

Full Length Research Paper

Studies on the diversity, abundance and succession of hydrocarbon utilizing micro organisms in tropical soil polluted with oily sludge

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A research was carried out in a tropical region to study the population of hydrocarbon utilizers in soil polluted with oily sludge. Plots were prepared to receive treatments with neat and emulsified oily sludge. These plots were further treated with fertilizer and bioaugmented with a consortium of hydrocarbon utilizers for six months. Results obtained indicated that, the presence of oily sludge in soil caused the growth of diverse genera of oil degraders. The major genera of bacteria active in polluted soil were *Pseudomonas*, *Bacillus* and *Acinetobacter*, while fungal general were *Aspergillus*, *Penicillium*, and *Mucor*. Abundant microbial growth was observed during the first 60 days. Some organisms such as *Pseudomonas*, *Bacillus*, *Penicillium*, and *Aspergillus* were present in polluted soil throughout the experimental period, while others including *Candida*, *Sporobolomyces* and *Rhizopus* were found only during the first two months. Further analysis revealed that, succession of the hydrocarbon utilizers in polluted soil was subject to seasonal variations and depended primarily on the fraction of the oil being utilized at a specific time and also on the physiology of the micro organisms involved. In addition, the selective appearance and succession of hydrocarbon utilizers in the polluted soil were affected only by the presence of neat and emulsified oil in soil as compare to other treatment parameters. The practical implication of these findings suggests that reloading of oil in some treated plots could be carried out after the first 90 days. Molecular techniques are underway to provide a more comprehensive study on this successional trend.

Key words: Microbial succession and diversity, oily sludge, bioremediation, hydrocarbon utilizers.

INTRODUCTION

Hydrocarbonoclastic microbes play a paramount role in bioremediation. They include bacteria, fungi, yeasts and some algae. These organisms have been isolated from heavily oil-polluted deposits or in a variety of soils and water continuously exposed to hydrocarbon for several years (Ibe and Ibe, 1986). The importance of microorganisms in decomposing natural organic residues in soils,

sediments, and aquatic ecosystems had long been recognised. Microbial transformation of organic contaminants normally occurs because the organisms can use the contaminants for their own energy needs, growth and reproduction. The ability of certain microorganisms to degrade petroleum seems to be an adaptive process and is governed by environmental conditions. The presence of petroleum may also affect the microbial community through selection of species. In a study by Ibe and Ibe (1986) a variety of oil degrading microorganisms were isolated from oil polluted samples and among the bacte-

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ria, *Pseudomonas*, *Acetobacter*, *Chromobacterium* and *Corynebacterium* were isolated as dominant species. *Candida* species were the yeast commonly found while *Penicillium* and *Cladosporium* were common among the fungi. Shailubhai (1986) isolated the followings bacteria and fungi genera from oily sludge treated soil: *Pseudomonas*, *Arthrobacter*, *Bacillus*, *Actinomyces*, *Sarcina*, *Vibrio*, *Brevibacterium*, *Flavobacterium*, *Cylindrocarpon*, *Fusarium*, *Aspergillus* and *Rhodotorula*. A number of investigators have reported the distribution and abundance of petroleum hydrocarbon utilizing microorganisms present in oily sludge polluted environments (Prado et al., 1993 and Genouw et al., 1994; Alexander, 2000; Admon et al., 2001; David et al., 2001; Zucchi et al., 2003; Christopher and Kitts, 2004; Marc et al., 2005).

In the tropical region, works have been published to establish the diversity and abundance of hydrocarbon utilizing micro-organisms in oil polluted sites (Okpokwasili and Okerie, 1988; Prado et al, 1993; and Adams and Jackson, 1996); but works on the succession of hydrocarbon utilizing microbes on polluted site appeared to be at a pioneering stage. Knowledge of the succession of hydrocarbon utilizing micro-organisms in addition to their diversity and abundance during bioremediation in any given oil polluted environment is critical as it can help to monitor the efficiency of the process as well as identify the appropriate period for re-loading the oil in soil after the first treatment; also it might give an indication for proper composition of consortium of non-genetically engineered micro-organisms for microbial seeding during some bioremediation processes in areas where genetic engineered micro-organisms have not yet been accepted to be used in the environment due to regulations constraints. This study was carried out to serve these purposes with particular reference to a tropical area.

MATERIALS AND METHODS

Study area

This research work was carried out in Calabar, Nigeria. The city of Calabar resides in a coastal/estuarine zone that has an opening to the Atlantic Ocean; in addition, Calabar lies in the rainforest belt of South-eastern Nigeria; the soil characteristic is loamy sand. The study area witnesses in a year two seasons: the rainy and dry season. The rainy season expands from June to November while the dry season begins in December and terminates in March.

Preparation of experimental area

A simulation study was carried out with the aim to bioremediate an environment (soil) intentionally polluted with oily sludge. The experimentation was performed in a planting land. The land was covered with dense vegetation. An area of 10 m x 30 m (300 m²) was cleared to prepared plots for the experimentation during each season (rainy and dry). Nine (9) levees each of 5 m (length) x 2 m (width) x 0.2 m (depth) were prepared up. The distance between 2 consecutive levees was 1 m. On each of the split levee, an area of

1.5 m x 1.5 m was demarcated to receive treatment for the study.

Collection of materials

Oily sludge: The oily sludge was obtained from an open-to the-air-storage pond of a crude oil production Tank Farm of Elf Petroleum, Nigeria. Oil dispersant/emulsifier used to emulsified the oily sludge was the dispersant CNN₂₀₀₀, formulated by recycling tropical agricultural wastes; dispersant CNN₂₀₀₀ was tested for its toxicity potentials and was found to be environment friendly. Fertilizer NPK 15:15:15 was purchased from a local Agricultural shop.

Experimental layout

The experimental design consisted of nine (9) small plots (levees) and each plot received specific treatment options organized as follows: 1-Plot C: Control soil (free from oil pollution). 2-Plot S: Neat oily sludge (S). 3-Plot O: Oily sludge (S) and seeded with consortium of micro-organisms (O). 4-Plot E: Emulsified oily sludge (E). 5-Plot F: Oily sludge (S) and amended with Fertilizer (F). 6-Plot (O + F): Oily sludge (S) seeded with the consortium (O) and amended with fertilizer (F). 7-Plot (E+F): Emulsified oily sludge (E) and amended with fertilizer (F). 8-Plot (E+O): Emulsified oily sludge (E) and seeded with consortium of micro-organisms (O). 9-Plot (E+O+F): Emulsified oily sludge (E) amended with fertilizer (F) and seeded with consortium (O)

Treatment of plots with oily sludge/ emulsified oily sludge

In plots that received, non-emulsified oily sludge, four (4) kilograms of oily sludge were added and ploughed with soil in the demarcated areas of treatment. In plots that were treated with emulsified oily sludge, the same amount of oil was used and emulsified with dispersant CNN₂₀₀₀ at ratio of 1 g oil/ 1.5 ml of emulsifier .

Treatment of plots with consortium of micro-organisms for microbial seeding

Consortium of micro-organisms for seeding the oily sludge polluted soil was added at a predetermined amount of inocula. The average inoculum sizes were 32.1 x 10⁸ CFU / ml for *Bacillus* sp, 23.6 x 10⁶ CFU / ml for *Aspergillus* sp and 29.2 x 10⁶ CFU / ml for *Penicillium* sp. (Nkeng et al., 2005).

Treatment of plots with nutrient fertilizer

Fertilizer was added once every week for 1 month and then twice every 2 weeks for the remaining period of the study (Mentzer and Ebere, 1996).

Tilling exercise

All plots were tilled 4 times a week. Plots treated in dry season were moistened 3 times a week with of sterile distilled water.

Collection of samples

Soils in treated plots were properly mixed and 200 g were collected at the end of each month in sterile polythene bags and labelled.

Table 1. Diversity, abundance, and succession of hydrocarbon utilizing bacteria (HUB) and fungi (HUF) in tropical soil polluted with oily sludge in dry season

Month	Hydrocarbon utilizing bacteria			Hydrocarbon utilizing fungi		
	Unpolluted soil	S	E	Unpolluted soil	S	E
December	<i>Micrococcus+</i> <i>Bacillus+</i> <i>Acinetobacter+</i> <i>Serratia+</i> <i>Streptomyces+</i> <i>Chromobacterium</i> <i>Nocardia+</i>	<i>Bacillus+++</i> <i>Pseudomonas++</i> <i>Acetobacter+</i> <i>Streptomyces+</i> <i>Serratia+</i>	<i>Pseudomonas+++</i> <i>Bacillus+++</i> <i>Streptomyces++</i> <i>Flavobacterium+</i> <i>Acinetobacter+</i>	<i>Chrysosporium+</i> <i>Aspergillus+</i> <i>Penicillium+</i> <i>Fusarium+</i> <i>Mucor+</i> <i>Rhizopus+</i> <i>Cladosporium+</i>	<i>Aspergillus++</i> <i>Penicillium++</i> <i>Fusarium+</i> <i>Mucor+</i> <i>Candida+++</i>	<i>Aspergillus+++</i> <i>Penicillium+++</i> <i>Fusarium+++</i> <i>Candida+++</i> <i>Rhizopus+</i> <i>Paecilomyces+</i> <i>Verticillium+</i>
January	Same as above	<i>Bacillus+++</i> <i>Pseudomonas++</i> <i>Acetobacter+</i> <i>Streptomyces+</i> <i>Serratia+</i>	<i>Pseudomonas+++</i> <i>Bacillus+++</i> <i>Streptomyces++</i> <i>Flavobacterium+</i> <i>Acinetobacter+</i>	Same as above	<i>Aspergillus++</i> <i>Penicillium++</i> <i>Fusarium+</i> <i>Candida+++</i>	<i>Aspergillus+++</i> <i>Penicillium+++</i> <i>Fusarium+++</i> <i>Candida+++</i> <i>Rhizopus+</i> <i>Paecilomyces+</i>
February	Same as above	<i>Bacillus+++</i> <i>Pseudomonas++</i> <i>Acetobacter+</i> <i>Serratia+</i>	<i>Pseudomonas+++</i> <i>Bacillus+++</i> <i>Aeromonas+</i> <i>Flavobacterium+</i>	Same as above	<i>Aspergillus++</i> <i>Penicillium++</i> <i>Fusarium+</i>	<i>Aspergillus++</i> <i>Penicillium++</i> <i>Fusarium+</i> <i>Paecilomyces+</i>
March	Same as above	<i>Bacillus+++</i> <i>Pseudomonas++</i> <i>Serratia+</i>	<i>Pseudomonas+++</i> <i>Bacillus+++</i> <i>Aeromonas+</i>	Same as above	<i>Aspergillus++</i> <i>Penicillium++</i> <i>Fusarium+</i>	<i>Aspergillus++</i> <i>Penicillium++</i> <i>Fusarium+</i>

+++ : Profuse growth; ++ : Moderate growth; + : Scanty growth; S : Soil polluted with neat oily sludge; E : Soil polluted with emulsified oily sludge; HUB : Hydrocarbon Utilizing Bacteria; HUF : Hydrocarbon Utilizing Fungi.

They were sent to the laboratory and analysed immediately for the abundance, diversity and succession of hydrocarbon utilizers in a monthly basis.

Processing of the specimens

Isolation of hydrocarbon utilizing bacteria (HUB) and fungi (HUF) in soil samples was carried out following the method described by Amadi et al (1996). Fungi isolates were identified using the method described by Martha and Kathleen (1998) and bacteria were identified following the biochemical technique/scheme of API Bio-merieux Environmental.

RESULTS AND DISCUSSION

Tables 1 and 2 present the results of the diversity, abundance and succession of hydrocarbon utilizers in polluted soil samples in the dry and rainy seasons, respectively. We can observe from these Tables that diverse genera of hydrocarbon utilizers exist in the soil. Some of the organisms found in the control soil were also found in the polluted soils. The presence of oil and emulsified oil cause the selective appearance of hydrocarbon utilizers in soil. Abundant microbial growth was observed in the

polluted soil during the first 60 days. The diversity of micro-organisms reduced as the oil was being consumed in the soil. The diversity and successional trend experienced changes with seasonal variation. The major genera of bacteria active in polluted soils were *Pseudomonas*, *Bacillus*, *Serratia*, and *Acinetobacter*, while fungal genera were *Aspergillus*, *Penicillium*, and *Mucor*. Some organisms such as *Pseudomonas*, *Bacillus*, *Aspergillus*, and *Penicillium* were present in the polluted soil throughout the experimental period irrespective of the season. Meanwhile other organisms including *Candida*, *Sporobolomyces*, and *Rhizopus*, were present in polluted soils only during the first two months.

Figure 1 presents the composite dendrogram summarizing organisms taxonomy performed using hierarchical cluster analysis to illustrate linkage between groups (parameters). The dendrogram indicates that some of the organisms found in the control soil are also found in the other plots. Further more organisms found in test plots S, O, F, O+F present the same cluster distance; the same observation is seen with organisms in test plots E, E+O, E+F, E+O+F. This infers that the types of micro-organisms isolated in test plot S was the same isolated in plots O, F, and O+F, likewise those in test plot E were

Table 2. Diversity, abundance, and succession of hydrocarbon utilizing bacteria (HUB) and fungi (HUF) in soil polluted with oily sludge in rainy season in topical soil

Months	HUB			HUF		
	Unpolluted soil	S (neat oil)	E	Unpolluted soil	S	E
August	<i>Achromobacte+</i> <i>Bacillus+</i> <i>Micrococcus+</i> <i>Acinetobacter+</i> <i>Serratia+</i> <i>Actinomyces +</i> <i>Streptomyces+</i>	<i>Pseudomonas+++</i> <i>Bacillus+++</i> <i>Acinetobacter+</i> <i>Streptomyces+</i> <i>Serratia+</i>	<i>Pseudomonas+++</i> <i>Bacillus +++</i> <i>Nocardia+</i> <i>Streptomyces+</i> <i>Acinetobacter+</i> <i>Achromobacter+</i>	<i>Apergillus+</i> <i>Penicillium+</i> <i>Cladosporium+</i> <i>Absidia+</i> <i>Mucor+</i> <i>Alternria+</i> <i>Trichoderma+</i>	<i>Aspergillus++</i> <i>Penicillium+++</i> <i>Mucor+</i> <i>Candida+++</i>	<i>Aspergillus+++</i> <i>Penicillium+++</i> <i>Candida++</i> <i>Mucor+</i> <i>Sporobolomyces++</i> <i>Phialophora+</i>
September	Same as above	<i>Pseudomonas+++</i> <i>Bacillus+++</i> <i>Acinetobacter+</i> <i>Streptomyces+</i> <i>Serratia+</i>	<i>Pseudomonas+++</i> <i>Bacillus +++</i> <i>Nocardia+</i> <i>Streptomyces+</i> <i>Acinetobacter+</i>	Same as above	<i>Aspergillus++</i> <i>Penicillium++</i> <i>Mucor+</i> <i>Candida+++</i>	<i>Aspergillus+++</i> <i>Penicillium+++</i> <i>Candida++</i> <i>Mucor+</i> <i>Sporobolomyces++</i>
October	Same as Above	<i>Pseudomonas+++</i> <i>Bacillus+++</i> <i>Streptomyces+</i> <i>Micrococcus+</i>	<i>Pseudomonas+++</i> <i>Bacillus +++</i> <i>Flavobacterium+</i> <i>Streptomyces+</i>	Same as Above	<i>Aspergillus++</i> <i>Penicillium+++</i> <i>Mucor+</i>	<i>Aspergillus+++</i> <i>Penicillium+++</i> <i>Phialophora+</i>
November	Same as Above	<i>Pseudomonas++</i> <i>Bacillus++</i> <i>Micrococcus+</i>	<i>Pseudomonas+++</i> <i>Bacillus +++</i> <i>Flavobacterium+</i>	Same as Above	<i>Aspergillus++</i> <i>Penicillium+++</i> <i>Mucor+</i>	<i>Aspergillus+++</i> <i>Penicillium+++</i> <i>Phialophora+</i>

+++ : Profuse growth; ++ : Moderate growth; + : Scanty growth; HUB: Hydrocarbon Utilizing Bacteria; HUF: Hydrocarbon Utilizing Fungi; S: Soil polluted with neat oily sludge; E: Soil polluted with emulsified oily sludge.

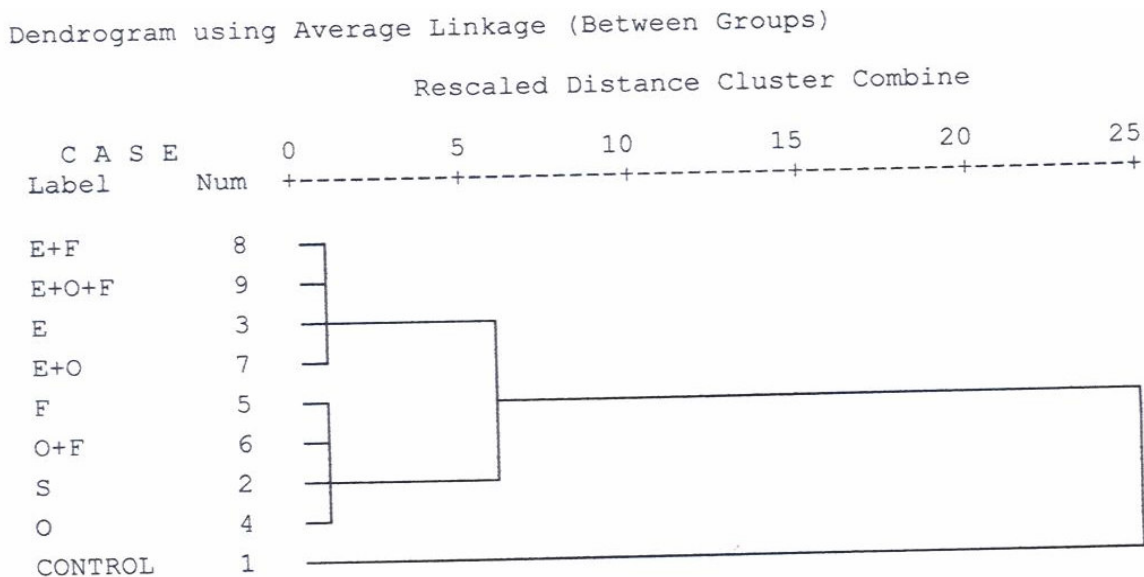


Figure 1. Dendrogram summarising taxonomy performed using hierarchical cluster analysis

the same in E+O, E+F and E+O+F. These observations indicate that the addition of Fertilizer (F) and Organisms (O) did not appear to have affected the appearance,

distribution and succession of micro-organisms in the test plots, rather only the presence of oil and emulsified oil did.

The various bacteria and fungi isolated in this study have been reported by many authors and the physiology of some of these petroleum hydrocarbon utilizers have been extensively studied. *Candida sp* are known to best metabolized n-alkanes than the aromatic. Thus, the profuse growth of these yeast cells in the beginning of the incubation period in polluted test plots could be associated with the utilization of the n-alkane fractions of the oily sludge. When the concentration of n-alkane depleted significantly after 2 months, there was little substrate for their growth, so they disappeared from the polluted soil and were absent among the isolates by the third months (Tables 1 and 2). Many enzymes and electron carriers involved in the n-alkanes metabolism have been reported in some species of *Candida*. A number of enzymes and electron carriers linked to the hydroxylation of n-alkanes to produce n-alkane-1-ol have been isolated in *Candida spp* (Singer and Finnerty, 1984a; Watkinson and Morgan, 1990). Other microorganisms isolated in the test plots have been reported as hydrocarbon utilizers. For example, *Pseudomonas putida* was found by Britton (1988) to produce enzyme mono-oxygenase linked to electron carrier rubredoxin during hydroxylation of n-alkane to produce n-alkane-1-ol. A species of *Pseudomonas fluorescens* capable of producing dehydrogenase enzyme that attacks aromatic naphthalene to produce catechol had been reported by Mark (1990). Weissenfels et al., (1990) isolated pure cultures of *Pseudomonas paucimobilis* and *Pseudomonas vesicularis* capable of degrading polyaromatic hydrocarbons. The gram-positive bacteria have also been isolated, thus Heitkamp and Cerniglia (1989) isolated a single gram-positive strain capable of the biodegradation of naphthalene, phenanthrene, fluoranthene and pyrene. Some strains of *Bacillus sp* and species of fungi including *Aspergillus spp.* and *Fusarium sp* capable of initiating the degradation of n-alkanes by sub terminal oxidation have been reported (Watkinson and Morgan, 1990).

Bacteria succession had been studied by a number of authors. Some reports described the structure and dynamics of bacterial community involved in bioremediation of crude oil (Alexander, 2000; Admon et al., 2001); in this study a few group of bacteria were observed to increase in abundance in response to oil contamination. In a study on bacterial succession on polyaromatic hydrocarbons (PAHs)-contaminated soil, David et al. (2001) reported in USA, that the intrinsic biodegradative potential of an environmental site can be derived from the polyphasic characterization of the in situ microbial community. During another study on bacterial community on oil polluted soil, Zucchi et al., (2003) in Italy, reported that successive phases of activation on bacterial population occur during bioremediation treatment of oil in soil.

Christopher and Kitts (2004) in a study on bacteria succession in a land treatment unit in the USA, observe

that the specific phylotypes of bacteria were associated with different phases of petroleum degradation, a sharp increase in plate counts was reported during the first three weeks indicating increase in biomass associated with petroleum degradation, the dominant phylotypes in sample during total rapid petroleum degradation were *Flavobacterium*, *Pseudomonas*, *Methylococcus* and *Methylobacter*. After the total petroleum hydrocarbon (TPH) degradation rate slowed, four other phylotypes *Rhodanobacter*, *Thermomonas*, *Xanthomonas* and *Sternotrophomonas* gained dominance in the community while *Pseudomonas* and *Flavobacterium* decreased in abundance. Marc et al (2005) in Spain observed that at the early stage of polycyclic aromatic hydrocarbon biodegradation in soil, the genera *Sphingomonas*, and *Azospirillum* were the dominant group; at a later stage the genera of *Xanthomonas*, *Sphingomonas*, *Alcaligenes*, and *Achromobacter* were the dominant group. It could be inferred from this development above that geographic variation affects the microbial diversity and succession.

Conclusion

Successional trend suggests that the initial first degradation is mediated by microbial utilisation of bioavailable compound, governed by microbial physiology and is subject to geographical variation. The practical inference that could be derived is that emulsified plots could be reloaded after the first three months, since microbial growth pattern indicates the oxidation process which was very active in the first 90 days and depleted tremendously by the fourth month. Allochthonous micro-organisms capable of efficiently oxidizing petroleum hydrocarbons could be selected and used for microbial seeding during bioremediation of oil polluted site, in such area where regulatory constraints are still preventing the use of genetically engineered microbes during oil pollution abatement programme. This study has given a typical example in a tropical area.

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