^{-1}-10^{-2}) were inoculated onto mineral salt agar supplemented with different antimicrobial agents as mentioned above for selective enumeration of kerosene utilizing bacteria (KUB), fungi (KUF) and actinomycetes (KUA), respectively. Sterile Whatman No. 1 filter paper was saturated with 0.5ml of kerosene. This was placed aseptically onto the inside lid of the plate, and taped round with masking tape. This kerosene became the sole source of carbon and energy for the growth of microorganisms through vapor phase transfer. The plates were prepared in triplicates, inverted and incubated at room temperature. KUB and KUF counts were recorded after 4 to 7 days incubation while KUA count was recorded after 14 days incubation.

Characterization and identification of microorganisms from kerosene polluted soil.

Identification of microbial isolates was based on their morphological and biochemical characteristics as described by Buchanan and Gibbons (1974) and Domsch and Gams (1972).

Soil particle size analysis

Particle size distribution analysis was determined by standard Bouyoucos-type hydrometer method as described by Gee and Bauder (1986).

Soil pH

The actual acidity of the soil (in distilled water) was determined by adding 10g of air dried and sieved soil in 25ml distilled water. pH was measured as described by Alef and Nannipieri (1995).

Total nitrogen

Total nitrogen was estimated by macro-Kjeldahl digestion method (Juo, 1979)

Organic Carbon

This was determined using modified Walkley-Black method (Alan and Nannipieri, 1995)

Heavy metal analysis

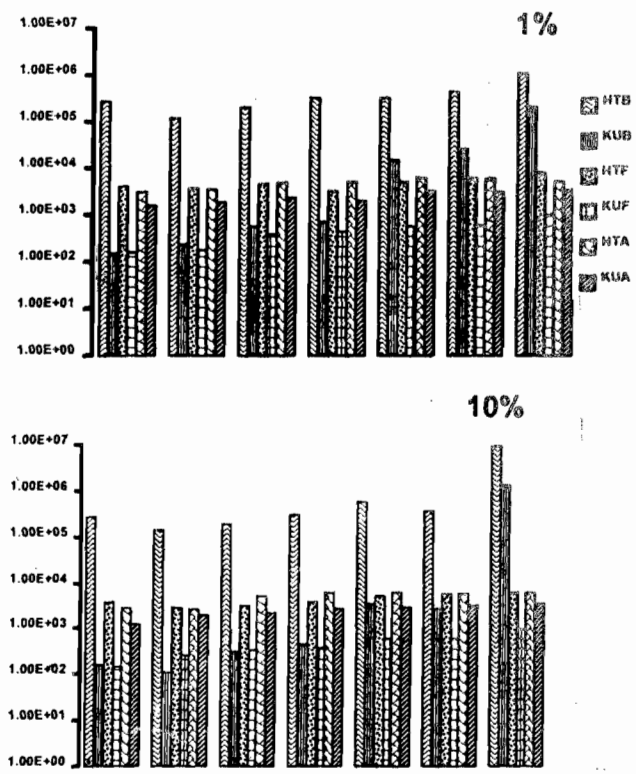
This was carried out by aqua regia digestion method adopted by Etesin (2002). After digestion, heavy metals analysis was performed by atomic absorption spectroscopy using UNICAM-939/959 model (Wavelength range: 200-1000nm) of atomic absorption spectrophotometer (Offenbach, Germany). The Heavy metals determined were Lead (Pb) Chromium (Cr), Zinc (Zn), Copper (Cu), Nickel (Ni), Manganese (Mn), Cadmium (Cd), Cobalt (Co) and Iron (Fe).

Analysis of data

Data were analyzed with statistical software. Correlation analysis to show the relationship between microbial population and duration of pollution was performed using SPSS version 10.0 (SPSS Inc. USA), while a two-way analysis of variance to establish the effect of pollution levels and duration on the data obtained was carried out using Microsoft EXCEL 2003(Microsoft Corporation. USA)

RESULTS

Microbial counts at different levels of kerosene pollution are presented in Figure 1. Heterotrophic bacteria (HTB), heterotrophic fungi (HTF) and heterotrophic actinomycetes (HTA) counts before pollution and in all levels of pollution were in the range of 10^3-10^7 colony-forming units (CFU/g) while kerosene utilizing bacteria (KUB), kerosene utilizing fungi (KUF) and kerosene utilizing actinomycetes (KUA) were in the range of 10^3-10^5 CFU/g. In all the levels of kerosene pollution, there were positive correlations between the microbial population and duration of pollution (Table 1). KUF and KUA populations showed significant correlation ($P< 0.01$) in all the levels of pollution. HTA population showed positive significant correlation ($P<0.05$ and $P<0.01$) in 10% and 20% levels of kerosene-polluted soil.



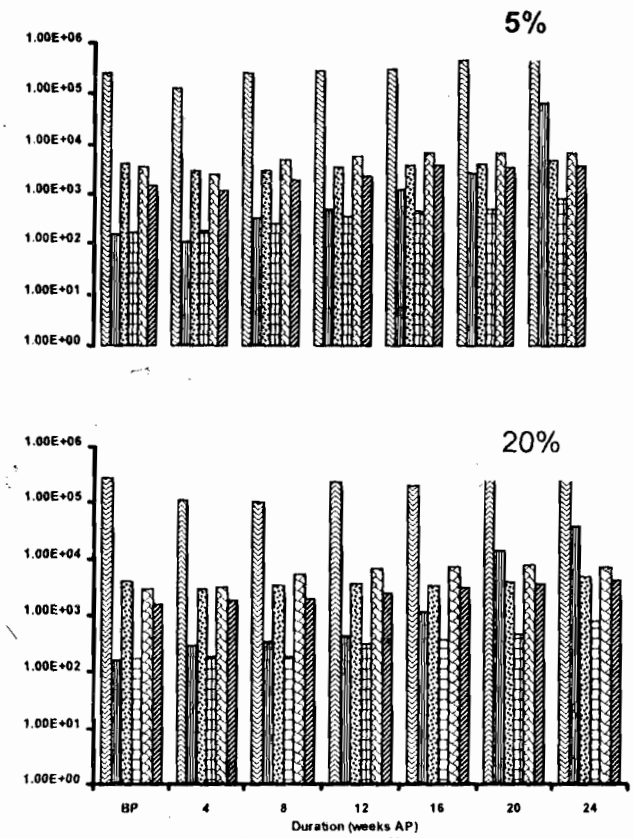


Figure 1: Microbial counts at different levels of kerosene pollution over time

(HTB= Heterotrophic Bactena, KUB= Kerosene Utilizing Bacteria, HTF=Heterbtrophic Fungi, KUF= Kerosene Utilizing Fungi, HTA= Heterotrophic Actinomycetes, KUA= Kerosene Utilizing Actinomycetes, BP= Before Pollution, AP= After Pollution)

In the 1% and 5% levels of kerosene-polluted soil, there was positive significant correlation (P<0.05) between HTA, HTF and HTB populations and duration of pollution. HTB and KUB also showed significant correlation (P<0.05) in 10% and 20% levels of kerosene polluted soils respectively.

Comparing variations in microbial populations over the duration of pollution and among levels of pollution, HTB and KUA populations varied significantly (P<0.01) over the duration of pollution but the variations among levels of pollution were not significant. KUB population variation over the duration and among levels of pollution was not statistically significant. HTF, KUF and HTA populations varied significantly over the duration of pollution and among levels of pollution (P<0.01) (Table 2). In spite of these variations heterotrophic microbial population densities at 24 weeks after pollution were typically but not significantly greater than that of the pristine garden soil.

The predominant microbial genera isolated from the polluted soils were *Pseudomonas, Flavobacterium, Serratia, Acinetobacter, Bacillus, Micrococcus, Aspergillus, Penicillium, Rhizopus, Fusarium, Mucor* and *Streptomyces*.

The soil in study was sandy loam (Sand-81.8%, Silt-7.4%ad Clay-10.8%). The pH value of soil before pollution was 4.74(Table 3). The values in kerosene polluted soils ranged from 4.82 to 6.46 (table 4) and varied significantly at p<0.05 over time (Table 5). There were no significant variations among the levels of pollution.

The total nitrogen of the pristine soil was 0.20 %(Table 3), the values ranged from 0.06 to 0.18% in kerosene-polluted soils (Table 4). Significant variations (P<0.05) were observed in duration of pollution with no significant variation among levels of pollution (Table 5).

Organic carbon content value of 3.42% as obtained in pristine soil (Table 3). The values ranged between 5.54% and 9.92% in kerosene-polluted soil (Table 4). There was also significant variation (P<0.05) in organic C contents among different pollution levels of the soil (Table 5).

The total nitrogen and Organic C in pristine soil resulted in C:N ratio of 17.1 (Table 3), the value ranged from 32.6 to 178.9 in the kerosene polluted soils (Table 4). These values varied significantly (P<0.05) with duration of pollution and among levels of pollution (Table 5). Decreased total nitrogen and increased organic carbon were observed over the period of study. The organic carbon contents of the polluted soils were greater than that of the pristine soil.

A total of nine heavy metals were analyzed for in pristine and kerosene polluted soils respectively (Table 6). In the pristine soil seven heavy metals were detected. Among these heavy metals Fe, Mn and Cr had high concentrations of 96.082ppm, 5.578ppm and 2.602ppm respectively. These heavy metals were also found to be in high concentrations in kerosene-polluted soils. All the nine heavy metals analyzed for were detected in the pollutant (kerosene). Their concentrations in kerosene were significantly lower than that of the pristine soil except for Cd and Co, which were below detection limit (BDL) in the pristine soil. The concentration of these heavy metals after 24 weeks of pollution increased in some cases slightly with the levels of pollution.

Table 1: Coefficients of correlation (r) relating duration of pollution to microbial population at the different levels of kerosene-polluted soils

DURATION	HTB	KUB	HTF	KUF	HTA	KUA
1%K	.771*	.694	.798*	.945**	.816*	.931**
Sig. (2-tailed)	.042	.084	.031	.001	.025	.002
5%K	.633	.614	.861*	.949**	.853*	.979**
Sig. (2-tailed)	.127	.142	.013	.001	.015	.000
10%K	.807*	.638	.513	.942**	.881**	.920**
Sig. (2-tailed)	.028	.123	.239	.004	.009	.003
20%K	.731	.767*	.571	.912**	.919**	.982**
Sig. (2-tailed)	.062	.044	.180	.004	.003	.000

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Table 2: Soil microbial population variation over time and at the different levels of kerosene pollution

	Source of Variation			
	Duration of pollution		Levels of pollution	
Microbial population	*F*	*P- value*	*F*	*P- value*
HTB	29.197	$2.4x10^{-8}$*	2.657	0.079
KUB	1.695	0.179	0.899	0.461
HTF	8.682	0.00016*	5.325	0.0083*
KUF	71.316	$1.5x10^{-11}$*	9.024	0.0007*
HTA	43.219	$1.0x10^{-9}$*	5.523	0.0072*
KUA	29.113	$2.5x10^{-8}$*	0.656	0.586

Critical F value for Duration of pollution = 2.661
Critical F value for Levels of pollution = 3.159
* = Significant

Table 3 : Some physico-chemical properties of pristine garden soil

Parameters	Value*
pH	4.74
Organic C (%)	3.42
Total N (%)	0.20
C:N ratio	17.1
BS (%)	87.8
Sand (%)	81.8
Silt (%)	7.40
Clay (%)	10.80

*Values are mean of duplicate determination

Table 4: pH, organic carbon, total nitrogen and C:N ratio of kerosene polluted soils at interval of eight weeks

Duration (Weeks)	pH	Org. C (%)	Total N(%)	C:N ratio
		1% Kerosene		
8	4.82	5.54	0.17	32.6
16	6.39	5.66	0.13	44.2
24	6.46	5.96	0.09	66.9
		5% Kerosene		
8	4.95	7.63	0.16	48.2
16	5.30	7.99	0.14	57.3
24	6.01	7.46	0.10	75.2
		10% kerosene		
8	5.12	9.71	0.18	54.1
16	6.38	9.76	0.11	90.7
24	6.39	9.65	0.06	164.2
		20% kerosene		
8	5.65	9.65	0.13	76.9
16	5.76	9.74	0.10	97.9
24	6.40	9.92	0.06	178.9

Values are mean of duplicate determinations

Table 5: Some soil physico-chemical properties variations over time and at different levels of kerosene pollution

	Source of Variation			
	Duration of pollution		Levels of pollution	
Soil parameters	*F*	*P- value*	*F*	*P- value*
pH	11.312	0.009*	1.515	0.303
Org. C	0.669	0.546	287.938	$7.16x10^{-7}$*
Total N	34.521	0.0005*	4.211	0.063
C:N ratio	10.309	0.011*	6.994	0.022*

Critical F value for Duration of pollution = 5.143
Critical F value for Levels of pollution = 4.757
* = Significant

Table 6: Heavy metals concentrations in pristine and kerosene-polluted soil samples

Samples	Pb	Cr	Zn	Cu	Ni	Mn	Cd	Co	Fe
	(ppm)								
Pristine soil	0.546	2.602	0.384	0.074	0.288	5.578	BDL	BDL	96.082
Kerosene	0.043	0.424	0.079	0.002	0.004	0.001	0.001	0.001	0.051
Kerosene-polluted soils 24 weeks after pollution									
1%K	0.278	1.360	0.448	0.046	0.154	2.256	BDL	BDL	100.081
5%K	0.300	2.340	0.438	0.048	0.158	2.610	BDL	BDL	96.080
10%K	0.320	3.542	0.451	0.066	0.198	2.990	BDL	BDL	100.101
20%K	0.376	3.003	0.466	0.072	0.218	2.774	BDL	BDL	100.102

BDL= below detection limit ppm= parts per million

Values are mean of duplicate determinations

DISCUSSION

There were positive correlations between the microbial population and duration of pollution. KUF and KUA populations showed positive significant correlation ($P<0.01$) in all levels of pollution. HTA populations showed positive significant correlation ($P<0.05$) in 10% and 20 % ($P<0.01$) levels of kerosene-polluted soils. These positive significant correlations suggest that the introduction of kerosene into the soil stimulated the microbial populations that were able to metabolize kerosene. The non-significant positive correlations of some microbial populations with the duration infer that these microbes, following kerosene contamination, were not stimulated. This finding agrees with a report by Foght and Westlake (1987) which states that, following the introduction of petroleum hydrocarbon, some microbial species flourish while others diminish due to cytoxicity.

The soil in this study was sandy loam (Sand-81.8%, silt-7.4% and Clay-10.8%). Soil has a powerful effect on its microflora on the basis of different particle size distribution by providing a specific habitat that selects a specific microorganism (Garbeva *et al.*, 2004). Though the texture was not expected to change in a short term but kerosene pollution had profound effects on microflora of the sandy loam soil in this study.

The pH value of soil before pollution was 4.74(Table 3). The values in kerosene-polluted soils ranged from 4.82 to 6.46 (Table 4) and varied significantly at $P<0.05$ over the duration of pollution (Table 5). Soil pH affects the abundance of microorganism, as bacteria are generally more prevalent in alkaline soil while fungi are more prevalent in acidic soils. This is important because microbes are responsible for the cycling of nutrients. The most diverse and numerous microbial populations are found in the near-neutral soils. In agreement with this, kerosene-polluted soil in this study showed microbial diversity and abundance as the pH increased towards neutrality over time.

The total nitrogen and organic carbon in the pristine soil resulted in C:N ratio of 17.1(Table 3) while the value ranged from 32.6-178.9 in the kerosene polluted soils (Table 4). This indicates that 1g biomass of microorganisms would require 33.6g to 179.9g of N (may be from fertilizer) for effective bioremediation of the kerosene-polluted soil. C:N ratio values varied significantly ($P<0.05$) with duration of pollution and levels of pollution. A high C:N ratio encourages proliferation with a resultant immobilization of not only mineral nitrogen but also other nutrient elements in the soils by microbes. Most agricultural soils have a C:N ratio of about 10-12 and if the C:N ratio is above this value, there will be chances of nutrient being immobilized by microbes and rendered unavailable to plants (Okolo *et al.*, 2005; Odu, 1981). C:N ratio values obtained in the polluted soil in this study suggest the unsuitable nature of kerosene-polluted soil for agricultural purposes except after proper bioremediation.

Some heavy metals (Fe, Mn and Cr) were found to be present in higher concentrations in both pristine and kerosene-polluted soils. Many heavy metals are toxic to plants and animals if absorbed in excessive amount. Some of the heavy metals namely Cr, Mn, Cu Ni, Pb detected in this study are considered hazardous. Compounds of cadmium, nickel and some forms of chromium (chromates) are carcinogenic as well (Mishra *et al.*, 2001).

Most heavy metals act as enzyme inhibitors and disrupt the metabolic processes of organisms. Concerns over the presence of heavy metals in an environment arise from the fact that they cannot be broken down to non-toxic forms (Mishra *et al.*, 2001).

properties of kerosene-polluted soil intended for agricultural purpose is essential, as kerosene pollution could trigger soil microbial response thereby adversely affecting soil physicochemical properties. In view of this, there is a need for nutrient amendment in a previously contaminated soil.

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