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Bacterial population dynamics in a crude oil polluted soil undergoing bioremediation in a screen house

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A study was carried out in a screen house to determine the bacterial population dynamics in a crude oil soil undergoing bioremediation. The agricultural soil samples were polluted with different concentrations (5, 8 and 11%) of crude oil. Physiochemical and microbiological analyses were carried out on the polluted and unpolluted soil samples at various intervals beginning from two weeks after the pollution of the soil samples to the fourteenth week. The soil samples were found to be acidic (5.40) and rich in phosphorous (26.52 mg/kg). There were no significant differences in the pH, organic matter, sodium, potassium and magnesium of the unpolluted and the polluted soil samples. The microbial loads of the polluted soil samples were lower than the unpolluted soil samples throughout the study. The highest bacterial loads (27.00^{d}±2.20, 19.40^{c}±1.80, 8.70^{b}±0.50 for 5, 8 and 11% crude oil concentration respectively) were observed, when *Ewingella americana* (bacterium with known crude oil degrading ability) was inoculated into the polluted soil samples. The bacterial species responsible for the bioremediation from the polluted soil samples were identified using conventional techniques. The bacterial species isolated and identified were *Pseudomonas aeruginosa, Bacillus subtilis, Bacillus cereus, Proteus vulgaris* and *Staphylococcus aureus. Bacillus* spp has the highest percentage frequency (*Bacillus subtilis* = 30.95% and *Bacillus cereus* = 21.43%). The consistent isolation of these bacteria shows that they could survive the crude oil pollution and possibly utilize the crude oil, thereby making the crude oil less harmful to the environment.

Key words: Bacteria, bioremediation, bacterial species, crude oil.

INTRODUCTION

Crude oil is the major source of energy for industries and homes. Crude oil and petroleum products are very complex and consist of mixtures of thousands of individual compounds that exhibit a wide range of physical properties (Leahy and Colwell, 1996). Understanding these properties is important in determining behaviour of spilled oil and the appropriate response option. Petroleum may be classified into four major groups based on their different solubility in organic solvents and their chemical composition (Leahy and Colwell, 1996). They are saturated and unsaturated hydrocarbons, which are usually the most abundant constituents in crude oils.

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Aromatic hydrocarbons include monocyclic aromatics (benzene and toluene) and polycyclic aromatic hydrocarbons (PAHs) (naphthalene and anthracene). PAHs are of particular environmental concern because they are potential carcinogens.

Bioremediation as defined by American Academy of Microbiology is the use of living organisms (especially microorganisms) to reduce or eliminate environmental hazards resulting from accumulations of toxic chemicals or other hazardous waste (Gibson and Sayler, 1992). The microorganism may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site. Contaminant compounds are transformed by living organisms through reactions that take place as a part of their metabolic process (Snape et al., 2001). Bioremediation of a compound is often as a result of the actions of multiple organisms. For bioremediation to be effective, microorganisms must enzymatically attack the pollutants and convert them to harmless products. Bioremediation can be effective, only where environmental conditions permit microbial growth and activity (Vidali, 2001). Its applications often involve the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate (Snape et al., 2001; Aichberger et al., 2005). Bioremediation has been employed to attack oil spill contaminants. Multiple techniques including the addition of fertilizers to facilitate the decomposition of crude oil by bacteria in bioremediation had been reported (Atlas, 1995). Bioremediation has helped in cleaning up of oil spills, pesticides, and other toxic materials. For example, accidents involving huge oil tankers regularly result in large spill that pollute coastlines and harm wildlife. Bacteria and other microorganisms can convert the toxic material in crude oil into less toxic ones (*Snape et al., 2001*). The microorganisms involved in bioremediation are aerobic and anaerobic bacteria. Examples of species of aerobic bacteria recognized for their degradative abilities are *Pseudomonas, Alicaligenes, Sphingomonas, Rhodococcus* and *Mycobacterium, Bacillus, Flavobacterium*. These microbes have often been reported to degrade pesticides and hydrocarbons, both alkanes and polyaromatic compounds. Many of these bacteria use the contaminant as the sole source of carbon and energy (April et al., 2000; Zhang et al., 2006). Anaerobic bacteria are not as frequently used as aerobic bacteria (Okoh and Ezeronye, 2002). Other examples of bacteria that have been implicated in crude oil biodegradation include; *Alicaligenes, Sphingomonas, Rhodococcus*, *Mycobacterium* and *Flavobacterium* (April *et al.,* 2000; Okoh and Ezeronye, 2002; Zhang *et al.,* 2006).

The aim of this study therefore, was to inoculate crude oil polluted soil with known bacterium with a high biodegradative ability obtained from previous research work, and monitor the bacterial population dynamics of the soil while undergoing bioremediation.

MATERIALS AND METHODS

Collection of sample

Agricultural soil samples were collected using soil auger from the back of E-Test Centre, Obakekere, Federal University of Technology, Akure (FUTA), Nigeria and taken to the screen house in the Department of Crop Science and Pest Management, FUTA. Polluted soil samples were collected at intervals from the screen house and analyzed in the Department of Microbiology, FUTA. These were done using methods described by Voroney (2006) and Ibitoye (2008) for collecting of soil sample.

Crude oil used

Bonny light oil is a high grade of Nigerian crude oil with high API gravity (low specific gravity), produced in the Niger Delta basin and named after the prolific region around the city of Bonny. It has low sulphur content and this makes it a highly desired grade for its low corrosiveness to refinery infrastructure and the lower environmental impact of its by-products in refinery effluent.

Pollution of sample

Ten kilograms (10 kg) of the soil sample was weighed into 4 sterile buckets of 16 L each. The first was the control (unpolluted agricultural soil), the second was polluted with 5% crude oil, the third was polluted with 8% crude oil and the last was polluted with 11% crude oil. The crude oil used for this soil sample pollution is the bonny light. The polluted soil sample was left for two weeks in the screen house before further analyses. The conditions in the screen house are natural conditions (prevailing weather of FUTA, which is similar to the prevailing tropical weather).

Inoculation of polluted soil samples

Broth culture of bacterium (*Ewingella americana*) with known high degradative ability from stock culture of a previous work, was introduced into the polluted soil samples in the screen house after 8 weeks of pollution. The inoculated soil samples were also left in the screen house and were monitored under natural conditions for biological changes. Bacterial population and bacterial types were monitored during the bioremediation process, by conventional methods.

Isolation and identification of associated microorganisms

One gram (1 g) of the polluted soil samples each were weighed into 9 ml of sterile distilled water and diluted separated and serially for unpolluted and each polluted soil samples. Then 1 ml each of dilution factors of 10^4 was pipetted into sterile Petri dish for each soil samples. Thereafter, 20 ml of nutrient agar was cooled to 45°C, poured separately and aseptically into each plate. The plates were swirled and allowed to solidify. The solidified plates were incubated for 24 h at 37°C. The colonies were counted and associated microorganisms were isolated, characterized and identified according to the techniques described by Holt et al. (1994) and Fawole and Oso (2007). These microbial analyses were carried out, 2^{nd}, 8^{th}, 11^{th}, 13^{th} and 14^{th} weeks after the crude oil pollution.

Monitoring bacterial load

Using total plate count, plates in triplicates from unpolluted soil sample and polluted soil samples were observed 2nd, 8th, 11th, 13th and 14th weeks for their bacterial loads and the values were recorded.

Physiochemical parameters

The physiochemical parameters measured are; temperature, pH (Hendershot et al., 1993), organic carbon determination, organic matter (Schnitzer, 1978), total phosphate determination, nitrogen determination (Ademoroti, 1996) and metal determination in soil samples (Lacatusu, 2000).

Statistical analysis of data

Data obtained were subjected to one way analysis of variance (ANOVA) and Duncan's new multiple range test at 95% confidence level using SPSS 16.0 version. Differences were considered significant at P≤0.05. Data with the same superscript along the same column are not significantly different while data with different superscript along the same column are significantly different.

Table 1. Initial physicochemical characteristics of unpolluted soil sample.

Physicochemical parameters	Soil sample
pH	5.40
Moisture content (%)	8.60
Organic carbon (%)	2.89
Organic matter (%)	4.98
Phosphorus (mg/kg)	26.52
Potassium (mg/100 kg)	0.32
Sodium (mol/kg)	0.40
Calcium (mg/100 g)	5.90
Magnesium (mg/kg)	1.10

RESULTS AND DISCUSSION

The pH of the results (Tables 1 to 3) showed increments (5.13^{a}±0.175 - 6.09^{a}±0.48). These pH values are similar to the range (5.3 - 7.8) that has been documented to favour biodegradation of hydrocarbons (Stephen and Egene, 2012, Stephen et al., 2013). The significant difference in the organic carbon and the organic matter between the polluted and unpolluted soil samples (control) could be due to the presence of crude oil in the polluted soil (April et al., 2000; Zhang et al., 2006). However, it was observed that there was no significant difference between the organic carbon and the organic matter of the control and polluted soil samples after inoculation with *Ewingella Americana.* This may be due to higher microbial activities and growth in polluted soil samples (Aichberger et al., 2005; King et al., 2007). The phosphorus concentrations in polluted soils were lower than the control even after inoculation with significant difference between the polluted and the control samples (Tables 2 to 3) probably due to the fact that it is the limiting element in soils because of it high demand by plant and microorganisms (Norman and Hunner, 2008).

The unpolluted soil samples had the highest microbial load throughout the period under investigation (fourteen weeks) as shown in Figure 1. The microbial loads of a given habitat will relatively be the same as long as the conditions (such as food, nutrients, moisture content, pH, etc) are relatively constant over the period in view. The microbial load of a given habitat can also be affected by change in any of the environmental conditions, microbial succession and pollution (Atlas and Bartha, 1992; Nester et al., 2001; Banat, 2004).

The bacterial population of the polluted soil samples varied throughout the investigation period as shown in Figure 1. It was observed that the bacterial loads in the different concentrations of crude oil (bonny light) polluted soil were high and in an inverse proportion. That is, the higher the level of pollution, the lower the microbial load and verse versa. This corroborates the findings of Cooney (1984). Soil samples with the lowest concentration of pollution had the highest microbial loads throughout the study.

Comparing the results of the unpolluted soil sample to that of the polluted soil samples, the bacterial populations of the polluted soil samples were on the decreasing side in the course of the study, while the unpolluted soil samples' bacterial populations were relatively stable, these could be due to the inability of the indigenous bacteria to adapt to the crude oil pollution. This was also in conformity with those of Atlas and Bartha (1992).

The highest values of bacterial loads were observed after broth culture of *E. americana* with known crude oil degrading ability (published elsewhere) was inoculated into the polluted soil samples and there was a sharp fall in the microbial load afterwards, which could have resulted from bacterial competition between the indigenous bacteria and the inoculated bacterium for hydrocarbons in the polluted soil samples as source of carbon, for nutrients and energy. This is in agreement with the findings of Ijah and Antai (2003).

The bacterial population dynamics of the crude oil polluted soil undergoing bioremediation in a screen house, showed that there were five bacteria that were dominant throughout the research period (Table 5). They are: *P. aeruginosa, B. subtilis, P. vulgaris, B. cereus* and *S. aureus*. Bacteria from the unpolluted soil sample were *S. aureus, M. luteus, B. megaterium, C. sporogenes* and *B. cereus* (Table 4).

Bacillus megaterium, Clostridium sporogenes and *Micrococcus luteus* were isolated from the unpolluted soil sample but were not present in the polluted soil samples. This is probably due to the presence of crude oil

Table 2. Physicochemical characteristics of soil sample two weeks after pollution.

TRT	pH	Organic matter (%)	Organic carbon (%)	Phosphorus (mg/kg)	Sodium (mol/kg)	Potassium (mg/100 kg)	Magnesium (mg/kg)	Calcium (mg/100 kg)
Control	5.44^{a}±0.48	12.63^{d}±0.01	7.62^{d}±0.39	24.05^{c}±0.14	0.44^{b}±0.03	0.35^{b}±0.07	1.00^{c}±0.06	3.05^{b}±0.08
5%	5.31^{a}±0.29	1.97^{a}±0.26	1.28^{a}±0.16	20.25^{b}±0.13	0.29^{a}±0.08	0.34^{ab}±0.07	0.96^{c}± 0.06	2.84^{b}± 0.48
8%	5.35^{a}±0.10	3 .61^{b}±0.42	2 .32^{b}±0.24	19.12^{a}±0.39	0 .40^{ab}±0.09	0 .24^{a}±0.05	0 .47^{a}±0.12	1 .11^{a}±0.64
11%	5.13^{a}±0.17	9.23^{c}±0.07	5.10^{c}±0.37	20.04^{b}±0.14	0.36^{a}b±0.08	0.32^{ab}±0.04	0.75^{b}±0.06	1.43^{a}±0.24

TRT, Treatment; control, unpolluted agricultural soil.

Table 3. Physicochemical characteristics of soil sample two (2) weeks after inoculation.

TRT	pH	Organic matter (%)	Organic carbon (%)	Phosphorus (mg/kg)	Sodium (mol/kg)	Potassium (mg/100 kg)	Magnesium (mg/kg)	Calcium (mg/100 kg)
Control	6.09^{a}±0.48	1.89^{a}±1.21	2.19^{c}±0.39	23.35^{c}±0.14	0.29^{a}±0.03	0.21^{a}±0.07	1.77^{a}±0.06	3.97^{b}±0.08
5%	5.54^{a}±0.29	2.27^{a}±0.31	1.73^{b}c±0.16	19.37^{b}±0.13	0.26^{a}±0.08	0.23^{a}±0.07	1.99^{a}±0.06	3.99^{b}±0.48
8%	5.47^{a}±0.10	2.17^{a}±0.29	1.45^{ab}±0.24	18.39^{a}±0.11	0.31^{a}±0.09	0.18^{a}±0.05	1.66^{a}±0.12	2.15^{a}±0.64
11%	5.45^{a}±0.17	1.86^{a}±0.23	1.00^{a}±0.37	19.60^{a}±0.14	0.26^{a}±0.08	0.23^{a}±0.04	1.52^{a}±0.06	2.18^{a}±0.24

TRT, Treatment; control, unpolluted agricultural soil.

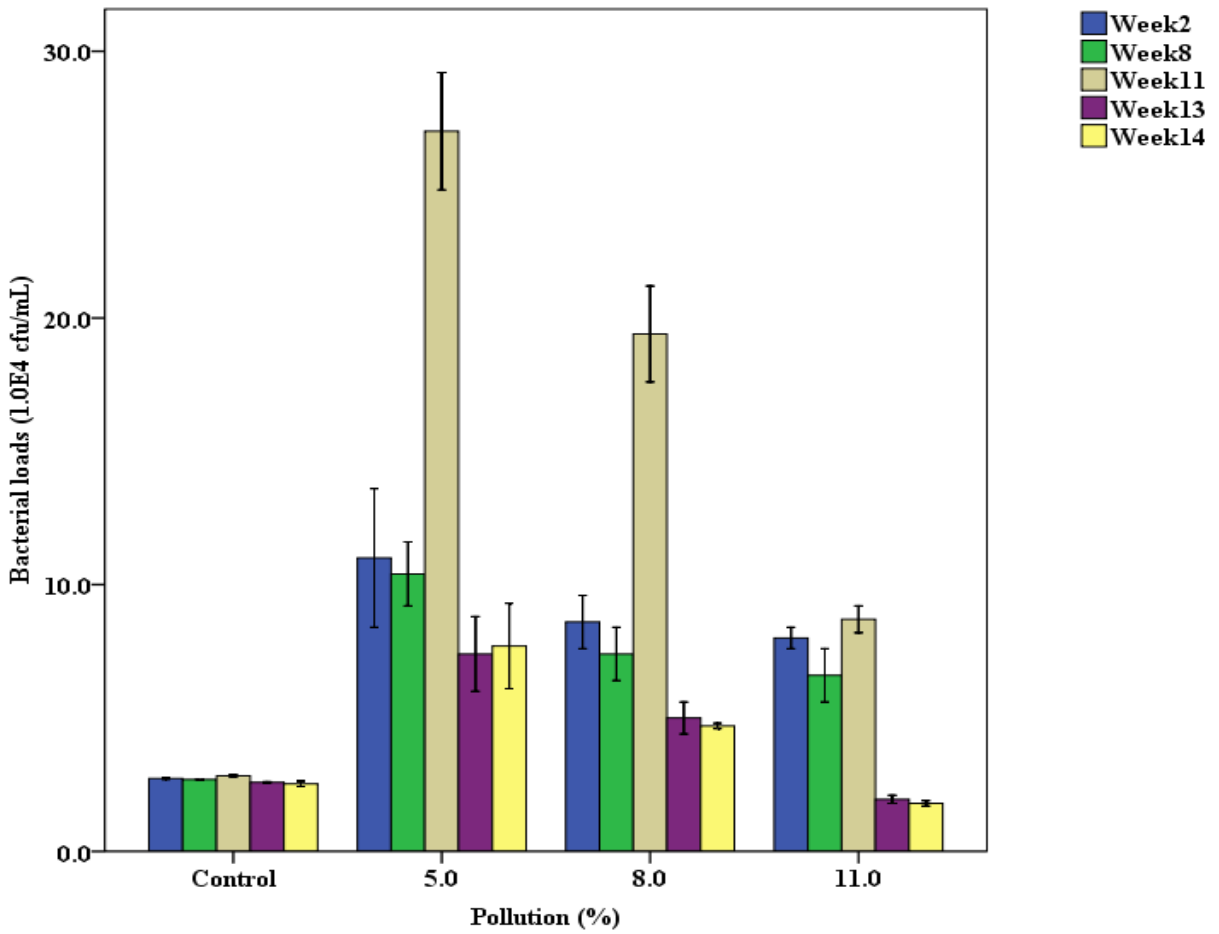

Figure 1. Bacterial loads of polluted and unpolluted soil. Control, Unpolluted agricultural soil.

Table 4. The morphological and biochemical characteristics of isolates in the unpolluted soil sample.

Dominant isolates	1	2	3	4	5
Pigmentation	Yellow	Cream	White	Cream	Colorless
Edge	Entire	Entire	Undulate	Irregular	Undulate
Elevation	Raised	Raised	Flat	Raised	Flat
Surface	Smooth	Smooth	Smooth	Smooth	Woolly
Shape of cell	Cocci	Cocci	Rod	Rod	Rod
Gram's reaction	+	+	+	+	+
Spore Stain	-	-	+	+	+
Motility	-	-	+	+	+
Catalase	+	+	+	+	+
Coagulase	+	-	-	-	-
Indole	-	-	-	-	-
Citrate		+	-	-	-
Starch Hydrolysis	+	+	+	+	+
Oxidase		-	-	+	-
Methylred	-	-	+	-	-
Vogas proskeruer		-	-	-	+
Sugar utilization					
Glucose		-	-	AG	A
Fructose	A	A	A	-	AG
Sucrose	A	A	AG	-	A
Maltose	A	A	A	A	A
Lactose	-	A	A	-	-
Mannitol	A	A	-	A	-
Galactose	AG	-	-	-	AG
Sorbitol		A	A	-	
Probable organism	*Staphylococcus aureus*	*Micrococcus luteus*	*Bacillus megaterium*	*Clostridium sporogenes*	*Bacillus cereus*

+, Positive; -, Negative; A, acid production; AG, acid and gas production.

in the polluted soil which may be harmful to those bacteria or rather, they were unable to use the crude oil as source of carbon and energy (Atlas and Bartha, 1992).

The occurrence of the isolates varied as shown in Table 6, with *B. subtilis* having the highest occurrence of 13 (39.95%) and *S. aureus* had the lowest occurrence of 5 (11.9%). The high occurrence of *Bacillus* spp. could be related to their high adaptability to different environments (Perfumo et al., 2007; Ashlee et al., 2008; Alfreda and Ekene, 2012).

Bacillus spp. has been known to be related to carbon mineralization of crude oil; some have been isolated from soil polluted by crude oil or petroleum products; and also known as one of the commonly found rod bacteria in the soil (Perfumo *et al.,* 2007; Alfreda and Ekene, 2012). This may be responsible for their consistency in these soil samples polluted with bonny light crude oil.

Pseudomonas spp. with percentage occurrence of 19.05 is also known to be related to crude oil in various ways such as carbon mineralization of crude oil. It is present in most terrestrial crude oil spillage in Nigeria, and they are commonly found in the soil (Zhang et al., 2006 and Perfumo et al., 2007). Their consistency in this study buttresses their adaptability to different environments.

S. aureus were observed initially at the beginning of the investigation but were absent in the course of the research work (Table 5). This might be due to their inability to adapt to the change in their environment (crude oil pollution). It was later observed that *Staphylococcus aureus* reoccurred in the course of the investigation (week 13^{th} and 14^{th}) (Table 6). This could be as a result of re-colonization by *S. aureus* (Okoh, 2003).

The continuous presence of the pollution, could probably result in the enzymes of these microbes adapting to degrade/utilize the crude oil and also the presence of the pollution might have stimulated the bacteria to elaborate enzymes from their constitutive enzymes to degrade/utilize the crude oil (Snape et al., 2001; Stephen et al., 2013).

Table 5. The morphological and biochemical characteristics of dominant isolates in the polluted soil samples.

Dominant isolates	1	2	3	4	5
Pigmentation	Yellow	Green	Cream	Colorless	Colorless
Edge	Entire	Undulate	Undulate	Undulate	Undulate
Elevation	Raised	Flat	Flat	Flat	Flat
Surface	Smooth	Smooth	Rhizoid	Woolly	Woolly
Shape of cell	Cocci	Rod	Rod	Rod	Rod
Gram's reaction	+	-	-	+	+
Spore Stain	-	-	-	-	+
Motility	-	+	+	-	+
Catalase	+	+	+	+	+
Coagulase	+	-	-	-	-
Indole	-	-	-	-	-
Citrate		+	+	+	-
Starch Hydrolysis	+	-		+	+
Oxidase		+	-		-
Vogas proskeruer		-			+
Sugar utilization					
Glucose		-	AG	A	A
Fructose	A		-	A	AG
Sucrose	A	-	A	A	A
Maltose	A	-	AG	A	A
Lactose	-	-	-	AG	-
Mannitol	A	-	AG	-	-
Galactose	AG	-	AG	A	AG
Sorbitol		-			
Probable organism	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Proteus vulgaris*	*Bacillus subtilis*	*Bacillus cereus*
Weeks isolate were found	2^{nd}, 13^{th}, 14^{th}	2^{nd}, 8^{th}, 11^{th}, 13^{th}, 14^{th}	2^{nd}, 8^{th}, 11^{th}, 13^{th}, 14^{th}	2^{nd}, 8^{th}, 11^{th}, 13^{th}, 14^{th}	2^{nd}, 8^{th}, 11^{th}, 13^{th},14^{th},

+, Positive; -, Negative; A, acid production; AG, acid and gas production.

Table 6. Frequency of occurrence of the consistence probable isolates.

Isolate	Week 2	Week 8	Week 11	Week 13	Week 14	Total	% Frequency
Staphyloccocus aureus	2	-	-	1	2	5	11.90
Proteus vulgaris	1	1	2	1	2	7	16.67
Pseudomonas aeruginosa	1	2	2	1	2	8	19.05
Bacillus cereus	2	2	1	2	2	9	21.43
Bacillus subtilis	3	2	3	2	3	13	30.95

In nature, bioremediation of crude oil typically involves a succession of species within the consortium of microbes present. Microorganisms classified as non hydrocarbon utilizers may also play an important role in the eventual removal of petroleum from the environment (Teas et al., 1989). Degradation of petroleum involves progressive or sequential reactions in which certain organisms may carry out the initial attack on the petroleum constituent. This, produces intermediate compounds that are subsequently utilized by a different group of organisms, in the process that results in further degradation (Teas et al., 1989; Okoh, 2003; Banat, 2004; Olukunle and Boboye, 2013).

Conclusion

The population dynamics of bacteria in this study shows that they could survive the crude oil pollution and possibly utilize the crude oil, thereby making the crude oil less harmful to the environment. The bacteria obtained in this

research work could be employed in bioremediation of crude oil polluted soils. Further research could be conducted on the synergy of *Ewingella americana* and the bacteria obtained in this study for bioremediation of crude oil polluted sites.

Conflict of interests

The authors did not declare any conflict of interest.

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