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Conditioners and significance of t-RFLP profile of the assemblage of prokaryotic microorganisms in crude oil polluted soils

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Following an increased interest in management practices designed to reduce posed phytotoxicity during phytoremediation experiments, crude oil polluted soil and variants from conditioners-aided phytoremediation experiment were subjected to terminal restriction fragment polymorphism (t-RFLP) to evalute the biodiversity of bacterial microflora of polluted soil and amendments conditions. Genetic fingerprinting showed that hydrocarbons stress led to depletion of the genetic resources of soil microflora and to a radical change in its qualitative composition. The amended stressed soils not only has a greater number of species present, but the individuals in the community are distributed more equitably among these species. Non-uniform marginal regain of community was clear with applied conditioner. Positive associations, however were observed with conditioner and phyto-assisted clean-up attempts.

Key words: Crude oil pollution, soil conditioners, microbial diversity, phytoremediation, environment.

INTRODUCTION

The oil industry has been a key sector of the Nigerian economy for over 50 years, but many Nigerians have paid a high price (UNEP, 2011) for this sector. It may therefore become pertinent to provide the foundation upon which necessary actions will follow to remedy the complex environmental and sustainable development issues facing people in the Niger Delta. A dynamic age with concomitant increases in crude oil exploration and production across the globe amidst other alternative sources of energy, requires an understanding of microbial diversity to design appropriate and sustainable polluted soil remediation techniques. Techniques devoid of basic considerations for microbial community structure are flawed (McArthur, 2006). Nwaichi et al. (2011) described the rhizospheric interactions with phytoremediation, the use of plants to remove, degrade or separate hazardous substances. Biodiversity is the extent of variation of life forms within a given ecosystem and are interrelated with genetic diversity. It is often used as a measure of the health of biological systems and has been employed to explain natural environment, nature conservation and extinction concern observed in the last decades of the

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Table 1. Initial soil character.

Identity	Polluted	Unpolluted (control)
PAHs (mg/kg)	0.424±0.009	0.077±0.002
THC (mg/kg)	378.3±5.001	64.8±0.821
BTEX (mg/kg)	<0.001	<0.001
As (mg/kg)	1.25±0.002	0.49±0.002
Cd (m/kg)	17.20±0.211	<0.001
Cr (mg/kg)	30.00±0.184	18.70±0.007
Fe (mg/kg)	20,642,50±18.652	16,657.50±24.127
Cu (mg/kg)	9.30±0.072	7.75±0.004
Pb (mg/kg)	86.20±4.440	45.30±2.008
TOC (%)	3.08±0.001	0.96±0.005
Cl^-	400±8.561	80±3.332
NO_3^- (mg/kg)	7.75±0.124	26.25±3.005
SO_4^{2-} (mg/kg)	215.00±5.112	305.00±8.190
N (%)	0.64±0.009	2.13±0.005
P (mg/kg)	0.67±0.002	26.30±2.883
K (cmol/kg)	1.612±0.087	0.068±0.001
Ca (cmol/kg)	<0.01	<0.01
Mg (cmol/kg)	4.721±0.461	0.682±0.011
Na (cmol/kg)	0.346±0.006	0.118±0.002
pH	3.85±0.052	5.45±0.088
Temp. (°C)	28.2±2.991	27.2±3.122

20th century. The word 'biodiversity' may have been coined by Rosen (1985) while planning the National Forum on Biological Diversity organized by the National Research Council (NRC) which was to be held in 1986, and first appeared in a publication in 1988 when entomologist E. O. Wilson used it as the title of the proceedings of that forum (Biodiversity, 2013).

Robe et al. (2003) described prokaryotes as the most ubiquitous organisms on earth, represented in all habitats, including soil, sediment, marine and terrestrial subsurface, animals and plant tissues. The further underscored their key role in the biogeochemical cycles of the biosphere and represent an enormous reservoir of novel valuable molecules for health or industry. Ecological diversity measurements (Sahney et al., 2010) are necessary to understand survival and adaptability of species.

Crude oil polluted soils suffer compaction (Nwaichi et al., 2010), which impedes growth, decreasing the ability of plants to take up nutrients and water (Parrish et al., 2004; Rezek et al., 2009). Soil conditioners, which are products added to soil to improve the soil's physical qualities, especially their ability to provide nutrition for plants, have relieved such soils as reported by Johnson et al. (2009) and Rezek et al. (2009), and in particular, may stimulate microbiological activity, increase nutrient levels, add more loft and texture to soil and improve plant survival rates to sustain natural cycle. Similar studies have few documentation on the fate on phytoremediation, especially of hydroarbon polluted sites.

Given recorded successes from molecular biological methodologies in microbial ecology studies, we attempted to utilize t-RFLP to characterize community dynamics and changes in community structure in response to changes in prevailing physicochemical parameters due to crude oil pollution and accompanying conditioners at phytoremediation sites.

MATERIALS AND METHODS

Description of study area

The study area consists of a two week old crude oil spilled (which caught fire afterwards) soil in Oshie community in the Niger Delta region of Nigeria. This community is a host to a major oil company in Nigeria. Eye witnesses suspect sabotage as the cause of spill. Soils (20 cm depth) from this site were randomly collected and bulked following a field survey, characterized and set up in a 90 days phytoremediation pot experiments (in replicates of three) using *Vigna subterranean* (Bambara), *Hevea brassilensis* (Rubber), *Cymbogonium citratus* (Lemon grass) and *Fimbristylis litoralis* (Fimbristylis). An agricultural soil in the same region with history of no pollution constituted control regimes. Chemical and physical characterization of control and polluted soils (Table 1) were done before the start of the experiment to determine appropriate soil conditioners to be used. Watering was based on need. At 90 days, plants were harvested and rhizopheric soils were collected for laboratory analyses. These samples were transported in ice chest coolers to Institute of Agrophysics Lublin Poland for analysis. Watering was on need basis. Different soil regimes ranging from planted polluted and unpolluted, and organic manure - amended

variants were subjected to preparations for various analyses discussed below.

Soil conditioning

Organic manure (poultry dung) and inorganic manure (NPK 20:10:10) were added for augumentation and comparison, and the amount was determined using the method of Akobundu (1987), C = (R × A)/Q (where C = amount of amendment, R = 2 = a constant, A = weight of soil, and Q = product weight of substance. Initial Physicochemistry for polluted and unpolluted soils. Air - dried screen (2 mm) soils were subjected to gas chromatographic (HP Gas Chromatograph 5890 Series ii, using dichloromethane as extraction solvent) analysis for Polycyclic aromatic hydrocarbons (PAH), Atomic absorption spectrophotometric analysis for heavy metals, Kjeldahl method for Total Nitrogen and portable meters as reported by Nwaichi et al. (2011).

Extraction of soil DNA

To avoid cross-contamination, Pre- and Post- activities including sample sorting and handling, DNA extraction, bench preparation for PCR, PCR implementation, its product visualization and storage, were physically seperated throughout the study period. Several attempts were made to optimize nucleic acid extraction method, which is important for description of microbial diversity. DNA was isolated from soil samples using FastDNA® SPIN kit for Feces and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA) following manufacturer's protocol. DNA concentration thereafter was determined using Nano Drop 2000 Spectrophotometer (Thermo Scientific). This kit included physical disruption method, glass bead homogenization and freezing-thawing, thus rendering confined bacteria available for lysis treatments (Frostegard et al., 1999), for a better DNA yields.

PCR for AOA (polymerase chain reaction - ammonia oxidizing archea)

Dilution of DNA to concentration of 2 ng/µl in nuclease free water was made and samples centrifuged in 'short' mode at 12000rcf. Into 0.1 ml PCR tubes, 28 µl reaction mix (15 µl SIGMA ReadyMixTM REDTaq® PCR Reaction mix with $MgCl_2$, 0.5 µl Primers AOA 19F and 0.5 µl AOA A643R and 12 µl kit water) was added to 2 µl diluted DNA, then centrifuged. A fluorophore, 6 - carboxyfluorescein (6 FAM) was used to label primer for AOA amplification. For effective hybridization of the PCR amplified 16S rDNA gene, PCR conditions on AB Applied Biosystems Veriti 96 Well Fast Thermocycler, were set to: Stage 1(Denaturation) -95^{o}C, 5 min; Stage 2 - 92°C, 45 s (Primer Annealing), 59°C, 30 s, 72°C, 1 min (Primer Extension) x 35cycles; Stage 3(Cycling concludes with final extension) - 72°C, 7 min, 4.0°C and ∞) was set. Sample was loaded, and run completely, then visualized on Agarose gel electrophoresis (AGE) as described by Traugott (2006). Samples reamplifications were done when some signs of contamination were observed.

PCR products purification

ExoSAP-IT® for ABI Affymetrix® USB® products (Source: Exonuclease 1- Recombinant) supplied in special buffer, was added to amplicones in the ratio of 2:5 in 0.1 ml PCR tubes and vortexed. In thermal cycler (37°C, 15 min, 80°C, 15 min), incubation was done. Centrifugation at 750 rcf for 4 min was performed before and after product transfer into catridges. Catridges were discarded and purified DNA (Young et al., 1993) was ready. DNA concen-ration was again measured to work out volumes.

Digestion of amplicons using restriction endonuclease

To 0.1 ml tube, 5µl of amplicone, 0.6 µl of Csp enzymes and 0.6 µl of TANGO buffer were added (following master mix method). Nuclease free water was added to 10 µl reaction volume while pipette - mixing and short centrifuged. Incubation in thermal cycler (37°C, 2 h: digestion; 65°C, 20 min: inactivation) followed.

Terminal restriction fragment length poly- morphism (t-RFLP) AOA

This was done using a DNA sequencer (AB Applied Biosystems HITACHI 3130). Master mix of 9 µl HiDi formamide and 0.5 µl Liz standard was centrifuged. To MicrAmpTM Optical 96-well Reaction Plate, 9.5 µl mix and add 1 µl of products after restriction were mixed, centrifuged at 800 rpm for 3 min to clear bubbles. Products were cooled on ice after incubation in Thermal block (95 C, 3 min for denaturation). Sequencer manufacturer's protocol was followed and all experiments were done in replicates of three. The size, in basepairs, of terminal restriction fragments (T-RFs) was determined thereof. T-RFs with a size < 40 bp and 1% area were excluded from analyses.

Statistical analyses

ANOVA, data (n=3) mean comparisons, Principal component and factor analyses were elaborately done using STATISTICA v 10.

RESULTS AND DISCUSSION

Initial soil asessment revealed that PAHs, Cd, As and Pb polluted soil and phytoremediation technique recovered the soil to a certain degree (data not shown) for plants and microorganisms to survive and thrive. Data obtained for control soil further shows that most soils in the Niger Delta are contaminated residually even when there is no history of pollution in the area (Table 1). High Cd levels may have arisen from complex nature of spill, and later, fire due to perceived sabotage. Genetic diversity, the level of biodiversity, refers to the total number of genetic characteristics in the genetic makeup of a species. Multivariate analysis of resolved t-RFLP fingerprints (Figure 1) showed nine active communities in Bambara - cultivated control soils and these were drastically reduced to 3 with crude oil pollution under same condition. LaMontagne et al. (2002) described its relevance in visualising relationships among fingerprints as done in this study. Although organic amendments only marginally increased community by one, species benefited in terms of growth. Presence of an internal standard (GeneScan-1000 ROX) in each sample, was useful in quantifying variation, not only in terms of the size, in basepairs of terminal restriction fragments, but also in terms of the relative proportions of each fragment in a community profile. For Rubber - cultivated regimes, species were severely impacted and a community shift was observed.

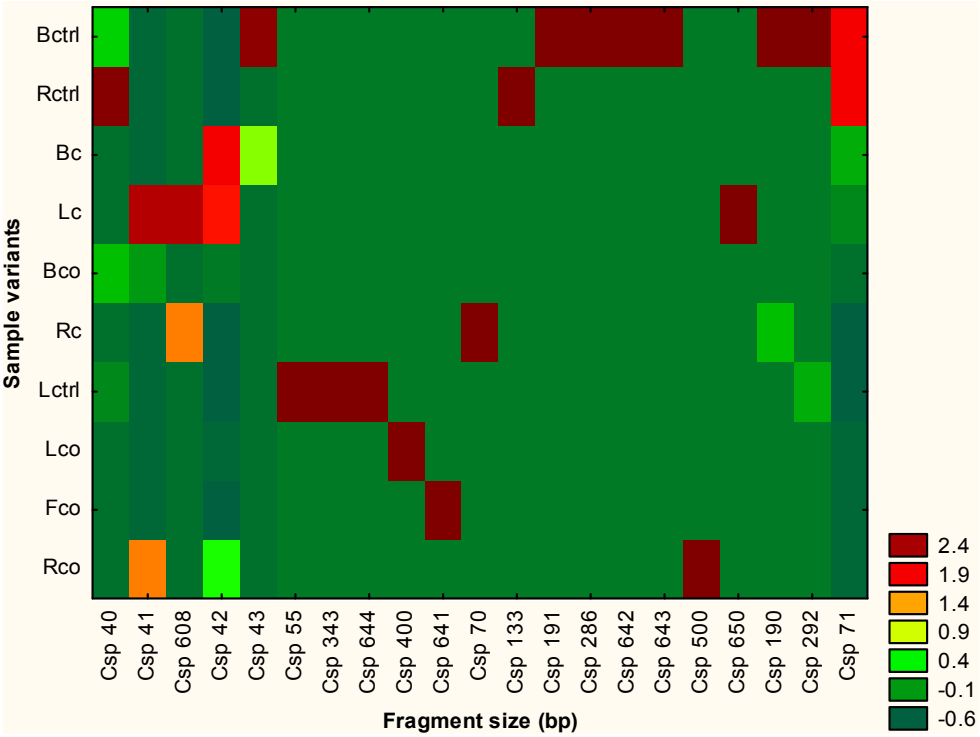

Figure 1. Multivariate analysis of t-RFLP profile. F, B, R and L denote soils planted with Fimbristylis, Bambara, Rubber and Lemon grass plants; attached ctrl, c, and co represent uncontaminated, contaminated and unamended, and contaminated and organic manure amended soils, respectively.

Lankau (2007) of the National Science Foundation found that diversity within a species is necessary to maintain diversity among species, and vice versa. Application of organic amendments indicated regain of a soil microbial community within 90 days. This could lead a loss of biological diversity (NBII, 2013). A significant reduction in community was also observed with contamination for Lemon grass nutrient - unamended cultivated soil but regain of community with organic conditioner, was marginal (10%) at 90 days. Consequently, the structure of the soil bacterial assemblage changes with hydrocarbon stress and a change of dominant forms occurs and the genetic diversity of prokaryotes decreases (Rivera and Lake, 2004). Depending on choice of plant for phytoremediation of polluted soil, considerable regain of communities could be established as seen with *C. citratus* and *F. Littoralis* - cultivated soil communities. For unamended and control regimes, fragments for Fimbristylis -cultivated soils were either < 40 bp or < 1% area and were excluded for analysis.

A more detailed analysis of the ecological parameters of the assemblage of prokaryotes (Figure 2) showed the highest genetic diversity, determined by Shannon's H index in control soil where Lemon grass was grown and the lowest in the contaminated but amended soil cultivated with Fimbristylis. To make these index numbers more biologically sensible (Tuomisto, 2010), they were converted to the effective number of types (ENT), which is the real biodiversity to allow for comparison of the biodiversity with other communities. This means that these extreme communities with Shannon index of 1.66 and 0.68 have equivalent diversity as communities with 5 and 2 equally-common species, respectively. Consistent high Shannon H values for Bambara cultivated rhizospheric soils may be be related to its family, leguminosae. From Figure 2, our results show that the diversity and evenness in organic manure amended contaminated regimes are much higher than in the unamended counterpart under similar conditions. Equitability index, EH, which is interpreted as the number of equally abundant species necessary to produce the same diversity as observed in a sample, showed dissimilar number of individuals in study community. Obasi et al. (2013) related high equitability values or Pileon's evenness index to relatively low human activities.

Nutrient recharge (Table 2) of the community had a beneficial effect and agrees with the findings of Patyka and Kruglov (2008), who identified increased species richness with systematic increase of manure. The amended stressed soils not only has a greater number of species present, but the individuals in the community are distributed more equitably among these species. Pearson's

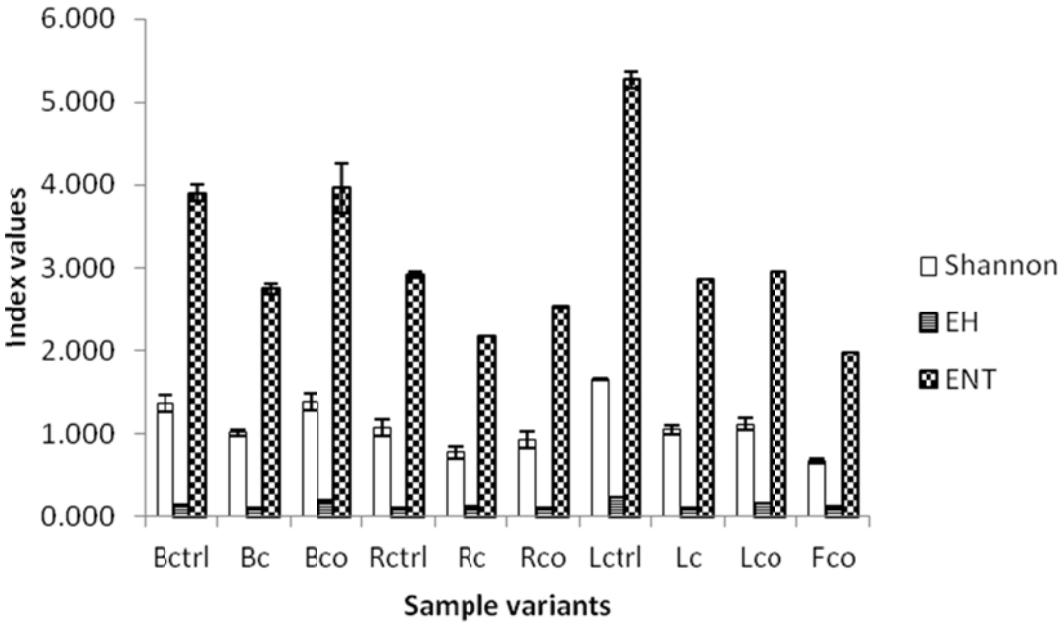

Figure 2. Ecological parameters of prokaryote assemblage of tested soils. EH, ENT, B, L, R and F denotes Equitability index, effective number of types of species, Bambara, Lemon grass, Rubber, and Fimbristylis - cultivated; attached ctrl, c, and co denotes control (unpolluted), crude oil polluted and organically amended - crude oil polluted agricultural soils respectively.

Table 2. Mean level of primary nutrients after phytoremediation.

Nutrient	Soil variants											
	Unpolluted				Polluted and unamended				Polluted but amended			
	F	B	R	L	F	B	R	L	F	B	R	L
NO_3-N (mg/kg)	10.02	21.40	10.98	10.66	26.40	44.20	26.15	10.45	32.42	40.60	32.89	18.00
P (mg/kg)	7.13	17.10	31.39	9.22	18.33	30.67	16.00	13.42	40.15	40.00	38.00	43.33
K (cmol/kg)	0.60	0.89	0. 86	1.49	0.39	0.54	1.94	1.06	0.99	1.57	2.28	2.14

B, L, R and F denotes Bambara, Lemon grass, Rubber and Fimbristylis - cultivated.

Table 3. Pearson's correlation coefficient of Cr removal versus total Expected number of species types, ENT.

Cr removal	ENT			
	Fimb	Bam	Rub	Lem
Fimb	-0.55			
Bam		-0.56		
Rub			-0.58	
Lem				-0.97

Fimb, Bam, Rub and Lem represent *F. Littoralis, V. Subterranea, H. Brasilensis* and *C. Citratus* respectively.

correlation coefficient of Cr removal versus total Expected number of species types, ENT (Table 3) shows moderate negative correlation *for F. Littoralis, V. Subterranea* and *H. Brassilensis* - cultivated soils, and near perfect negative correlation for soils cultivated with *C. citratus*. This implies that increase in performance of plants at remediation of polluted soil is tantamount to increased expected number of types of species. In other

words, increased contaminant load reduced the expected number of types of species in a soil community.

Conclusion

Different levels of disturbance gave different effects on abounding microbial diversity. In order to preserve biodiversity in a given distorted environment, it is important to understand the levels and types of available nutrients and inherent limitations. Abundance and evenness of the species present in study site, given different life - lines (organic amendments and phytoremediation) as indicated by Shannon index were minimally regained. We believe that there is a high tendency of genetic characteristics to vary with time. Natural nodule - level association could be explored in disturbed soil recovery. Generally, low equitability index, EH indicates that all species in the community are represented by a dissimilar number of individuals.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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