SAWDUST ASSISTED BIOREMEDIATION OF PMS HYDROCARBON IMPACTED AGRICULTURAL SOIL IN NIGER DELTA NIGERIA

AMECHI S. NWANKWEGU[1*]

[1]*Department of Applied Microbiology and Brewing, Faculty of biosciences, Nnamdi Azikiwe University PMB, 5025, Awka, Anambra State, Nigeria*

ABSTRACT

Sawdust evaluation in bioremediation of PMS hydrocarbon polluted agricultural soil was carried out. The effect of different parameters; total petroleum hydrocarbon, dehydrogenase activity, optical density and pH on bioremediation performance were evaluated. Studied parameters such as microbial dynamics and hydrocarbon removal were found to be higher in sawdust amended system and differed significantly with control at $p < 0.05$. Constant removal efficiency (96.0 %) was observed in SA option from week 6 while NA proceded relatively slow and produced 71.0 % on week 8 of the study. First order kinetic gave high r^2 values (0.945), first order degradation constant (0.47 d^{-1}), and shorter degradation half-life (1.50 d). *Micrococcus luteus,* and *Rhizopus arrhizus* were isolated in the study which displayed extreme PMS hydrocarbon utilization. The use of sawdust greatly increased biodegradation rate and resulted in effective PMS hydrocarbon clean up. Therefore, sawdust provides alternative source of bioremediation material for abundant hydrocarbon spill.

KEYWORDS: Sawdust, Total petroleum hydrocarbon, Premium motor spirit, Pollution, ALARP point.

AMECHI S. NWANKWEGU
Department of Applied Microbiology and BrewingFaculty of biosciences, Nnamdi Azikiwe University PMB,5025, Awka, Anambra State, Nigeria

*Corresponding Author

INTRODUCTION

Premium motor spirit (PMS) is one of the most widely used petroleum products. It is a mixture of volatile, flammable liquid hydrocarbons derived from petroleum that is used as fuel for internal combustion engines. The boiling point range of premium motor spirit falls between 10° C and 210° C and has the potential to contain several hundred isomers of various hydrocarbons. It was first produced from light naphtha batch distilled from crude oil and liquid condensate from natural gas production. Nowadays, it is prepared by mixing various components produced by refining processes such as atmospheric distillation, polymerization, isomerization, catalytic reforming etc. These are however, important environmental pollutants and sequel to the dangers and high cost inherent in physicochemical cleanup strategies such as evaporation, dispersion, dissolution and photo-oxidation in environmental remediation following concomitant hydrocarbon spill on the lithosphere have gingered the scientific curiosity into a search for new approaches that are cost effective, eco-friendly as well as offers the optimum propensity of complete mineralization of the hydrocarbon contaminants.[1, 2, 3] Hydrocarbon removal using different amendments of both organic and inorganic origins has so far been evaluated.[4] Most of these amendments are not without their limitations and have been found to cause some physical and chemical changes including changes in pH, salinity as well as toxicity to both plants and microbial cells (microbiota) consequently resulting in poor bioremediation outputs.[5,6] Apart from these, some amendments such as inorganic fertilizers [Nitrogen Phosphorus and Potassium (N.P.K), Diammonium tetraoxosulphate iv $(NH_4)_2SO_4$, Ammonium chloride (NH_4Cl)] and other nitrogenous based amendments have been found to cause eutrophication especially in aquatic ecosystem and such high nutrients load phenomenon often provides less opportunity for the hydrocarbon contaminants to be recognized as food source by microorganisms.[7,8] At present there is considerably little information on the use of sawdust in the bioremediation of premium motor spirit contaminated soil. Therefore, the aims of the present study were to investigate the use of saw dust in bioremediation of PMS impacted agricultural soil and optimize the process conditions such as initial pH, dehydrogenase activity, optical density and percentage PMS degradation.

MATERIALS AND METHODS

Sample collection

Hydrocarbon polluted agricultural soil sample from Agip Nigeria limited, Okoroma/Tereke, Nembe Local Government area Bayelsa state, Niger Delta, Nigeria was collected for the entire study. The soil was sieved with I mm sieve and used at 1:1 ratio for the preparation of the composite samples. The soil was further polluted with premium motor spirit (PMS) to adequately instigate a scenario of abundant hydrocarbon spill. Sawdust obtained from commercial wood-workshop in Awka, Nigeria was used as biostimulant and microbial cell immobilizing agent. Soil sample was divided into two parts sawdust amended (SA) and natural attenuation

(NA) which served as the control. 1000 g of the soil each was then polluted with 400 ml PMS obtained from local petrol station inAwka, Nigeria. 500 g of sawdust was amended to SA group only making 50 % sawdust or 2:1 soil- sawdust ratio. No sawdust was added to the NA option. Experiment was set up in aluminum pans of surface area 1253.73 cm^2 and volume 4225.11 cm^3. The experimental design set up is shown in Table 1.

Soil physicochemical assay

The physicochemical characteristics of the soil such as cation exchange capacity (CEC) (mEql/100g), total organic carbon (TOC) (%), particle sizes (%) (sand, clay and silt) and pH prior to further contamination with the PMS hydrocarbon were determined (Table 2). Briefly, the ethylene glycol and pure ethanol method was used in cation exchange capacity determination.For the detection of soil total organic carbon, standard methods of APHA5310 was used. Briefly, 5.0 g weight of soil was heated with an excess volume of standard $K_2Cr_2O_7$ in the presence of Con. H_2SO_4. The soil was slowly digested at the low temperature by the heat of dilution of H_2SO_4 and the organic carbon in the soil was thus oxidized to CO_2. The highest temperature attained by the heat of dilution reaction produced with the addition of concentrated H_2SO_4 was approximately 12°C which is sufficient to oxidize the active forms of the soil organic carbon but not the more inert forms of carbon that may be present. The excess of $K_2Cr_2O_7$ not reduced by organic matter was titrated against a standard solution of ferrous ammonium sulphate in the presence of phosphoric acid and diphenylamine as indicator. Actual measurement of oxidizable organic carbon was converted to percentage organic matter using a constant factor numerically equal to 1.334 and expressed as total organic carbon . The articles sizes analysis and using deionized water, ASTM Type I grade, Amyl alcohol, sodium hexametaphosphate (HMP), 5% dispersing solution was adopted [14].The soil pH was measured using digital pH meter model UB-10A (Denver Instruments, USA).[9-14]

Microbial enumeration

Standard spread plate method using 10-fold serial dilution with 1 g of PMS contaminated soil was used. 0.1 ml aliquots of 10^{-5} dilutions were spread on triplicates of prepared mineral salt agar.[15, 16, 17] This was slightly modified by adding neutral red colour indicator for microbial PMS hydrocarbon degraders. Incubation lasted for 1 – 3 and 1 – 5 days for bacteria and fungi respectively at 28±2°C. Active microbial growths were indicated by zones of clearance and colour changes from yellow to red and were expressed in colony forming unit per gram (CFU/g). Six microbial isolates designated as (ISA - ISF) were obtained. The isolates were subjected to screening assessment for 21days in a prepared mineral salt medium placed in an electric supplied orbital shaker during which isolates (ISD and ISE) gave the best result of PMS degradation and survival. Optical density (at absorbance of 560 nm), total viable count (in colony forming unit per gram CFU/g) and pH were the monitored parameters at every 7 days interval.[18] The two isolates were characterized biochemically in the laboratory and further identified to species level at Centre for Agriculture and Biosciences

International (CABI) microbial identification services (Bakem Lane, Egham Surrey TW20 9TY, United Kingdom) using partial 16S rDNA and ITS rDNA sequencing analyses respectively.

Molecular identification of isolates

Briefly, total of two microbial isolates (bacterium ISD and fungus ISE) were submitted to CABI for microbial identification. A unique CABI reference number (IMI number) was assigned to each of the samples. All the original samples were subjected to purity check. All procedures were validated and processing undertaken in accordance with CABI's in – house method as documented in TPs 61 – 68 and TP70. Procedures involved the following steps: Molecular assays were carried out on each sample using nucleic acid as a template. A proprietary formulation [microLYSIS®-PLUS (MLP) Microzone, UK)] was subjected to the rapid heating and cooling of a thermal cycler, to lyse cells and release deoxyribonucleic acid (DNA). Following DNA extraction, polymerase chain reaction (PCR) was employed to amplify copies of the rDNA in vitro. The quality of the PCR product was assessed by undertaking gel electrophoresis. PCR purification step was carried out to remove unutilized dNTPs, primers, polymerase and other PCR mixture compounds and obtained a highly purified DNA template for sequencing. This procedure also allowed concentration of low yield amplicons. Sequencing reactions were undertaken using BigDye® Terminator v3.1 kit from Applied Biosystems (Life Technologies, UK) which utilizes fluorescent labeling of the chain terminator ddNTPs, to permit sequencing. Removal of excess unincorporated dye terminators was carried out to ensure a problem-free electrophoresis of fluorescently labelled sequencing reaction products on the capillary array AB 3130 Genetic Analyzer (DS1) DyeEx™ 2.0 (Qiagen, UK). Modules containing prehydrated gel-filtration resin were optimized for clean-up of sequencing reactions containing BigDye® terminators. Dye removal was followed by suspension of the purified products in highly deionised formamide Hi-Di™ (Life Technologies, UK) to prevent rapid sample evaporation and secondary structure formation. Samples were loaded onto the AB 3130 Genetic Analyzer and sequencing undertaken to determine the order of the nucleotide bases, adenine, guanine, cytosine, and thymine in the DNA oligonucleotide. Following sequencing, identifications were undertaken by comparing the sequence obtained with those available in European Molecular Biology Laboratory (EMBL) via the European Bioinformatics Institute (EBI).

TPH quantification

3.0 g of soil each withdrawn from SA and NA were sampled in week 0, 1, 2, 4, 6 and 8 for total petroleum hydrocarbon (TPH) using gas chromatography. Briefly, following soil extraction in dichloromethane, the extract was analyzed by gas chromatography using HP Agilant 6890 gas chromatography (Agilant technologies, 610 wharfdale Road, Wokingham Berkshire, United Kingdom) equipped with flame atomization Detector (FID), an Agilent 7673 autosampler and 5 capillary column (15 x 0.25nm), with a normal film thickness of 0.25um, splitless injection method (all in batch). Injection volume was 1uL and injection temperature $350°C$. Helium was used as carrier gas (2ml/mn) and hydrogen used for combustion in FID. Mean TPH values were expressed in mgkg^{-1}. Results are shown in Table 7.

Dehydrogenase activity

This was used to monitor the bioremediation effectiveness as indicator of overall soil microbial activity. Tripenyl tetrazolium chloride (TTC) was used (11). Briefly, the absorbance of formazan was read spectrophotometrically at 485 nm. 1 g of sieved soil was placed in test tubes (15 x 100 mm), mixed with 1 ml of 3% aqueous (w/v) 2,3,5-triphenyl tetrazolium chloride and stirred with a glass rod. After 96 h of incubation (28±2°C), 10 ml of ethanol was added to each test tube and the suspension was vortexed for 30 s. The tubes were then incubated for 1 h to allow suspended soil to settle. The resulting supernatant (5 ml) was carefully transferred to clean test tubes using Pasteur pipettes. Absorbance was read spectrophotometrically at 485 nm. The concentration of formazan was evaluated using extinction coefficient of 15433 Mol cm^{-1}.

Statistical analysis

Each set of data in the experiments conducted was collected in three replicates and the analytical result was the mean of three data sets. The standard deviations (error bars) and statistical differences (5% level of significance) were analyzed by using GraphPad Prism 6® software (full version) (GraphPad Software, CA, USA).

RESULTS

Physicochemical analyses

Parameters analyzed prior to the soil pollution with PMS confirmed that the soil is a good agricultural soil (loam) with optimum pH (6.90±0.80), and effective total microbial activities. The cation exchange capacity which is a measure of the total exchangeable cations in the soil and an index of soil fertility was remarkably high 22.71±1.01 meq/100g, total organic carbon (31.49±0.41 %), total heterotrophic microbes, physical characteristics of the sawdust (pH 8.9±0.23) and PMS (pH 5.1±0.71) as well as soil particle size distributions were eavaluted (Table 2).

Effect of PMS concentrations on microbial growth dynamics

Introduction of the premium motor spirit (PMS) hydrocarbon into the soil microcosms (SA and NA) brought about loss in the microbial cell number between weeks 0 – 2. Active microbial activities resumed between week 4 through week 6 and then decreased from week 8 (Table 3). Screening for isolates with excellent ability in PMS hydrocarbon biodegradation selected ISD and ISE during the 21 days screening study. ISD and ISE produced remarkable absorbance at 560 nm Optical densities (Table 4). ISD and ISE also showed high total viable count relative to the other four isolates. Results are shown in Table 5.pH determination (table not available) of the 21 days screening also corroborated the biodegradative potentials of ISD and ISE. The 16S and ITS rDNA sequencing analyses of two microbial isolates (ISD, a bacterium and ISE, a fungus

respectively) reported that IMI 505079 Identified as *Micrococcus luteus* by 16S rDNA sequence analysis using the FASTA algorithm with the Prokaryote database from EBI. Top matches were made to *Micrococcus luteus* and the validated type strain sequence (AJ536198) gave a match of 99% and IMI 504617 identified as *Rhizopus arrhizus* by ITS rDNA sequence analysis using the FASTA algorithm with the Fungus database from EBI. The sequence obtained from this sample showed top matches at 100% identity to multiple sequences of *Rhizopus arrhizus* and synonyms of this species, including published sequences from reference culture collections e.g. JN942919 (DAOM 178621) published in Schoch C.L. et al. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences of the United States of America 109(16):6241-6. Tables 6 and 7 show the results of biochemical characterization of the selected isolates ISD and ISE prior to molecular identification.

TPH degradation

Residual Total petroleum hydrocarbon (TPH) concentrations monitored gas chromatographically reported least TPH residual from 109663.12 $mgkg^{-1}$ – 4197.89 $mgkg^{-1}$ in SA option throughout the study period where as a larger TPH fraction (31964.46 $mgkg^{-1}$) was still present in the NA system after week 8 (Table 8). Facilitated PMS hydrocarbon biodegradation in SA system began from weeks 4 – 8 and produced 80.0 % and 96.0 % removal efficiencies respectively. The initial biodegradation percentage in SA within week 0 – 2 was observably low thus preceded with 25.0 % and 34.0 % for week 1 and 2 respectively but was yet faster than NA microcosm where extremely low percentage

degradation (12.0 %, 23.0 % and 26.0 %) was reported in week 1 – 4. It is also striking then that the removal efficiency (80.0 %) of SA by week 4 was remarkably higher than the removal efficiency (71.0 %) produced by the NA on the week 8. A very low rate of biodegradation was therefore reported of NA throughout the study period as shown in figure 1.The residual TPH concentration therefore, followed a trend proportional to the microbial dynamics so that at higher bioload level larger fraction of TPH was removed.

Effect of pH on PMS biodegradation

Plan SA maintained slightly alkaline pH while plan NA slightly acidic throughout the study period. pH variations in the studied systems might have been due to the pH status of the different soil additives. It is important to note that the pH of the PMS hydrocarbon was 5.1±0.71 (Table 2) and upon soil pollution, the pH dropped from 6.90±0.80 (pH of typical agricultural soil used in the experiment) to 5.3±0.17 in NA week 0. Again, the saw dust biomass had the pH of 8.9±0.23 thus; the saw dust amended plan SA had the pH of 7.5±0.24 by week 0. Saw dust addition to the polluted soil microcosm resulted to slightly elevated pH relative to the (NA) control (Table 9).

Kinetic parameter

Kinetic modeling using first and second orders was used to evaluate the rate of chemical reaction in term of TPH degradation/ removal with time in the studied systems. Kinetic model of hydrocarbon biodegradation has also been described by.[18, 19, 20, 21, 22] A given experimental study is expected to conform to either of the kinetic orders (first or second). Mathematically, kinetic models are expressed as follows; For the first order reactions Differential rate law.

$$\text{Rate} = \frac{-dC}{dt} = kC^1 = kC$$

Integral rate law

$$\text{Rate} = \frac{-dC}{dt} = kC$$

Rearranging

$$= \frac{dC}{C} = -kdt$$

$$\ln\left(\frac{C_t}{C_0}\right) = -kt \qquad (1)$$

$t_{1/2}$ is a timescale by which the initial concentration is decreased by half of its original value. This can be represented by the following equation.

$$C = \frac{1}{2}C_0$$

After a period of one half-life, t = $t_{1/2}$

$$\frac{C_0/2}{C_0} = \frac{1}{2} = e^{-kt_{1/2}}$$

Taking log of both sides, ($\ln e^x = x$)

$$\ln 0.5 = -kt$$

$$t_{1/2} = \frac{\ln 2}{k} = \frac{0.693}{k} \qquad (2)$$

$$-\frac{dC}{dt} = kC^2$$

Rearranging, $\frac{dC}{C^2} = -kdt$

$$\frac{1}{C_t} - \frac{1}{C_0} = kt$$

$$\therefore \frac{1}{C_t} = kt + \frac{1}{C_0}$$

(3)

In the present study, we estimated the first and second order kinetic model using linear data plots of $\ln C_o/C_t$ and $1/C_t$ versus time (t) derived from equations (1) and (3), respectively (Figs. 2 and 3). The correlation coefficient (r^2 =0.945) value of the first order kinetic ($\ln C_o/C_t$) obtained for treatment plan SA was relatively high approaching 1 with a shorter half-life ($t_{1/2}$ = Ln2/K) of 1.50 days indicating an enhanced bioremediation condition (Table 10). R^2 values generated from the first order kinetic for both treatment plans (SA and NA) were all higher than those of their respective second order kinetics. Hence, first order kinetic generated data which conveniently fitted into the present study. Data plots of $\ln Co/C_t$ and $1/Ct$ versus time in week generated linear regression curves. The first order rate degradation half-life (1.50 days) in the plan SA system at k = 0.47 day^{-1} was absolutely short that it was 2.90 times faster than NA at k = 0.16 day^{-1} and ti_{12} = 4.30 days. The second order degradation half-life calculated from the rate equation $t_{1/2}$ = (1/KC$_o$) where K = biodegradation constant for second order (gmg^{-1}day^{-1}) and C_0 = initial contaminant (PMS hydrocarbon) concentration (mg/kg) revealed that half the PMS concentration was removed in 3.10 days and 26.0 days at k = 3.40 × 10^{-5} gmg^{-1}day^{-1} and 2.80 × 10^{-6} gmg^{-1}day^{-1} for plan SA and NA respectively. Second order rate in SA was therefore, 8.4 times faster than the order rate in NA of the same kinetic order. The first and second order kinetic linear regression curves are shown in figures 2 and 3.

Effect of PMS on Dehydrogenase activity (DHA)
DHA as monitored by absorbance of formazan (pink colour) produced from tripheny tetrazolium chloride (TTC) reduction due to microbial respiration quantified spectrophotometrically at 485 nm indicated that bioremediation was absolutely effective. The 2, 3, 5-tripheny tetrazolium chloride (redox indicator) was enzymatically reduced to red 1, 3, 5-triphenyl formazan by the PMS hydrocarbon degraders' living tissues due to activities of oxidoreductase enzymes class called dehydrogenases. Optimum DHA values in SA were reported from week 4 – 8 (7.90±0.43 mgg^{-1} dry soil /96h, 8.10±0.47 mgg^{-1} dry soil /96h and 8.30±0.33 mgg^{-1} dry soil /96h) respectively. By week 8, saw dust amended SA plan had a DHA of 8.30±0.33 mgg^{-1} dry soil /96h which interestingly had a numerical proximity to the DHA of the uncontaminated agricultural soil (8.60±0.56 mgg^{-1} dry soil /96h). This however, suggested that the biological behaviour of the soil which was previously lost due to PMS hydrocarbon pollution has been restored as early as week 4 through week 8 and significantly aided by the saw dust biomass. The dehydrogenase activity (5.80±0.66) of NA system in week 8 was still very low relative to the typical agricultural soil and this suggested a considerable loss of important soil biological activity in the microcosm. Results are shown in Table 11.

Table 1
Bioremediation design

Experimental group	description
SA	1000 g soil + 400 ml PMS + 500 g sawdust
NA	1000 g soil + 400 ml PMS+ no sawdust added (control)
Each experimental group established in three replicates.	

Table 2
Baseline physicochemical properties of composite samples

Parameter	value
Soil pH	6.90±0.80
Soil dehydrogenase activity DHA (mgg^{-1} dry soil/ 96h)	8.60±0.56
Soil particles sizes	
Sand (%)	1.20±0.13
Clay (%)	77.30±0.51
Silt (%)	21.50±0.42
Texture	loam
Cation exchange capacity CEC (meq/100g)	22.71±1.01
Soil total organic carbon (% TOC)	31.49±0.41
Soil total heterotrophic bacteria THB (10^5 CFU/g)	4.50±1.11
Soil total heterotrophic fungi THF (10^5 CFU/g)	2.0±0.14
Sawdust pH	8.9±0.23
Sawdust total heterotrophic fungi	8.7±0.31
Premium motor spirit (PMS) pH	5.1±0.71
Values are mean of replicate analyses ± S.D	

Table 3
Microbial hydrocarbon degraders

	Week		Plan	
	SA	NA	SA	NA
	(Sawdust)	(Control	(Sawdust)	(Control)
	bacterial count (10^{-5} CFU/g)		*fungal count (10^{-5} CFU/g)*	
0	3.40 ± 0.66^a	2.10 ± 0.56^a	4.80 ± 0.66^a	1.10 ± 0.42^b
1	3.50 ± 0.34^b	2.80 ± 0.71^a	4.80 ± 0.99^a	1.00 ± 0.63^a
2	3.90 ± 0.22^a	2.80 ± 0.16^b	5.60 ± 0.26^b	1.30 ± 0.00^a
4	5.40 ± 0.27^a	3.00 ± 0.11^a	7.40 ± 0.13^a	2.40 ± 0.31^a
6	6.8 ± 0.11^a	3.40 ± 0.13^a	7.80 ± 0.33^a	2.50 ± 0.23^a
8	5.30 ± 0.65^b	3.10 ± 0.14^a	6.80 ± 0.64^b	2.20 ± 0.43^b

Values are mean of replicate analyses ± S.D.
Mean superscripts with different letter differ significantly (P < 0.05)

Table 4
Screening for isolates with PMS degradative potential (OD at 560 nm)

Isolate	Day		
	7	14	21
ISA	0.11 ± 0.01^a	0.10 ± 0.13^a	0.12 ± 0.96^b
ISB	0.21 ± 0.14^a	0.32 ± 0.86^b	0.11 ± 0.14^a
ISC	0.01 ± 1.12^b	0.13 ± 0.31^a	0.15 ± 0.19^a
ISD	0.84 ± 0.15^a	0.92 ± 0.15^a	0.98 ± 0.17^a
ISE	0.79 ± 0.99^b	0.94 ± 0.16^a	0.99 ± 0.23^a
ISF	0.31 ± 0.89^b	0.29 ± 0.51^a	0.34 ± 0.33^a

Values are mean of replicate analyses Mean
superscripts with different letter differ significantly (P < 0.05)

Table 5
Total Viable counts (10^4 CFU/g) as obtained for PMS degraders' screening

Isolate	Day		
	7	14	21
ISA	1.1 ± 0.33^a	1.0 ± 0.23^a	1.3 ± 0.34^a
ISB	1.4 ± 0.14^b	1.5 ± 0.32^a	1.2 ± 0.19^a
ISC	1.0 ± 0.36^a	1.4 ± 0.51^a	1.6 ± 0.66^a
ISD	4.0 ± 0.13^b	$4.8\pm0.56a$	5.6 ± 0.31^a
ISE	5.1 ± 0.61^b	5.3 ± 0.27^a	6.2 ± 0.43^a
ISF	2.1 ± 0.18^b	1.7 ± 0.24^a	2.2 ± 0.37

Values are mean of replicate analyses ± S.D. Mean
superscripts with different letter differ significantly (P < 0.05);
ISD and ISE were selected for further studies.

Table 6
Bacterial genera characterizations

Test	Isolate
	ISD
Morphological&Gram reation	cocci+
Motility	-
Citrate	-
Catalase	+
Indole	-
Methyl red	+
Voges Proskauer	-
Starch hydrolysis	-
Oxidase	-
Urease	-
Glucose	+
Lactose	+
Surcrose	+
Xylose	+
Arabinose	+
Maltose	+
	Micrococcus sp

+ = positive, - = negative, ISD = bacterial PMS hydrocarbon degrader further
identified to species level by CABI microbial identification services United Kingdom via
16S rDNA sequencing analysis as Micrococcus luteus (AJ536198).

Table 7
Identity of fungal genera

Test	Isolate
ISE	
Macroscopic	White and raised cottony mycelia
Microscropic	non- septate hyphae
	Rhizopus sp

ISE = fungal PMS hydrocarbon degrader further
identified to species level by CABI microbial identification services
UK via ISTS rDNA sequencing as Rhizopus arrhizus.

Table 8
TPH quantification (mgkg^{-1})

Week	Treated option	
	SA	**NA**
0	109663.12±0.66^a	109663.12±0.65^a
1	82247.34±0.43^b	96503.55±0.71^b
2	72377.66±0.22^a	84440.60±0.10^a
4	21932.62±0.27^a	81006.12±0.71^b
6	4386.52 ±0.21^a	39478.72±0.76^b
8	4197.89±0.71^b	31964.46±0.31^a

Values are mean of replicate analyses ± S.D . Mean superscripts with
different letter differ significantly (P < 0.05)

Table 9
Weekly pH determination

Plan					Week	
	0	1	2	4	6	8
SA	7.5±0.24^a	7.8±0.31^b	8.0±0.16^a	8.2±0.11^a	8.2±0.14^a	8.5±0.11^a
NA	5.3±0.17^a	5.4±0.44^a	5.6±0.35^b	5.5±0.43^a	5.9±0.41^b	6.3±0.18^a

Values are mean of replicate analyses ± S.D. Mean superscripts with different letter differ
significantly (P < 0.05)

Table 10
Kinetic model and linear regression analysis

Plan	K (day-1)	r^2	t1/2(days)	K_2 (gmg^{-1}day^{-1})	r^2	$t_{1/2}$(in days)
SA	0.47	0.945	1.50	3.4×10^{-5}	0.858	3.10
NA	0.16	0.925	4.30	2.8×10^{-6}	0.78	26.0

K - First order kinetic biodegradation constant obtained as slope of the plot of LnC$_o$/C$_t$,
K$_2$ - Second order kinetic biodegradation constant
obtained as plot of reciprocal of C$_t$ i.e. (1/C$_t$), Co - Initial contaminant concentration, C$_t$-
Contaminant concentration at time t in week

Table 11
Dehydrogenase activity (mgg^{-1} dry soil /96h)

Plan				Week		
	0	1	2	4	6	8
SA	4.20±1.90^b	4.70±0.32^b	6.70±0.46^a	7.90±0.43^b	8.10±0.47^b	8.30±0.33^b
NA	2.70±0.81^a	3.30±1.23^b	3.80±0.099^b	4.60±0.65^b	5.4±0.73^a	5.80±0.66^b

Values are mean of replicate analyses ± S.D.
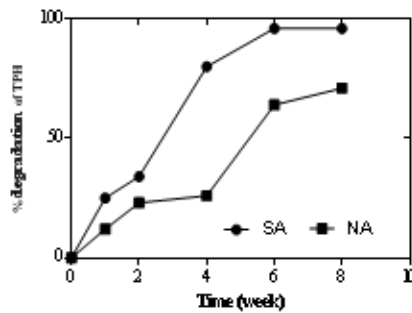*Mean superscripts with different letter differ significantly (P < 0.05)*

Figure 1

TPH removal efficiency versus time (in week). % PMS degradation calculated by =

$$\frac{TPH_{CO} - TPH_{CT}}{TPH_{CO}} \, x \, \frac{100}{1}$$ *Where TPH_{CO} = TPH concentration in mgkg^{-1} at time (week 0), TPH_{CT} = TPH concentration in mgkg^{-1} at time t (week)*
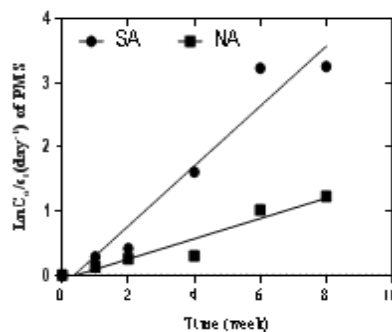

Figure 2
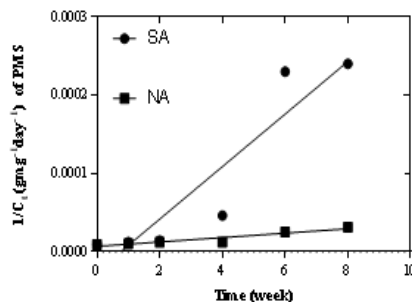*First orders kinetic versus time (in week)*

Figure 3
Second orders kinetic versus time (in week)

DISCUSSION

Microbial interactions to chemicals in petroleum hydrocarbon fractions constitute the pivot of hydrocarbon biodegradation. Observed loss of microbial cell number within weeks 0 – 2 of the study period suggested that not all the microorganisms present in the soil ecosystem are hydrocarbon degraders. It has been stated that in terrestrial ecosystems, spilled oil adsorbs to the soil particles, forming a cohesive, toxic mixture that is deleterious to some of the autochthonous/indigenous microorganisms which are probably non-hydrocarbon degraders.[15] Therefore, the hydrocarbon contaminants tend to exert either a microbastatic or microbacidal activities on the non – hydrocarbon degraders shortly after a contamination event occurred resulting in microbial population

decrease than a typical soil could bear [23]. The survived hydrocarbon degraders after a short time of microbial inactivity (lag) recognized the PMS hydrocarbon by probably biosurfactants and bioemulsifiers thus secreted their enzymes and metabolized the hydrocarbon as source energy and carbon. The utilization of the PMS hydrocarbon resulted in increased microbial growth within weeks 4 – 6 of the study. The saw dust biomasses which were predominantly fungi due to cellulosic content of the saw dust may have led to increased fungal population in the SA amended system [24]. Polluted soil amendment with limiting factors (nutrients) often results to logarithmic microbial multiplication.[25, 26] The higher percentage degradation in SA treatment system was attributed to the increased microbial activities brought about by the elevated fungal population in saw dust thus effective substrate (PMS hydrocarbon) utilization. To facilitate a system

undergoing bioremediation, it often requires methods that can dissociate the petroleum hydrocarbon fractions and create conditions for effective mass transfer process.[27] Sawdust biomass used in the present study effectively confirmed this hydrocarbon biodegradation target. It is important however to note that removal efficiency (96.0 %) of the PMS hydrocarbon was the same in week 6 and 8 indicating that further degradation study by even week 9 (week not studied in the present study) would still give 96.0 %.In this connection, this weekly percentage consistence effective from week 6 could be described as the ALARP point of PMS hydrocarbon biodegradation. ALARP point simply means "as low as reasonably practicable" and it has been defined as the point at which no further significant reduction or breakdown in the total petroleum hydrocarbon (TPH) fractions can be achieved both economical and substantially. According to some investigators, bioremediation does not always guarantee or result in complete mineralization of organic pollutants.[28, 29,] Many of these compounds are naturally transformed into metabolites of unknown persistence and toxicity. [25] The longer the presence of abundant hydrocarbon spill in the environment (soil and water), the more recalcitrant its hydrocarbon fractions become. It is for this reason that the paramount target for polluted soil amendments with materials such as sawdust is to increase the biodegradation rate following a fresh hydrocarbon spill The essence of bioremediation is to reduce the total petroleum hydrocarbon to a level that is undetectable or if detectable, but to a level that is generally regarded as safe by regulatory agencies. The 4.0 % TPH remnant fraction of PMS (ALARP point) though present in the soil could not negatively affect the microbiota and that was why even with the presence of this hydrocarbon fraction in the experimental soil, an optimum DHase activity equal to that of slightly contaminated agricultural soil was produced on week 8 suggesting that the soil does not recognized the 4.0 % portion as a hydrocarbon threat any longer due to its little or no bioavailability to microbiota. The total removal of this recalcitrant fraction would occur over the years via the combined interactions of natural processes as well as traditional physicochemical strategies like evaporation, dissolution, photo-oxidation including adsorption within organic polymeric matrices which could not immediately and effectively set-in during the heavy spill events due to its phenomenal slow nature. Again, the rationale behind bioremediation effort is usually the acceleration of natural processes.[15].The pH conditions in both plans were all within the optimum range for effective microbial activities and the sawdust pH condition slightly elevated the pH of amended SA plan. The pH of the studied options significantly varied with the control at $p<0.05$. Change in soil physicochemical status such as soil pH and dehydrogenase activities due to hydrocarbon introduction into the soil has also been previously reported. [30]. Bioremediation proceeds in such a manner that pH variations do not follow regular trend due to metabolite production. The overall success of bioremediation strategies has been reinforced by the integration of the natural biodegradation half-life, ($t_{1/2}$) and biodegradation kinetic orders. It is a linear regression / mathematical modeling which employs such parameters as; biodegradation kinetic constant (K day^{-1}), r^2, value (correlation coefficient) and biodegradation half-life in accessing the hydrocarbon spill impact on soil ecosystem. Kinetic parameters such as degradation constant K (day^{-1}) and r^2 value (Table 10) have been reported to vary proportionately with hydrocarbon biodegradation in the present study thus, increase in these parameters resulted in faster and more effectiveness of bioremediation. This linear relationship as evidenced by the first order kinetic (Fig. 2) conformed to the present study. The half-life of degradation mathematically expressed as ($t_{1/2}$ = Ln2/K and Ln2 numerically equals 0.693 thus rewritten as $t_{1/2}$ = 0.693/K) otherwise known as the reaction time has been found to vary directly with the contaminant decay/biodegradation which resulted in much of the contaminant (PMS) being removed at a shorter half-life (time interval) of 1.50 days in plan SA. Hence, the faster the biodegradative processes and higher the percentage degradation (removal efficiency), the shorter the Biodegradation half-life. Enzymatic activities are frequently used for determining the influence of various pollutants (PMS in this study) on microbial quality. Enzyme inhibitions on environmental samples such as soil depend on the nature and concentration of pollutants. Hydrocarbon and its derivatives inhibit enzymatic activities by interfering with enzymes –substrates complexes, denaturing microbial enzyme proteins and interfering with the active sites. Dehydrogenases (intracellular enzymes) play a significant role in the biological oxidation of soil organic matter (OM) by transferring hydrogen from organic substrate (PMS hydrocarbon) to inorganic acceptors (molecular oxygen).[30]Many specific dehydrogenases transfer hydrogen to either nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP).[31]Some basic steps that may be necessary for a successful bioremediation project will include compliance analysis, site characterization, method selection / feasibility studies, remediation proper and end project analysis [26]. Site characterization using dehydrogenase activity (DHA) confirmed the high fertility status of the agricultural soil. High DHA were obtained in plan SA system with a particular attention on the value obtained on the 8th week (8.30±0.33 mgg^{-1} dry soil /96h) which almost equalized the DHA of the typical agricultural soil before severe pollution(8.60±0.56 mgg^{-1} dry soil /96h) used in the present study. This was attributed to the effective pollutant degradation brought about by the enhanced microbial activities created within the soil matrices of the SA system acting in concert (consortium). This degree of success in the bioremediation effort suggests a significant soil fertility recovery to warrant immediate cultivation. [31, 32] The DHA of the control soil (soil not amended with sawdust) was 2.50 mgg^{-1} dry soil /96h (30.10 %) less than the sawdust amended plan SA thus, confirmed that bioremediation was not effective within the study period in plan NA system.

CONCLUSION

Sawdust derived from commercially available wood-workshops is a promising bulking agent in bioremediation of PMS hydrocarbon soil pollution. This study confirmed that sawdust enhances fungal growth

more than bacterial growth. We report that parameters such degradation constant (K), r^2 values, and DHA increase with increasing removal efficiency of hydrocarbon contaminants. Hence, the use of sawdust in bioremediation is encouraged and can offer an alternative source of remediation material which is cost effective and generally eco-friendly. Sawdust however, is generated waste by wood workers (furniture makers) usually awaiting disposal either in municipal dumpsites or by incineration. Meanwhile, remedial application of sawdust in filed study would really involve no initial capital expenditure.

CONFLICT OF INTEREST

Conflicts of interest declare none.

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