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Comparative Effectiveness of Activated Soil in Bioremediation of a Farmland Polluted Soil by Polyaromatic Hydrocarbon in the Niger Delta

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Abstract

Polyaromatic hydrocarbon contamination in terrestrial ecosystem is evidently one of the most crucial ecological threats in the Niger Delta as a result of their toxic, mutagenic and carcinogenic properties. Laboratory treatability study was carried out on soil from Ngia Ama in Tombia Kingdom impacted by artisanal oil refining operations, to evaluate the bioremediation effectiveness of activated soil from Bomu in Ogoni against other bioremediation techniques. Bioremediation study was monitored in 5 microcosms designated: biostimulation (BST) biostimulation-bioaugmentation (BSAG), bioaugmentation (AGN), bioattenuation (BAT) and sterilized control (STL) for 30 days. All treatments contained 1 kg (amendments inclusive) of Ngia Ama soil (PSN) sample. The biostimulation agent was inorganic fertilizer (NPK 20: 10: 10) while Bomu sample served as the bioaugmentation agent. Spread plate technique was employed to isolate and enumerate total heterotrophic, hydrocarbon utilizing and PAH-degrading bacteria (THB, HUB and PDB respectively). PAH-degrading bacteria were identified using molecular analysis while polymerase chain reaction (PCR) technique was employed to detect catabolic genes using specific gene primers. Gas chromatography-flame ionization detector (GC-FID) was employed to ascertain PAH concentration losses. The baseline PAH-degrading bacterial counts for Bomu and Ngia Ama samples were 4.6×10^6 CFU/g and 4.9×10^4 CFU/g respectively. The percentage losses of total PAH and TPH as analyzed by GC-FID were 68.7%, 70.0% (BST), 66.4%, 72.6% (BSAG), 68.4%, 64.7% (AGN), 63.0%, 67.0% (BAT) and 43.4%, 47.3% (STL), though there was no statistical differences between the various treatment setups at $p \leq 0.05$. Genera of *Enterobacter, Shewanella, Burkholderia, Pseudomonas, Bacillus, Acinetobacter, Exiguobacterium* and *Stenotrophomonas* were isolated from both samples. The PDB sequences have been deposited in GenBank under the accession numbers KX754444 to KX754459. The activated soil from Bomu showed effectiveness as biotsimulation thus can be used to remediate hydrocarbon polluted sites in the Niger Delta as a cheap *in-situ* option.

Keywords: Activated soil; Bioremediation; Polyaromatic hydrocarbons; PAH-degrading bacteria; Polymerase chain reaction

Introduction

Background

Niger Delta Region of Nigeria is one of the top countries in the world with huge oil and gas reserve. The exploration of this crude resource gives rise to hydrocarbon pollution through routine oil operation, vehicular accident and illegal petroleum refining activities. The latter is currently the leading source of oil pollution in the region and the likelihood of putting a stop to this act is very slim because of the economic benefits enjoyed by the perpetrators and their collaborators. The hydrocarbon wastes emanating from these refineries are of high molecular weight including polyaromatic hydrocarbons (PAHs). The eco-toxicological impacts already in manifestation include air borne black soot, changes in soil physicochemical features, depletion in biodiversity, ground water contamination, bioaccumulation in environmental receptors and cancer related diseases and deaths [1].

Polyaromatic hydrocarbons (PAHs) are a group of organic pollutants that consist of two or more aromatic rings arranged in different configurations [2]. They are ubiquitous [3], persistent due to their inert nature [4], and are highly insoluble in water [5]. According to Kumar et al. [6], there are not less than 30 parent PAHs in crude oil out of which 16 have been considered as priority pollutants by the United State Environmental Protection Agency (US EPA), World Health Organisation (WHO) and European Union (UN) [7] due to their mutagenic, carcinogenic and teratogenic nature.

Remediation of these contaminants is necessary for environmental safety, sustainable development and human health. The mechanistic principles of PAHs' remediation are physical, chemical and biological methods [8]. Physical and chemical methods are preferred to biological method when exigency calls, however they are capital intensive and require high energy with huge consumption of synthetic chemicals which may pose additional environmental challenge [9]. These and other reasons such as simplicity of technology, minimal site disruption, flexibility to be combined with other physicochemical methods (treatment train) of remediation are reasons bioremediation has become an attractive technology these days. The most critical advantage is that the biodegradation agents are hugely unlimited, renewable, versatile, flexible and ubiquitous [10].

Bacteria stand out to be the most resourceful organisms when it comes to bioremediation. Bacteria can survive anaerobic and prohibitive environments such as acid mines, heavy metal and radioactive dumps. Bacterial adaptive physiological and degradative

competence makes them more versatile than any other group of organisms. More so, they respond to selective pressure [11] stimulation and genetic recombination far more than any other microorganism, thereby producing requisite biomass, biosurfactants, exopolysacharrides, enzymes and catabolic genes to degrade bioavailable pollutants [12].

These aforementioned biotic factors are expressed in basic forms of bioremediation such as bioattenuation, biostimulation and bioaugementation. Bioattenuation relies on natural processes to deplete contaminants through biological transformation [9]. Biostimulation requires the introduction of nutrients and/or oxygen to a polluted site as to encourage the growth of naturally occurring pollutant-degrading microorganisms. Bioaugmentation involves the seeding of allochthonous wide type or genetically modified microorganisms to polluted sites in order to accelerate the removal of hazardous compounds of interest [13].

Biodegradation of PAHs can be carried out aerobically and anaerobically [14] via metabolic and co-metabolic pathways [10]. Aerobic biodegradation of PAHs are well studied with established pathways and signatory metabolites for each PAH molecule [15]. Two and three ringed PAHs degrade faster than the high molecular weight counterparts. During aerobic metabolism, PAHs by dioxygenase action changes to cis-dihydrodiols, the latter is transformed into dihydroxy-compounds through biochemical oxidation [16]. Catechol, gentisate, protocatachuate and phthalates are unique intermediates which are then subjected to ring cleavage (ortho or meta) pathways. The metabolites formed are then fed into the tricarboxylic acid cycle (TCA). Anaerobic biodegradation of PAHs is still at its developing stage. Thus its metabolic pathways are still on their nascent stage of study [17].

Biodegradation is the vehicle through which bioremediation is carried out. Bioattenuation, biostimulation and bioaugmentation are the three forms of bioremediations and has been employed in remediation of polluted sites in the Niger Delta. However, activated soil as an option of bioaugmentation is less reported even though it is arguably the cheapest method of bioremediation in the Niger Delta of Nigeria. The aim of this study is to carry out aerobic laboratory treatability study, under conditions that favour bacterial metabolism, to ascertain the effectiveness of activated soil in decontaminating PAHs against other bioremediation techniques.

Materials and Methods

Site description and collection

The soil samples for this study were collected from Ngia Ama (4.7947°N, 6.6831°E) Tombia in Degema Local Government Area of Rivers State where illegal refining history. The pollution in this island was only six months old with respect to the sampling time. The second sample was collected from Bomu (4.6340°N, 7. 3559°E) in Gokana Local Government Area of Rivers State. Pollution at this site dates back to 2007. Soil samples were collected in four different points at Bomu, merged to form a composite sample. At Ngia Ama, obviously polluted soil samples were collected at three spots to make a composite soil sample. The soil samples were collected with sterile hand trowel at 0-30 cm depth, put in a sterile polythene bag and transported to the laboratory within a time frame of 6 hours.

Soil samples preparation

The collected soil samples were processed, removing all non-soil matters. One kilogram of the contaminated soil samples were preserved at 4°C for later physicochemical and microbiological baseline studies [18]. The remaining soil samples were air-dried for a period of 16 h [19] in a clean ventilated laboratory. Each of the soil samples was pulverized and passed through (2 mm pore size) sieve [20]. The soil samples were thoroughly mixed to ensure proper mixing of the contaminant thereby achieving homogeneity. The processed soil samples were then kept in a sterile PVC bag at ambient temperature for later use.

Baseline study

The physicochemical and microbiological analysis of the soil samples, Bomu activated soil (ASB) and Ngia Ama polluted soil (PSN), were carried out according to Chikere et al. [3]. Both contaminated soil samples were obtained and analyzed to quantify THB, PAH, total petroleum hydrocarbon (TPH), organic carbon, nitrate, phosphate, nitrogen, total hydrocarbon content (TOC), pH, exchangeable ion conductivity and carbon-nitrogen ratio.

Physicochemical analyses

Total organic carbon (TOC) was determined according to Avramidis et al. [21]. To determine the total hydrocarbon content, ten grams of soil was added into an extracting flask followed by addition of 10 ml of n-hexane. The suspension was shaken for half an hour and filtered. The filtrate's absorbance reading was taken at 420 nm. The amount of total hydrocarbon content in the soil was ascertained through extrapolation of plotted graph using a reference curve prepared with Bonny light. Determination of total petroleum hydrocarbon (TPH) was carried out according to Romanus et al. [22]. Determination of polyaromatic hydrocarbons (PAHs) was analyzed according to Rasdy et al. [23]. The GC-FID analysis was carried out on baseline, day 0, 15 and 30.

Total (Kjeldahl) nitrogen was conducted according to Diekow et al. [24]; phosphorus was ascertained according to APHA (1998). Soil pH was carried out by adding 10 g of each sample into a 100 ml of clean beakers. Deionized water (20 ml) was added and the suspension was thoroughly stirred with glass rod for 30 minutes to obtain homogenous mixture, after which calibrated pH meter (pH tester 20) was dipped into the beaker containing the suspension and the pH value was recorded after a steady reading. Average pH value was taken from triplicate readings. Soil conductivity test was done by dissolving 10 g of soil sample in 20 ml of deionized water and left standing for half an hour. The slurry was then filtered. The conductivity of the filtrate was determined by Hanna digital conductivity meter. Nitrate was determined according to Igwo-Ezikpe et al.

Bioremediation experimental set-up

Five microcosms were set-up with Ngia Ama polluted soil. Microcosm for biostimulation was defined by an inorganic fertilizer (NPK 20:10:10). NPK 20:10:10 is an inorganic fertilizer that has 20% nitrogen, 10% phosphorus (P_2O_5) and 10% potassium (K_2O). For bioaugmrntation/biostimulation, same fertilizer and activated soil from Bomu was used. To simulate bioaugmentation microcosm the activated soil was mixed with sterilized Ngia Ama sample. Sample of Ngia Ama with no amendment was used as bioattenuation microcosm while sterilized Ngia Ama sample was made as control. Plastic

container was used in all the set-ups. The set-up is summarized in Table 1.

Microcosm code	Description
BST	975 g of PSN+25 g of NPK (20:10:10)+5 ml of distilled water per day
BSAG	950 g PSN+25 g of NPK+25 g of activated ASB+5 ml of distilled water per day
AGN	975 g of PSN (sterilized)+25 g of activated ASB+5 ml of distilled water per day
BAT	1000 g of PSN+5 ml of distilled water per day
STL	1000 g of PSN (sterilized)+5 ml of distilled water per day

Table 1: Experimental set-up for soil bioremediation with monitoring on day 0, 15 and 30.

Microbiological analysis

Isolation and enumeration of total heterotrophic bacteria (THB) was done according to Agamuthu et al. [25] with some modifications. Hydrocarbon utilizing bacteria (HUB) was quantified using Bushnell Haas (BH) solid media supplemented with 0.05 g/ml of nystatin and 1% Bonny Light oil [26]. Aliquot of 0.1 ml from 10^{-4} and 10^{-5} dilutions were plated out in triplicates. The plates were incubated at 27°C for 7 days before colonies were counted. For the enumeration of PAH-degrading bacteria, one gram of soil sample from each treatment cell was added to 9 ml of BH broth supplemented with 1% mixed PAH (0.3 g/l) solution. An aliquot from 10^{-3} and 10^{-4} dilutions were spread on BH agar plate with 0.05 g/ml of nystatin in triplicates, incubated at room temperature for 7 days and enumeration done in colony forming unit per gram [27]. This analysis was carried out on each of the samples from the different treatment in time-course.

Tentative identification of isolates

Colonies formed on BH solid agar plates were sub-cultured on nutrient agar. Pure isolates retrieved from the microbial analyses were tentatively assigned identity based on their phenotypic and biochemical characterization [28].

Molecular analyses

DNA extraction: DNA extraction was carried out on pure isolates from ASB and PSN samples (baseline and day 30 respectively) using the ZP Fungi/Bacteria DNA MiniPrepTM supplied by Inqaba BiotecTM South Africa following the manufacturer's instructions. The DNA quantity and purity were ascertained with a NanodropR ND-1000 UV-Vis Spectrophotometer. The genomic DNA was stored at -20°C for PCR analysis [29].

PCR amplification of the 16S rRNA genes: The 16S rRNA regions of the isolates' rRNA genes were amplified using the 27 F and 1492 R primers on a thermal cycler for 35 cycles at a final volume of 50 microlitres. The PCR-mix contained X2 Dream taq Master mix (DNTPs, taq polymerase and MgCl), primers (at a concentration of 0.4 M) and extracted DNA (template). The thermocycling parameters followed thus: initial denaturation at 95°C for 5 minutes; denaturation at 95°C for 30 s; annealing at 52°C for 30 s; extension at 72°C for 30 s with 35 cycles and final extention at 72°C for 5 minutes before holding and cooling to 4°C [30]. Agarose gel electrophoresis was conducted according to Ding et al. [31].

Small subunit ribosome (16S rRNA) Gene Sequencing and Phylogenetic Analyses: The PCR amplicons from isolates of ASB and PSN samples were sequenced using a 3500 genetic analyzer. Bioedit algorithm was use to edit the resulting 16S rRNA sequence. BlastN was used to download similar sequences from the database of National Biotechnology Information Center (NCBI) to determine similarity between sequences and percentage similarities between matches [32]. ClustalX was employed to align the sequences. Neighborhood-Joining method (in MEGA 6.0) was used to generate the evolutionary tree [33] and analyzed by the bootstrap method [34]. The evolutionary distance computed by Jukes and Cantor [35] which are in the units of the number of base substitutions per site.

Statistical analyses

Data originating from this study was statistically analyzed using IBM SPSS Version 20 to determine the level of significance at $p \leq 0.05$. Chi-square and Post Hoc test were respectively tested for homogeneity and mean differences. A two-way analysis of variance (ANOVA) was used to analyze data from the bioremediation study taking into consideration the three different factors such as bacterial types, time and treatment and the interaction amongst these factors. For the loss of PAHs, a one-way ANOVA was used. Excel inbuilt statistical package (XLSTAT Ecology Version 2016.04.32525) was used for the graphical presentation of data.

Nucleotide sequence accession numbers

The draft nucleotide sequences described in this study have been submitted to GenBank under accession numbers KX754444 to KX7554459

Results

Baseline characteristics of ASB and PSN polluted soil samples

The values of the baseline physicochemical (pH, electrical conductivity, nitrate, phosphate, total nitrogen, total organic carbon, total petroleum hydrocarbon and polyaromatic hydrocarbon) and microbiological analysis of sample ASB and PSN are all shown in Table 2. All the physicochemical characteristics of ASB are appreciably lower in concentration and in value when compared to sample PSN.

On the other hand the bacterial count of total heterotrophic bacteria (THB), hydrocarbon utilizing bacteria (HUB) and PAH-degrading

bacteria (PDB) of sample ASB are higher than the PSN sample with a value between 1 to 2 order of magnitude.

Physicochemical parameter	Unit	ASB sample	PSN sample
Feelings to the hand		Grainy	Smooth
Colour		Dark	Light brown
pH	-	4.5	6.86
Electrical conductivity	µS/cm	12	174
Nitrate	mg/kg	0.9	4.36
Phosphate	mg/kg	0.1	0.76
Total nitrogen	mg/kg	-	0.241
Total organic carbon	%	0.15	0.95
Total petroleum hydrocarbon (TPH)	mg/kg	858 (C9)	2,067.72
Polyaromatic hydrocarbon (PAH)	ppm	Total: 5.84	Total: 193
Bacterial counts THB	CFU/g	$7.1 \times 10^9 \pm 8.2 \times 10^7$	$2.2 \times 10^7 \pm 8.2 \times 10^5$
HUB	CFU/g	$1.9 \times 10^6 \pm 8.2 \times 10^4$	$3.6 \times 10^5 \pm 8.2 \times 10^3$
PDB	CFU/g	$4.6 \times 10^5 \pm 8.2 \times 10^3$	$4.9 \times 10^4 \pm 8.2 \times 10^2$

Table 2: Baseline characteristics of activated soil from Bomu (ASB) and polluted soil from Ngia Ama (PSN).

Isolation and enumeration of total heterotrophic bacteria (THB), hydrocarbon degrading bacteria (HUB) and PAH degrading bacteria (PDB) counts in treated sample and the control

Microbiological counts were carried out in tandem with bioremediation monitoring over a period of 30 days in a laboratory condition. Evidence of microbial activities was ascertained by pure isolate and quantification of total heterotrophic bacteria (THB) counts, hydrocarbon utilizing bacteria (HUB) counts and PAH-degrading bacteria (PDB) counts in the various microcosms such as biostimulation (BST), bioaugmentation/biostimulation (BSAG), bioaugmentation (AGN) and a sterilized control (STL). Figure 1 shows the graphical presentation, comparatively.

Total heterotrophic bacteria (THB) population ranged from 5.2×10^3 CFU/g to 8.6×10^7 CFU/g across the microcosms. The highest THB value was recorded in BSAG at day 15 with a value of 8.6×10^7 CFU/g and highest in AGN at day 30 with a value of 4.0×10^6 CFU/g. The least count was recorded in STL at day 0. Hydrocarbon utilizing bacteria (HUB) count ranged from 8.2×10^3 CFU/g to 7.3×10^7 CFU/g across the different microcosms. The highest value was from BSAG (8.4×10^7 CFU/g) and least in STL (15 CFU/g) at day 30 and day 0 respectively. PDB count ranged from 2.2×10^3 CFU/g to 3.0×10^7 CFU/g across the microcosms. The highest and the lowest values were from AGN (3.0×10^7 CFU/g) at day 30 and STL (3.3 CFU/g) at day 0 respectively.

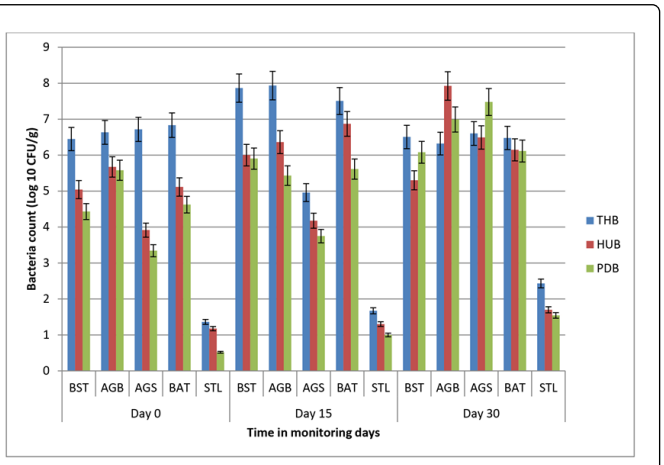

Figure 1: Comparison of growth response of total heterotrophic bacteria (THB), hydrocarbon degrading bacteria (HUB) and PAH degrading bacteria (PDB) over the 30 days monitoring period. THB, HUB and PDB are all homogenous since p-value >0.05. Post Hoc analysis showed that the different treatments reflect significant difference while the period only showed significant difference on day 30. Error bars represent 95% Cl; n3.

Biochemical characterization of PAH degrading bacteria (PDB)

Biochemical characterization of isolated PAH-degrading bacteria is shown in Tables 3 and 4.

	ASB1	ASB2	ASB3	ASB4	ASB5
Gram reaction	-	-	-	-	+
Cell morphology	Rods	Rods	Rods	Rods	Rods
	Pink	Creamy	Greenish	Pink	Creamy
	Circular	Circular	Circular	Circular	Irregular
Biochemical test					
Oxidase	-	+	+	+	-
Catalase	+	+	+	+	-
Citrate	+	+	+	+	+
Urease	+	+	-	+	-
Indole	-	-	-	-	-
Methyl red	-	-	-	-	+
Voges Proskauer	+	-	-	-	+
Motility	+	+	+	+	+
Lactose fermentation	+	-	-	-	+
H_2S production	-	+	-	-	-
Gas production	+	+	+	+	+
Spore test	-	-	-	-	+
Tentative identification	*Enterobacter sp*	*Klebsiella sp*	*Pseudomonas sp*	*Serratia sp*	*Bacillus sp*

Table 3: Biochemical characteristics of bacterial isolates from the Bomu activated soil (ASB).

	PSN-1	PSN-2	PSN-3	PSN-4	PSN-5	PSN-6	PSN-7
Gram reaction	-	-	+	-	-	-	-
Cell morphology	Rods	Rods	Rods	Rods	Rods	Rods	Rods
	Creamy	Greenish	Whitish	Greenish	Greenish	Greenish	Greenish
	Pin point	Medium	Small	Medium	Large	Large	Medium
	Dry	Mucoid	Dry	Mucoid	Mucoid	Mucoid	Mucoid
	Circular	irregular	Circular	Circular	Irregular	Circular	Irregular
Biochemical test							
Oxidase	+	+	+	+	+	+	-
Catalase	+	-	+	+	+	+	+
Citrate	+	+	+	+	+	-	+
Urease							
Indole	-	-	-	-	-	-	-
Methyl red	+	-	-	-	+	-	-
Voges Proskauer	-	+	-	+	+	+	-

Motility	+	+	+	+	+	+	+
Lactose fermentation	-	-	+	-	-	-	-
H2S production	-	-	-	-	-	-	-
Gas production	-	-	-	-	-	-	-
Spore test	-	-	-	-	-	-	-
Tentati identification	Alc. sp	Ps. sp	Artr. sp	Ps. sp	Ps. sp	Ps. sp	Ps. sp

Table 4: Biochemical characteristics of bacterial isolates from Ngia Ama polluted soil (PSN). Key: Alc.: *Alcaligenes*, Ps.: *Pseudomonas*, Artr.: *Arthrobacter*, sp.: species; +=positive to biochemical test; -=not positive biochemical test.

Physicochemical analysis of PSN sample

The pH highest value was 6.48 in AGN microcosm on day 0 and gradually declined to its lowest level (4.75) on day 30 in BST microcosm. Nitrogen content started with a peak of 4.27 mg/kg at day 0 in BSAG, got to its lowest level of 2.24 mg/kg on day 30 in BAT. Phosphate utilization started with its highest value (0.82 mg/kg) on day 0 in BSAG and got its lowest value (0.49 mg/kg) on day 30 in BAT. The trends of conductivity, total nitrogen, total hydrocarbon content, total organic carbon and carbon-nitrogen ratio are shown in Tables 5-7.

Physicochemical parameter	**BST**	**BSAG**	**AGN**	**BAT**	**STL**
pH	6.07	6.08	6.48	6.10	5.40
Conductivity (µS/cm)	186	168	160	166	154
Nitrate (mg/kg)	0.632	0.840	0.610	0.488	0.365
Phosphate (mg/kg)	0.78	0.82	0.76	0.66	0.62
Total nitrogen (mg/kg)	3.98	4.27	3.85	3.68	3.34
Total hydrocarbon content (%)	4.49	5.15	3.42	5.07	3.86
Total organic carbon (%)	1.39	2.20	0.87	2.84	1.54
Carbon-nitrogen ratio	5.345	6.215	3.152	9.896	5.811
TPH (mg/kg)	13.88	13.55	10.98	11.65	7.23
∑PAH (ppm)	99.268	108.933	98.425	78.010	77.908
Total PAH (ppm)	8.25843	10.23276	6.72978	3.54782	4.20712

Table 5: Day 0 physicochemical characteristics of treated Ngia Ama soil sample undergoing bioremediation.

Physicochemical parameter	**BST**	**BSAG**	**AGN**	**BAT**	**STL**
pH	5.36	5.45	5.10	4.80	5.46
Conductivity (µS/cm)	136	130	128	145	157
Nitrate (mg/kg)	0.287	0.354	0.276	0.265	0.345
Phosphate (mg/kg)	0.72	0.80	0.70	0.59	0.55
Total nitrogen (mg/kg)	2.85	3.56	2.84	2.35	3.00
Total hydrocarbon content (%)	4.09	4.70	3.94	3.77	3.05
Total organic carbon (%)	1.44	3.40	2.80	1.34	2.18
Carbon-nitrogen ratio	2.279	4.048	4.590	2.746	5.973
TPH (mg/kg)	9.37	8.00	7.54	6.10	5.10
∑PAH (ppm)	79.537	85.591	80.889	58.979	54.324
Total (ppm)	5.78818	8.77706	5.44378	1.93491	4.01295

Table 6: Day 15 physicochemical characteristics of treated Ngia Ama soil sample undergoing bioremediation.

Physicochemical parameter (ppm)	**BST**	**BSAG**	**AGN**	**BAT**	**STL**
pH	4.75	5.43	5.39	4.81	5.38
Conductivity (µS/cm)	255	177	224	185	188
Nitrate (mg/kg)	0.233	0.308	0.251	0.243	0.319
Phosphate (mg/kg)	0.69	0.76	0.62	0.49	0.50
Total nitrogen (mg/kg)	2.77	3.02	2.58	2.24	2.78

Total hydrocarbon content (%)	4.38	4.20	3.52	4.38	3.82
Total organic carbon (%)	1.62	1.60	2.00	2.40	1.80
Carbon-nitrogen ratio	3.857	3.704	3.846	3.857	4.390
TPH (mg/kg)	3.20	2.72	3.89	3.84	3.81
∑PAH (ppm)	62.575	54.907	60.213	45.609	48.019
Total PAH (ppm)	2.5894	3.4336	2.1293	1.3021	2.3786

Table 7: Day 30 physicochemical characteristics of treated Ngia Ama soil sample undergoing bioremediation.

Stoichiometric reduction of PAHs from crude oil polluted (PSN) soil sample

Chemical analysis of residual concentration of PAHs and TPHs using GC-FID revealed appreciable loss of PAHs and TPHs across all the microcosm setups within the prevailing laboratory conditions. ∑PAH concentration was between 103.878 ppm-68.8577 ppm on day 0 and reduced stoichiometrically to 60.1841 ppm-15.7733 ppm on day 30 across the microcosm setups. The percentage losses of total PAHs and TPHs as analyzed by GC-FID were 68.7 %, 70.0 % (BST), 66.4 %, 72.6 % (BSAG), 68.4 %, 64.7 % (AGN), 63.0 %, 67.0 % (BAT) and 43.4 %, 47.3 % (STL). Figure 2 shows the percentage loss of individual PAHs during the 30 days monitored period. The percentage degradation of the PAHs' ring in each microcosm and across the 5 microcosms is illustrated in Figures 3 and 4 respectively.

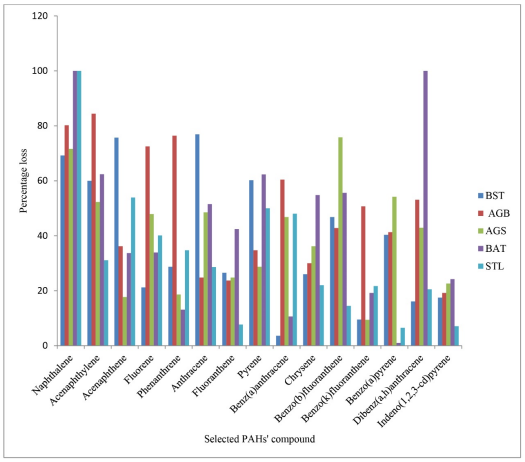

Figure 2: Percentage loss of PAHs' constituents during the 30 days bioremediation period. There was no significant differences amongst the treatments but each one of them shows significant difference against STL.

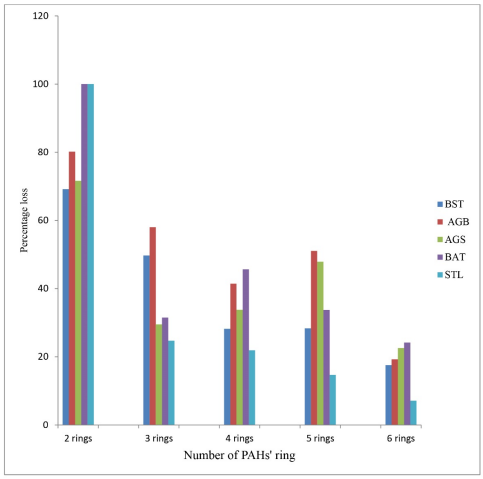

Figure 3: Percentage loss of mean value of PAHs' ring in each bioreactor after 30 days of monitoring.

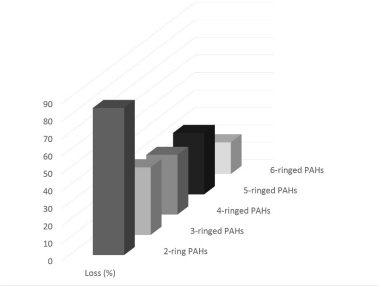

Figure 4: Percentage loss of PAH's rings across the 5 monitored microcosms after 30 days.

Molecular characterization of bacteria isolate from ASB and PSI samples

The Megablast search for 16S rDNA sequence similarity gave an exact match from the NCBI database. The least percentage similarity was shown to be 99% with respect to other species. The computed evolutionary distances were in harmony with the 16S rRNA phylogenetic placement of the isolates within the genera and revealed a high similarity to the species than other genera with in Table 8 shows the homoloy analysis of the PDB, Figure 5 displays the gel electrophoresis result. The results are based on comparison of SSU rRNA gene sequences of the isolates to the sequence that shows the highest sequence similarity to the isolate.

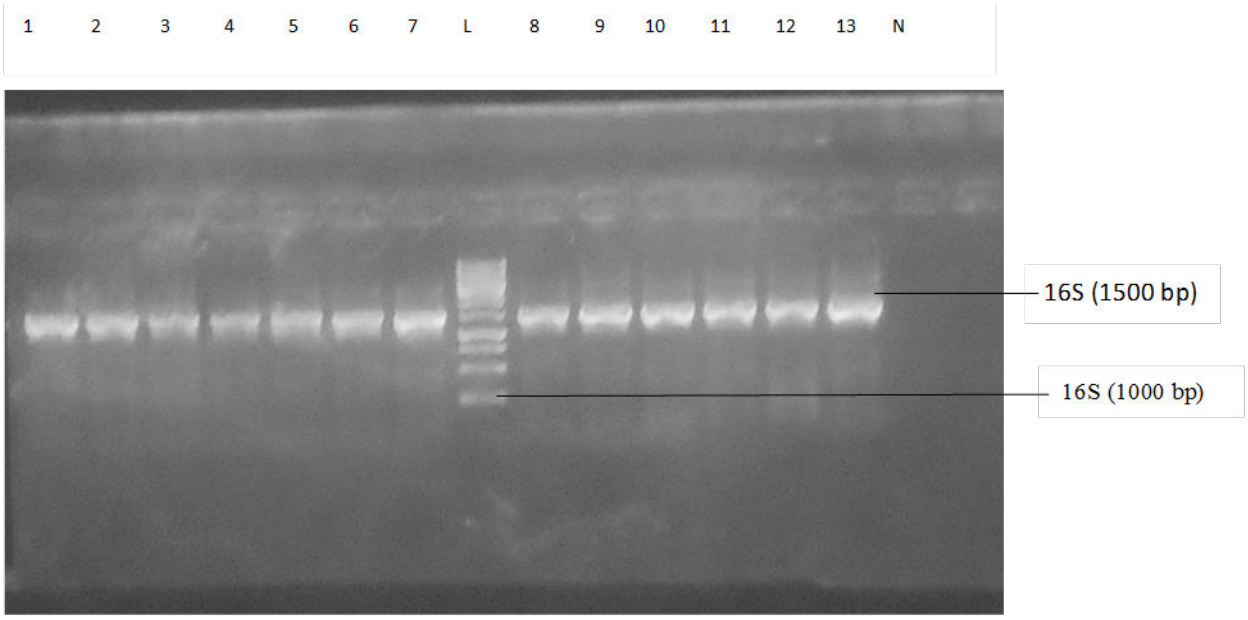

Figure 5: Agarose gel electrophoresis of 16S band (1500 bp) of some selected isolates. L: represents 1 kb ladder, lane 1-12 represent the 16S gene bands of the isolates while N represents the negative control.

S/n.	Isolate code	Closest strain	Percentage similarity	*GenBank Feedback	GenBank Accession no.
1	B1	*Enterobacter xiangfanensis* strain 9A	100	*Enterobacter xiangfanensis*	KX754444
2	B2	*Shewanella haliotis* strain 0315	100	*Shewanella haliotis* strain	KX754445
3	B3	*Pseudomonas denitrificans* strain Y-16	100	*Pseudomonas denitrificans*	KX754446
4	B4	*Burkholderia terrestris* strain R-233321	99	*Caballeronia terrestris*	KX754447
6	B6	*Acinetobacter calcoaceticus* strain N7	100	*Acinetobacter calcoaceticus*	KX754448
7	B9	*Acinetobacter pittii* strain AP_882	100	*Acinetobacter pittii* strain	KX754449
8	B11	*Pseudomonas nitroreducens* strain VITWW2	99	*Pseudomonas nitroreducens*	KX754450
9	B12	*Pseudomonas otitidis* strain IND2	100	*Pseudomonas otitidis*	KX754451
10	B13	*Stenotrophomonas maltophilia* strain C_	100	*Stenotrophomonas maltophilia*	KX754452
11	B15	*Pseudomonas aeruginosa* SJTD-1	100	*Pseudomonas aeruginosa*	KX754453
12	B16	Unidentified			
13	B37	*Pseudomonas fluorescens* strain KRST 01	100	*Pseudomonas fluorescens*	KX754454
14	B38	*Pseudomonas aeruginosa* SJTD-1	100	*Pseudomonas aeruginosa*	KX754455
15	B39	*Pseudomonas aeruginosa* SJTD-1	100	*Pseudomonas aeruginosa*	KX754456
16	B40	*Pseudomonas fluorescens* strain KRST 01	100	*Pseudomonas fluorescens*	KX754457
17	B41	*Exiguobacterium alkaliphilum* strain 12/1	99	*Exiguobacterium alkaliphilum*	KX754458

18	B42	*Pseudomonas fluorescens* strain KRST 01	100	*Pseudomonas fluorescens*	KX754459

Table 8: Homology analysis (with BLAST) of PAH-degrading bacteria isolate.

Discussion

This study focused on hydrocarbon pollution emanating from artisanal oil refining activities which is one major subset of humanly induced environmental stressors with heavy carbon fragment and polyaromatic hydrocarbons (PAHs). The latter class of hydrocarbon (PAHs) serves as the central focus for this study and justified by the fact that PAHs are recalcitrant in nature and chemically toxicogenic, thus required to be eliminated or reduced to harmless level to resuscitate stressed ecosystem.

The low physicochemical characteristics of ASB sample (especially pH of 4.5) reflect low physiological activity for bacterial biodegradation [36]. When added as a bioaugmentation agent into sample PSN (with a pH value of 6.8) it increased bacteria (from sample ASB) physiological activity to degrade TPHs and PAHs [37] in isolation or complementary to degradation role played by the indigenous bacteria. Positive results of microbiological analysis lend credence to metabolic adaptability of bacteria thereby utilizing the hydrocarbons as carbon and energy source for growth and replication [38] across microcosms, except the control. Darmayati et al. [39] had earlier used activated soil to remediate hydrocarbon polluted soil.

Serial dilution technique was employed to enumerate total heterotrophic bacteria (THB), hydrocarbon utilizing bacteria (HUB) and polyaromatic hydrocarbon degrading bacteria (PDB). Besides, the technique was also used provide an index which indicates the differing bacteria types from different treatments. Though serial dilution technique is a corner stone of quantitative microbiology [40], it causes differences in community structure and metabolic redundancy [41]. This implies that specialist genera hardly grow in higher diluents save the generalist microbes. Coupled to these limitations, 90 to 99% of constitute culturable but not viable bacteria [3].

The colonies per g of soil was calculated using the formula:

$$NB/g = MPC \times DF \times V1/V2 \times M$$

Where MPC=mean plate count; DF=dilution factor; V2=volume of original suspension; V2=inoculated volume; M=mass of soil added to V1.

Supported by this work, most literatures and research works have shown that Gram-negative bacteria are better degraders of hydrocarbons and dominate in processes involving crude oil degradation and remediation. Eze et al. [42] noted that Gram-negative bacteria predominate over Gram-positive bacteria in test samples due to the complexity of Gram-negative bacteria cell wall which hinders the penetration of certain substances and their entry into the cytoplasm. For instance Gram-negative bacteria cell walls possess porins which help in the selective uptake of substances by the cell and extrusion of others which may be harmful. Hydrocarbon degrading bacteria often display physiological responses that makes them insensitive to toxic effects, access insoluble hydrocarbons, or transfer large substances into the cytoplasm through biochemical mechanisms such as alteration of cell surface hydrophobicity, gaining of protection from hydrophilic lipopolysaccharide (specifically for Gram-negative bacteria) constituents and or using of repair mechanisms to compensate for losses in membrane as a result of lipophilic compound intercalation [12]. Gram-negative bacteria are less sensitive to the toxic effects of hydrocarbons compared to Gram-positive bacteria [43], hence dominate oil contaminated sites [44]. Most Gram-positive bacteria cannot counter the alteration of membrane architecture resulting from changes in protein conformation and fluidity due to insertion of hydrocarbons with the ultimate consequences of altering membrane-bound enzyme activities and disruption of the barrier and energy transduction roles [12].

Gamma-protobacteria, was the most dominant bacteria in this study. Vinas et al. observed in their study that different bacteria phylotypes of hydrocarbon degraders responded to different phases of bioremediation of PAHs and also in different nutrient amendments. According to them alpha-proteobacteria dominate in the early phase of bioremediation but when biostimulation is put into effect, beta-proteobacteria and alpha-proteobacteria co-dominated. Against these findings, it was shown in this study that the gamma-proteobacteria dominated in all phases of the bioremediation process and also in all amendment conditions which fully corroborates with Sara et al. [45] findings and partially to Fuentes et al. [46] which demonstrated gamma-proteobacteria bloom to support the ecological concept of "conditionally rare taxa" to mean rareness is a temporary state conditioned by environmental stressors.

Among the gamma-protobacteria, *Pseudomonas* species stand tall (as reflected in this work) when it comes to hydrocarbon degradation and bioremediation. This could be explained why *Pseudomonas* is accorded dominant reference in research field of Environmental Microbiology or in discuss involving bioremediation and biodegradation of crude oil or its products in the environment, be it in soil, aquatic media, sediments, mangroves or air. Apart from PAHs, *Pseudomonas* strains also degrade wide range of petroleum compounds, including S-and N-heteroatoms and resins [47,48]. Species that have been used for extensive studies in biodegradation and bioremediation are *Pseudomonas putida, Pseudomonas cepacia, Pseudomonas saccharophila, Pseudomonas xanthomarina, Pseudomonas aeruginosa, Pseudomonas pickettii* 56 [49,50]. They degrade wider spectrum of hydrocarbons by virtue of their catabolic genes for metabolic versatility [43], cell surface activities for improved uptake of hydrocarbon substrate and bioavailability (Johnsen et al.), enzymes for metabolic and co-metabolic functions [16], the flexibility to coexist with other organisms to enhance biodegradation [51] and the ability to survive and proliferate (as a r-strategists) in an environment stressed with constraints and take advantage of such opportunities [52].

The biodegradation activity of the bacteria reflects in the decrease of TPHs and PAHs residual concentrations across the monitored test microcosms. The TPHs and PAHs losses experienced in the sterilized control reflect abiotic influence such as volatilization [53]. Differences in the loss of total PAHs and TPHs was not so much pronounced across the microcosm setups. This implies that the rate of degradation is not statistically significant at $p \leq 0.05$ using analysis of variance (ANOVA). The 100% loss of naphthalene could be as a result of its solubility and volatility [19]. Pronounced loss between each ring category of PAHs means statistical significance at $p \leq 0.05$. This implies

that the ease of degradation is inversely proportional to the number of rings in PAHs.

Phylogenetic analysis based on the 16S rRNA is very useful and widely used due to its simplicity and allows to determine the structure of the bacterial population in soil metagenomics. The phylogenetic analysis was able to determine the taxonomic position of each of the isolate. The representation of a strain for more than two or more isolates with morphological dissimilarity as shown in Table 8 is an indication of phenotypic innovation. For instance isolates ASB37, ASB40 and ASB42 share same identity as *Pseudomonas fluorescence* strain KRST 01. This phenomenon could be attributed to cell's response to the adverse effects of contaminants.

Conclusion

Petroleum artisanal refining activities contribute to the spate of hydrocarbon pollution in the Niger Delta thus, unleash polyaromatic hydrocarbons to the ecosystem causing ecological constraints. The soil sample from Bomu has much reduced hydrocarbon content in comparison to the soil sample from Ngia Ama. The soil sample from Bomu has hydrocarbon degrading bacteria thus served as an activated soil which was used to remediate the Ngia Ama polluted soil. Comparatively, the activated soil proves effective against biostimulation, bioattenuation and a combination of biostimulation and bioaugmentation, though no evidence of statistical difference was evidenced. Analysis of bacterial SSU rRNA gene sequence revealed that *Enterobacter Shewanella, Pseudomonas, Burkholderia, Bacillus spp, Acinetobacter, Exiguobacter* and *Stenotrophomonas* are good candidate for PAH degradation.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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