academicJournals

Vol. 14(19), pp. 1632-1639, 13 May, 2015
DOI: 10.5897/AJB2015.14532
Article Number: C71707B52856
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Evaluation of soil microbial communities as influenced by crude oil pollution

Eucharia Oluchi Nwaichi[1]* and Magdalena Frac[1,2]

[1]University of Port Harcourt Port Harcourt Nigeria.
[1,2]Institute of Agrophysics, Felin Lublin Poland.

Received 27 February, 2015; Accepted 27 April, 2015

Impact of petroleum pollution in a vulnerable Niger Delta ecosystem was investigated to assess interactions in a first-generation phytoremediation site of a crude oil freshly-spilled agricultural soil. Community-level approach for assessing patterns of sole carbon-source utilization by mixed microbial samples was employed to differentiate spatial and temporal changes in the soil microbial communities. Genetic diversity and phenotypic expressions were measured for a more holistic perspective. The 5'-terminal restriction fragments generated after Csp digestion of 16S rRNA gene correlated with observed DNA concentrations in the community profile and revealed loss of diversity with pollution. Crude oil pollution significantly reduced phosphomonoesterases and respiratory activities and values were pH dependent. There were no expressed dehydrogenases activity in initial spill site but were enhanced with phytoremediation. Factor analysis of predictors and independent variables indicates that respiratory, alkaline phosphatase and β-glucosidase activities could be used to explain underlying factors. Positive soil – microbes - plant interactions were observed.

Key words: Species diversity, impact of crude oil pollution, soil – biota interactions, ecosystem monitoring, genetic diversity.

INTRODUCTION

Ecosystem functioning as described by Green et al. (2004), is incomplete without soil micorganisms, as they affect the chemical, biological and physical characteristics of soil. The anthropogenic influences, stemming from increased industrialization, oil exploration activities, sabotage and illegal refining of petroleum (Nwaichi et al., 2010) in oil – rich regions, on biogeochemical cycles could impede on soil quality evaluation (Li et al., 2005). Phytoremediation, the use of plants to remove, degrade or separate hazardous substances, has been described by Nwaichi et al. (2011) to rely simply on the plant capability to accumulate large quantities of a certain contaminants or to take up and transpire large amount of water from soil and

*Corresponding author. E-mail: nodullm@yahoo.com. Tel: +48605625137.

Abbreviations: TTC, 2,3,5-Triphenyltetrazolium chloride; **TPF,** triphenyl formazan; **PNP,** para-nitro phenyl phosphate; **PCR,** polymer chain reaction; **ENT,** effective number of types.

groundwater. They also reported the possible contributions from production and secretion of plant enzymes, or stimulation of microbial biotransformation within the rhizosphere to this emerging and environmentally – friendly technique. The study of complex diversity pattern of the microbial community in parched and inundated soil environment has proven to be a herculean task. This may have arisen from inadequate biological classification of such environment. Ovreas (2000) described species diversity to consist in species richness, total of species present, evenness, and the distribution and these complement genetic diversity and ecosystem biodiversity in understanding soil microbia diversity. A wholiststic understanding of soil microbial communities have been broadly defined by biochemical-based techniques and molecular-based techniques (Kirk et al., 2004; Abbasi et al., 2010). Biochemical methods accounted for their phenotypic expressions (e.g., respiration, enzymes and catabolic potential) while the use of signature lipid biomarkers (SLB), like phospholipid fatty acids (PLFA), and molecular biology (nucleic acid technologies) gives information on the microbial community composition based on groupings of fatty acids (Broughton and Gross, 2000) and genetic diversity, respectively. Molecular methods have the advantage of obtaining information about uncultivable organisms and can be applied to study complex trophic interactions in the field and to address underlying ecological questions.

In order to evaluate various strategies employed by microorganisms to adapt to changed environmental conditions under wide perturbations, this study seeks to evaluate the impact of crude oil pollution on soil microbial community, relevant biochemical indices, possible interactions thereof and the effects of phytoremediation on such ecosystem.

MATERIALS AND METHODS

The study area consists of a two – week old crude oil spilled (and burnt 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 susppects sabotage as the cause of spill. 20 cm depth soils from this site were randomly collected (using soil auger) and bulked following a field survey and set up in a phytoremediation pot experiments (in replicates of three) using *Vigna subterranea, Hevea brasilensis, Cymbopogom citratus* and *Fimbristylis littoralis*. *F. Littoralis* and *H. Brasilensis* were chosen for their prevalence in study area while the use of *V. subterranea* has been reported by Nwaichi et al. (2010; 2014). Black polyethylene bags were used for potting and free drains were made possible for the growth period by narrow perforations at the base, to avoid water logging in pots. pH was determined on site using Hanna micro pH meter by probe insertion in soil soil solution (1:5). Temperature was not regulated to mimick natural environments. Seedlings for *V. subterranea* and *H. brasilensis* were raised on moist cotton spread to exclude contamination while young *C. citratus* and *F. littoralis* were sourced from University of Port Harcourt botanical garden. An agricultural soil in the same region with history of no pollution constituted control regimes. Chemical and physical characterization of control and polluted soils were performed before the start of the experiment. After 90 days with watering, based on need, plants were harvested and rhizopheric soils were collected for laboratory analyses. These were transported in ice chests to Institute of Agrophysics Poland for analysis. Different soil regimes (2 mm screen and air dried) from planted and unplanted, polluted and unpolluted variants were subjected to preparations for various analyses discussed: Assay for protease activity was adapted from reported protocol by Alef and Nannipieri (1995). In order to determine the amino acids released after incubation of soil with sodium caseinate for 2 h at 50°C using Folin-Ciocalteu reagent, 5 ml of 1% substrate (sodium caseinate in TRIS HCl buffer pH 8.1 (prepared one night before and kept in fridge), was added into test only. Only 5 ml of TRIS HCl buffer pH 8.1 was added to control. After centrifugation for 2 min at 200 rpm, absorbance readings (578 nm) of 96 Corning plates containing 200 µl samples in a Spectrophotometer (Infinite M200 PRO TECAN) were taken with buffer as blank.

Measurement of dehydrogenase activity was adapted from reported protocol by Alef and Nannipieri (1995). This method is based on the estimation of the 2, 3, 5-Triphenyltetrazolium chloride (TTC) reduction rate to Triphenyl formazan (TPF) in soils after incubation at 30°C for 24 h. TTC and TPF are light-sensitive so beaker was shielded and all procedures were performed under diffused light. Results were corrected for control and calculation was done for ρ-nitrophenol per ml of the filtrate by reference to the calibration curve.

Phosphomonoesterases activity assay was adapted from reported protocol by Alef and Nannipieri (1995). The method is based on the determination of p-nitrophenol released after the incubation of soil with p-nitrophenyl phosphate for 1 h at 37°C. To 1 g dry soil, 0.25 ml Toluene was added, then 4 ml buffer after 10 min in a fume chamber for tests and control. To test, 1 ml substrate (para-nitro phenyl phosphate, PNP) was introduced and all samples incubated for 1 h at 37°C. Thereafter, 1 ml substrate was added to control. An aliquot of 1 ml 0.5 M $CaCl_2$ and 4 ml 0.5 M NaOH were added to test and control and shaken with Multi RS – 60 BIOSAN Programmable rotator-mixer/ shaker for 3 min and were centrifuged (Ependorf Centrifuge 5810R) at 4000 rpm for 10 min. Microplates containing 200 µl samples were read off in a spectrophotometer at 485 nm using buffer as blank. For buffer preparation, correction of 0. M HCl and 1 M NaOH mixture were corrected to pH 6.5 and 11 for acid and alkaline phosphatases, respectively.

Respiratory activity determination was adapted from reported protocol by Alef and Nannipieri (1995). Substrate (Glucose) induced method was employed. Evenly, 1 ml glucose solution was added onto the soil in respiratory flasks and autoclaved (Fedegari Autoclave AG and Classic Prestige Medical Autoclave) at 121°C for 20 min. Titration was done using 0.1 M HCl and Phenolphthalein indicator, while shaking and titre values were recorded for calculation.

Assay for B- glucosidase activity was adapted from reported protocol by Alef and Nannipieri (1995). This was based on the principle of released ρ-nitrophenol estimation, after soil incubation for 1 h at 37°C. Supernatant (200 µl) in well plates were read off spectrophotometrically at 400 nm. For buffer, 200 µl Modified universal buffer was used.

Community level profiling, CLLP was done using BIOLOG Microstation™ Biotek Instruments USA. One gram soil was added to 99 ml sterile peptone water and calibrated using a peristatic pump, and then autoclaved (SterilClave 18 BHD Caminox 2009) at 121°C for 20 min alongside tips and tubes. On 24 h intervals, 9 daily readings (27°C incubation) were taken. Carbon sources are as provided by manufacturer.

DNA extraction was done immediately following 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). These were stored at -80°.

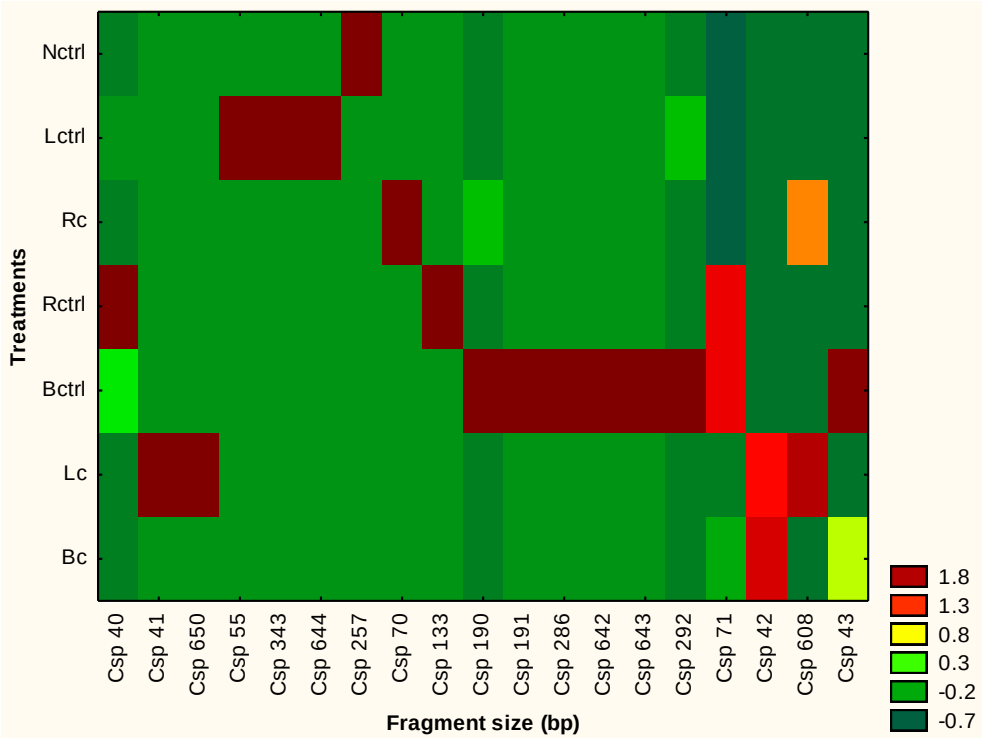

Figure 1. Cluster representation of tRFLP profiles from the study. Ctrl, c, and N denote control, crude oil polluted soils and 'no plant', while, B, L, and R represents Bambara, Lemon grass and Rubber plants respectively.

For PCR 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 12000 rcf. Manual specific instruction for AB Applied Biosystems Veriti 96 Well Fast Thermocycler was followed. ExoSAP-IT® for ABI Affymetrix® USB® products (Source: Exonuclease 1-Recombinant) was used for purification of products and kit procedure was followed. Restriction endonuclease kit protocol was followed for digestion. Optimization of samples were done as described by He et al. (1994), Roux (2003) and Traugott et al. (2006). Terminal restriction fragment length poly- morphism (t-RFLP) AOA was done using a DNA sequencer (AB Applied Biosystems HITACHI 3130) and manufacturer's protocol was followed. T-RFs with a size < 40 bp and 1% area were excluded from analyses.

Analysis of variance for data for triplicate determinations, principal component and factor analyses were elaborately done using STATISTICA v 10.

RESULTS AND DISCUSSION

From the results of the molecular technique based on polymer chain reaction (PCR), considerable differences between variants both with respect to the number of detected taxonomic units and to the structure of their distribution (Figure 1) were seen. In the polluted soils, there were fewer communities in comparison to unpolluted counterparts and could be employed in unravelling the numerical structure. Similar comunity distribution were seen for *H. brasilensis* cultivated soils, possibly because it is a native species to the study site where polluted soils were sourced. In a similar study but with PCB contamination, Patyka and Kruglov (2008) made similar observations. For comparison, the T- RFLP profile analysis of DNA extracted from control soil (Figure 1), in which levels of Hydrocarbons contaminants are negligible, differed considerably in both number and sizes of T-RFs and in their peak heights. Generally, DNA concentrations were observed to correlate with fragments present in the community profile, per treatment (data not shown).

Therefore, these peak heights, are reflective of the relative abundance of those ribotypes that are preferentially amplified during PCR and to an extent, may give an insight on the relative abundance of those ribotypes in the community. This is subject to any latent bias from PCR amplification. A more detailed analysis of the ecological parameters of the assemblage of prokaryotes showed the highest genetic diversity, determined by Shannon's index, in soil where Lemon grass was grown in an unpolluted soil and the lowest in the initial agricultural soil adopted as control, which was left unplanted. In this study, the equitability (evenness) index was relatively low and decreased with the area (Figure 1) occupied by the community. This means that the diversity and evenness in this experiment from the contaminants undisturbed habitat are much higher than in those from contaminants highly disturbed habitat. The

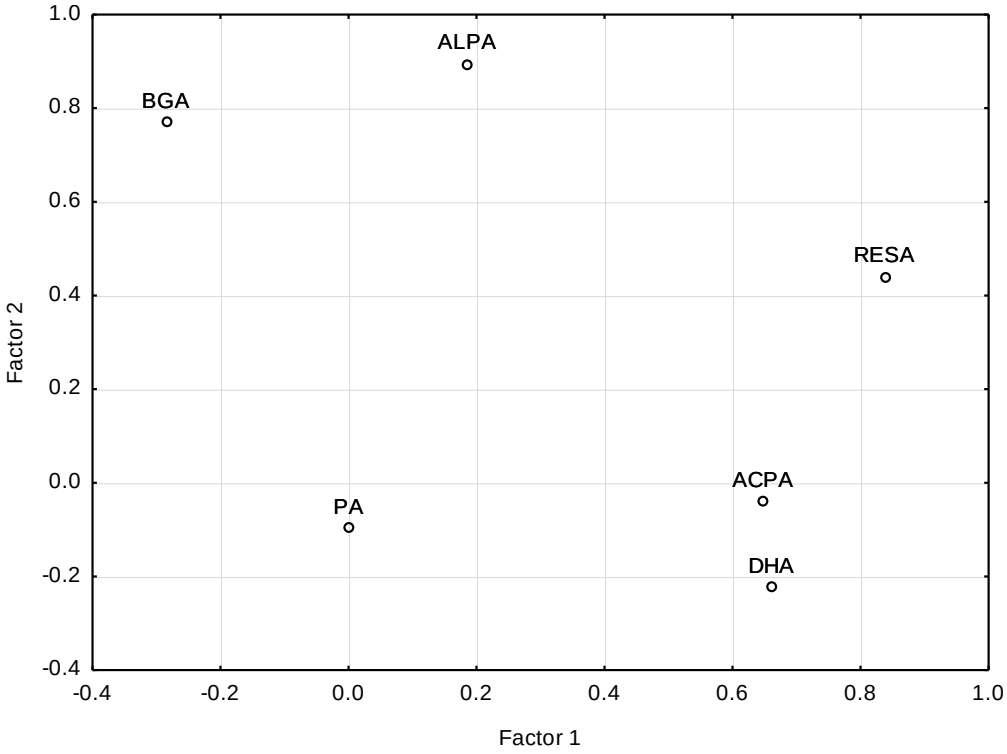

Figure 2. Factor analysis of predictors and independent variables for biochemical parameters. PA, DHA, ACPA, BGA, ALPA and RESA denote protease, dehydrogenase, acid phosphatase, β – glucosidase, alkaline phosphatase and respiratory activities respectively.

advantages with plant – soil - microbial interactions cannot be overemphasized. The unpolluted planted soils not only have greater number of species present, but the individuals in the community are distributed more equitably among these species. True diversity (Tuomisto, 2010) and effective number of types (ENT), revealed an equivalent diversity as a community with 4, 3 and 5 equally - common species for unpolluted soils cultivated with *V. subterranea, H. brasillensis* and *C. citratus*, respectively. There was a shift however to 3, 2 and 3 with pollution effects for *V. subterranea, H. brasillensis* and *C. citratus* – cultivated regimes, respectively.

Biochemical indices using factor analysis (Figure 2), which identifies "invisible" factors that represent the hidden organization or "organizing principle" of whatever is being measured with a number of observable measures (here dehydrogenases, proteases, phosphomonoesterases, β-glucosidases and respiratory activities) underscores the semblance of respiratory, alkaline phosphatase and β-glucosidase activities to hidden factors. Factor scores or "factor loadings" indicate how each "hidden" factor is associated with the "observable" variables used in the analysis (Tucker and MacCallum, 1997). Factor loading of 0.84 indicates that respiratory activity can be used to describe hidden Factor 1; in other words, Factor 1 has characteristics, very similar to respiratory activity. Other observable measures were not useful in describing Factor 1. Similarly, factor loadings of 0.89 and 0.77 indicate that Factor 2 has characteristics, very similar to alkaline phosphatase and β-glucosidase activities. At 95% confidence level, analysis of variance among plants and between treatments indicated significant differences between unplanted polluted and unpolluted soils, *H. brasilensis* and *V. subterranea* - planted (Figure 3) for respiratory activity. Observed acidic soil pH influenced recorded phosphomonoesterase activity. Uniform activity were recorded for dehydrogenases and were more marked between planted and unplanted regimes (Figure 4) in *H. brasilensis* - planted unpolluted soils. This again, is attributed to low pH values with least value recorded in unplanted polluted soil.

In the BIOLOG system, 95 different carbon sources were used to produce a metabolic profile of microorganisms. The profiles obtained using community samples were differentiated properly by statistical analyses. Diversity index measurements, as well as PCA analysis were done for a higher degrees of resolution (Balser, 2000) between soils in order not to loose some details. Statistical evalution of average well colour development shows significant differences in community spread (Figure 5) for polluted and unpolluted regimes especially between communities in planted and unplanted soils. Marked differences were recorded

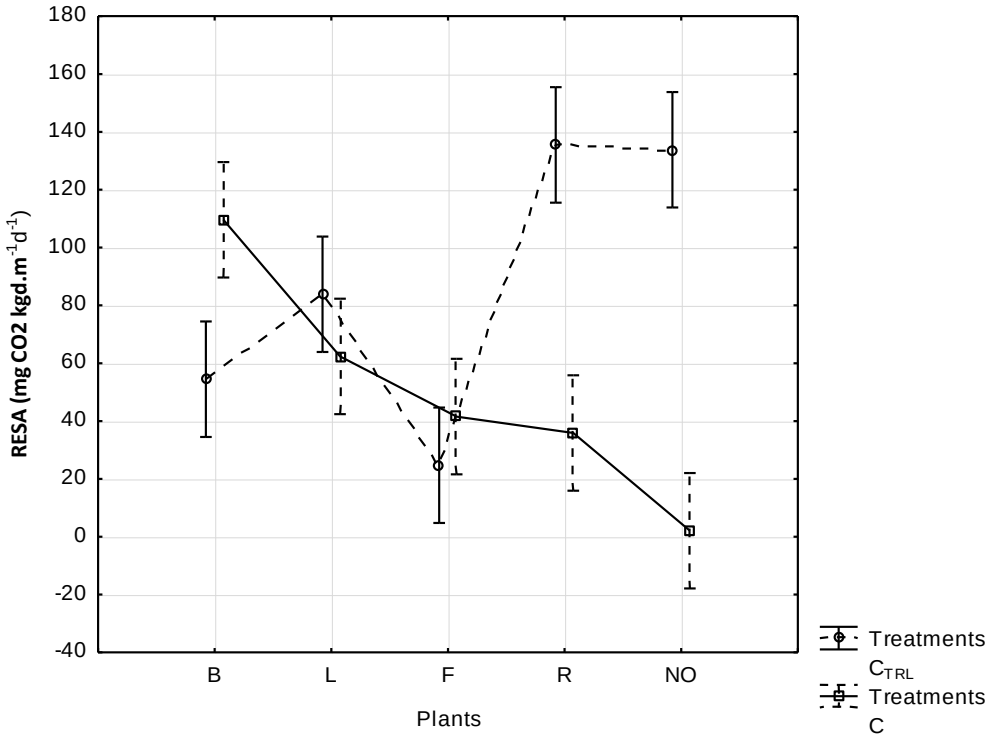

Figure 3. Respiratory activity among plants and between treatments. B, L, F, R, NO, and RESA denote Bambara, lemon grass, Fimbristylis, rubber, unplanted and respiratory activity.

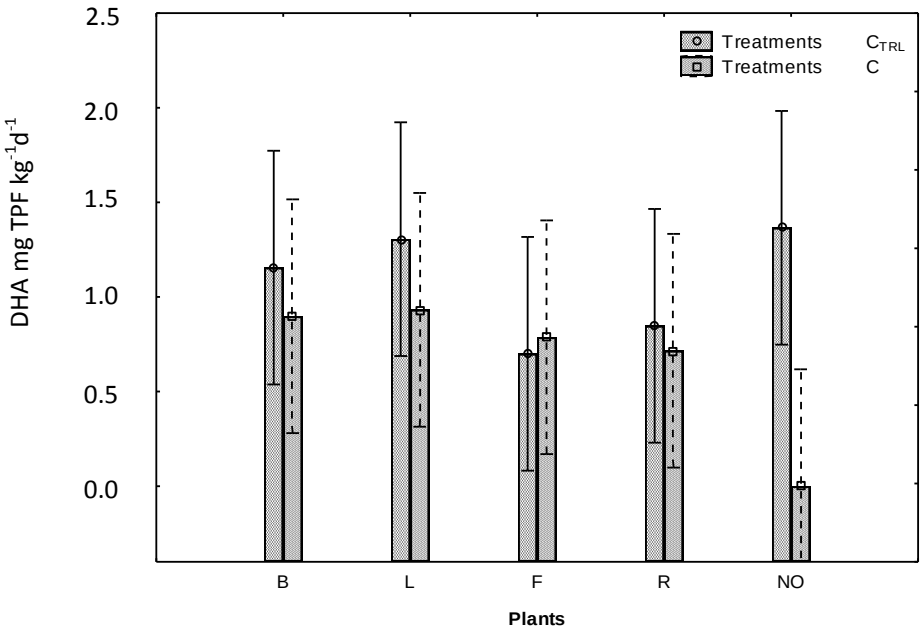

Figure 4. Dehydrogenase activity among plants and between treatments. B, L, F, R, NO, and DHA denote bambara, lemon grass, Fimbristylis, rubber, unplanted and dehydrogenases activity, respectively.

(Figure 6) among cultivated and uncultivated communities. The diversity (Figure 7) and evenness (not shown) of species in this study showed wide distortions due to contamination and plant type and were higher in

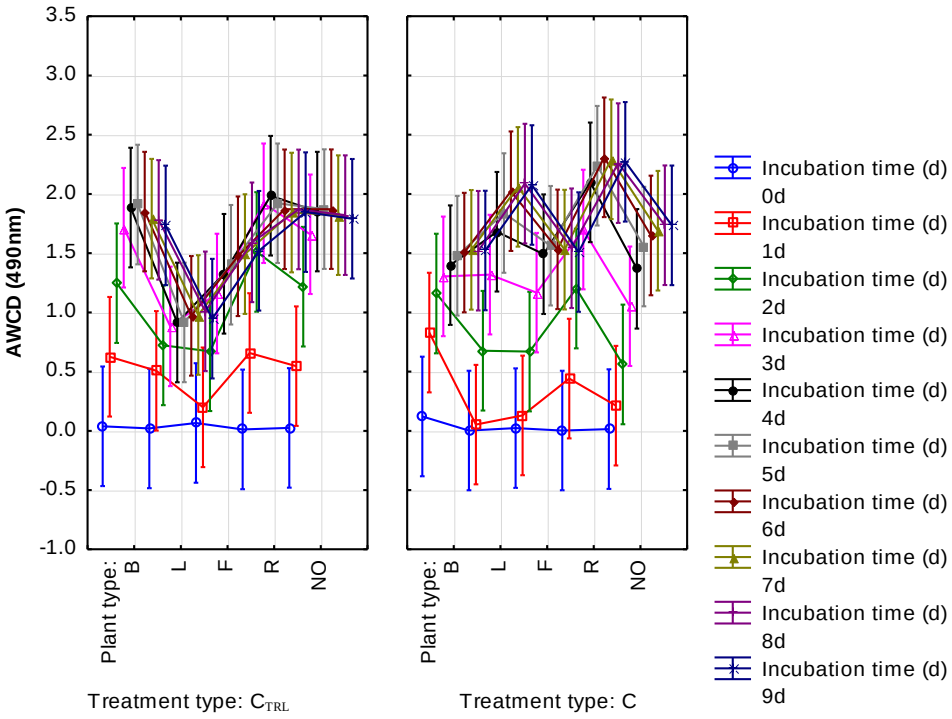

Figure 5. Variation of AWCD with time among plants and treatments. B, L, F, R, NO, and AWCD denote bambara, lemon grass, Fimbristylis, rubber, unplanted and average well colour development, respectively.

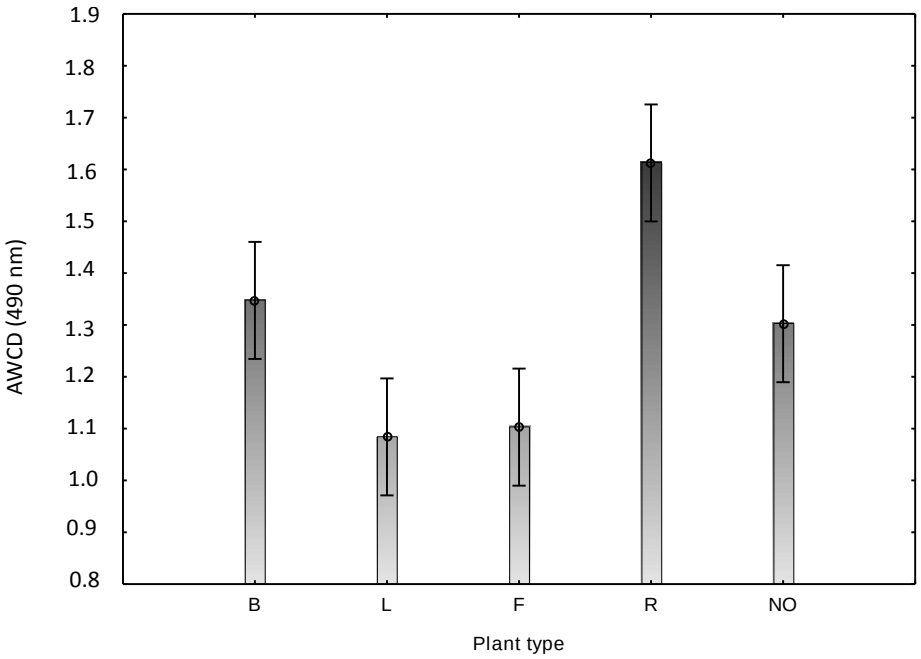

Figure 6. Colour development spread by plants. Vertical bars denote 0.95 confidence intervals. B, L, F, R, NO, and AWCD denote Bambara, Lemon grass, Fimbristylis, Rubber, unplanted and average well colour development, respectively.

communities from control planted soils closely followed by those of *H. brasilensis* and *F. littoralis* in polluted soils. It is worthy to mention that the two latter plants grow naturally in the polluted community, where test was

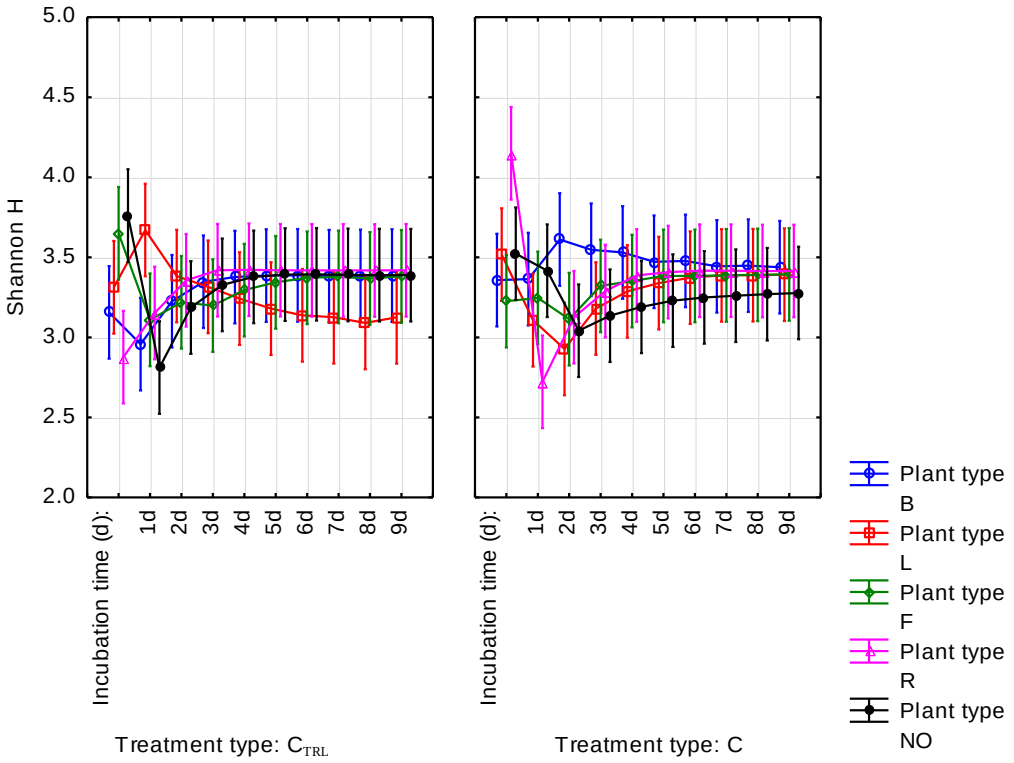

Figure 7. Species diversity characterization in a observed community. B, L, F, R, and NO denote bambara, lemon grass, Fimbristylis, Rubber, and unplanted regimes respectively.

sorced. Shape – wise, the dendrogram based on the result of the cluster analysis applied to the profiles of polluted soil and also appropriately reflects the difference in the origins of the samples (Figure 5). Near neutral buffered pH as reported by Liu et al. (2006), which is quite different from the acidic pH of study soils (Table 1) may limit some microorganisms that have adapted well to native soils. Such condition may have presented disadvantages in evaluation of soil microbial community structure. Although, retarded growth has been reported with crude oil pollution, Harper (1939) reported stimulated growth with low level of contamination. Given high percentage contaminant removal (data not shown) with phyto – assisted soil clean – up employed in this study, phenotypic growth, community increase and improved substrate consumption pattern in relatively cleaned soils corroborates this finding. These genomic data could be exploited to develop mass-target detection systems, that may enable identification of complete gamut of highly generalized biota in envrironmental biodiverse communities.

From data obtained from this study, there were marked shifts in the genotypic structure of soil microbial assemblage with crude oil pollution and diverse interactions were observed after a phytoremediation experiment. Community level physiological profiling generally, revealed significant changes due to contami-

Table 1. Chemical and physical characterization of control and polluted soils.

Identity	Polluted	Control
PAHs (mg/kg)	0.424	0.077
THC (mg/kg)	378.3	64.8
BTEX (mg/kg)	<0.001	<0.001
As (mg/kg)	1.25	0.49
Cd (mg/kg)	17.2	<0.001
Cr (mg/kg)	30.00	18.70
Fe (mg/kg)	20,642,50	16,657.50
Cu (mg/kg)	9.30	7.75
Pb (mg/kg)	806.20	400.30
TOC (%)	3.081	0.955
Cl^-	400	80
NO_3^- (mg/kg)	7.75	26.25
SO_4^{2-} (mg/kg)	215.00	305.00
N (%)	0.64	2.13
P (mg/kg)	0.67	26.30
K (cmol/kg)	1.612	0.068
Ca (cmol/kg)	<0.01	<0.01
Mg (cmol/kg)	4.721	0.682
Na (cmol/kg)	0.346	0.118
pH	3.45	5.45
Temp. (°C)	28.2	28.2

nation in metabolic diversity of mixed microbial communities. Limtations to this study may include PCR bias with DNA extractions and difficulty with quantifying some less dominant microorganisms in the community, as they might not be detected without fractionation. Also, the substrate concentration in the well of the BIOLOG plate may be much higher than that usually found in such parched environment.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

Acknowledgement is due to UNESCO LO'real International Fellowship FWIS 2013 for financing this project. We also appreciate the assistance from the Vice Chancellor of the University of Port Harcourt, Prof. J. A. Ajienka and the Director of the Institute of Agrophysics, Prof. Joseph Horabik.

REFERENCES

Abbasi FM, Ahmad H, Perveen F, Inamullah, Sajid M, Brar DS (2010). Assesment of genomic relationship between Oryza sativa and Oryza australiensis. Afr. J. Biotechnol. 9(12):1312-1316.

Alef K. and Nannipieri P (1995). B-Glucosidase activity. In: Methods in Applied Soil Microbiology and Biochemistry. Academic Press. Harcourt Brace & Coy publishers. London. pp. 350-352.

Balser TC (2000). .Analytical, methodological, and spatial variability in BiOLOG™ substrate utilization profiles of soil microbial communities. http://balserlab.wikispaces.com/file/view/TCB_ThesisCh2.pdf. Accesed 2 December 2013.

Broughton LC, Gross KL (2000). Patterns of diversity in plant and soil microbial communities along a productivity gradient in a Michigan old-field. Oecologia 125:420-427.

Harper JJ (1939). The effect of natural gas on the growth of microorganisms and accumulation of Nitrogen and Organic matter in the soil. Soil Sci. 48:461-466.

He Q, Marjamäki M, Soini H, Mertsola J and Viljanen MK (1994) Primers are decisive for sensitivity of PCR. BioTechniques. 17:82-87.

Liu B, Jia G, Chen J and Wand G (2006). A Review of Methods for Studying Microbial Diversity in Soils. Pedosphere lS(1):18-24.

Nwaichi EO, Frac M, Peters DE, Akpomiemie BO (2014). Conditioners and significance in t-RFLP Profile of the assemblage of prokaryotic microorganisms in a Niger Deltaic Crude Oil polluted soil. Afr. J. Biotechnol. 13(44):4220-4225.

Nwaichi EO, Onyeike EN, Wegwu MO (2010) Characterization and safety evaluation of the impact of hydrocarbons contaminants on ecological receptors. Bull. Environ. Contam. Toxicol. 85 (2):199-204.

Nwaichi EO, Osuji LC, Onyeike EN (2011).Evaluation and Decontamination of Crude Oil-Polluted Soils using Centrosema pubescen Benth and Amendment-support options. Int J. Phytoremediation 13(4):373-382.

Ovreas L (2000). Population and community level approaches for analyzing microbial diversity in natural environments. Ecol. Lett. 3: 236-251.

Patyka NV, Kruglov YV (2008). TRFLP Profile of the Assemblage of Prokaryotic Microorganisms in Podzolic Soils. Russ. Agric. Sci. 34(6):386-388.

Roux KH (2003). Optimization and troubleshooting in PCR. In: PCR Primer: A Laboratory Manual, 2nd edn (eds Dieffenbach CW, Dveksler GS), Cold Spring Harbor Laboratory Press, New York. pp. 35-41.

Traugott M, Zangerl P, Juen A, Schallhart N and Pfiffner L (2006). Detecting key parasitoids of lepidopteran pests by multiplex PCR. BioControl. 39:39-46.

Tucker L, MacCallum R (1997). Factor Extraction by Matrix Factoring Techniques. In: Exploratory Factor Analysis. UNC RCM. pp. 186-239.

Tuomisto H (2010). A consistent terminology for quantifying species diversity? Yes, it does exist. Oecologia 4:853-860.