Journal of Advances in Biology & Biotechnology

23(4): 23-38, 2020; Article no.JABB.58574
ISSN: 2394-1081

Biodiversity and Dynamics (Rate of Change) of Bacterial Communities Involved in the Biodegradation of Petroleum Refinery Sludge in Contaminated Soils

Tudararo-Aherobo Laurelta[1*], Atuayan Ernest[2], Adetutu Eric[3] and Ball Andrew[3]

[1]Department of Environmental Management and Toxicology, Federal University of Petroleum Resources, P.M.B. 1221, Effurun, Delta State, Nigeria.
[2]Department of Microbiology, University of Benin, Benin City, Nigeria.
[3]School of Applied Sciences, Centre for Environmental Sustainability and Remediation, RMIT University, Melbourne, Victoria, Australia.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JABB/2020/v23i430150
Editor(s):
(1) Dr. James W. Lee, Old Dominion University, Virginia.
(2) Dr. Fernando José Cebola Lidon, Universidade Nova de Lisboa, Portugal.
Reviewers:
(1) Amal Alsulaiman, Microbiology in Damascus University, Syria.
(2) S. Sudarsan, C. Abdul Hakeem College of Engineering and Technology, Affiliated to Anna University, India.
Complete Peer review History: http://www.sdiarticle4.com/review-history/58574

Original Research Article

Received 24 April 2020
Accepted 30 June 2020
Published 30 June 2020

ABSTRACT

Aim: The study assessed the bio treatability of the petroleum refinery sludge in contaminated soils by indigenous bacterial communities and the effects of the sludge contamination and bio stimulants on the biodiversity and dynamics (rate of change) of the bacterial communities involved in the biodegradation of the sludge, using the molecular biology technique, Denaturing Gradient Gel Electrophoresis (DGGE).
Study Design: The randomnized block design was used for the study.
Place and duration of the Study: The research was conducted in the biology laboratory of Flinders University, Adelaide, South Australia.
Methodology: The percentage of total petroleum hydrocarbons (TPH) degraded and the bacterial load in the test microcosms was assessed tri-weekly for 12 weeks. The percentage TPH was

**Corresponding author: E-mail: tudararo.aherobo@fupre.edu.ng;*

assessed using Gas chromatography, while the bacterial count was determined as gene copies using the culture independent molecular tool, quantitative real-time PCR (qRt-PCR) analysis. The effects of the experimental treatments on the biodiversity and dynamics (rate of change) of the bacterial communities involved in the biodegradation of the sludge in the soils was determined by the culture-independent molecular biology technique, DGGE. Moving Windows Analysis (MWA) and Shannon Weaver diversity index were used to determine the dynamics (rate of change) and biodiversity of the bacterial communities respectively.

Results: Results obtained for the Moving Window Analysis (MWA) which is used to determine the dynamics (Dy), or rate of change of the bacterial communities, showed that, the 1% and 5% sludge contaminated soils biostimulated with compost, recorded the highest Dy of 86.0 ± 1.90% and 87.0 ± 2.20% respectively.NPK biostimilated soil microcosms however recorded a lower Dy of 33.75± 3.20 and 32.50 ± 4.68% for 1% and 5% sludge contamination respectively. The biodiversity of the bacterial communities expressed as Shannon -Weaver index (H^1), recorded the highest value of 2.76 ±0.02 for the compost biostimulated microcosm in the 1% sludge treatment, while for the 5% sludge contamination, the treatment with NPK and surfactant enhanced the bacterial biodiversity most with a value of 2.76 ±0.07%. In the test soils with 1% sludge contamination, bio stimulation with NPK gave the highest % TPH degradation (78.25%) while the treatment with NPK and Triton-X 100 had the highest TPH degradation (46.55%) for the 5% sludge contaminated soils. There was insignificant difference in the % sludge degradation between the control and other treatments at $P > 0.05$ and F = 4.07 for the 1% sludge treated soils, while for the soils treated with 5% sludge there was significant difference between the control and other treatments at $P < 0.05$ and F= 4.07.

Conclusion: Bacteria species identified in the sludge by molecular biology techniques included; *Pseudomonas sp. ITRI77*, Uncultured *Thauera sp., Uncultured Pseudomonas sp.*, *Flavobacterium sp., Bacillaceae bacterium, Uncultured soil bacterium, Clostridium sp.,* most of which are Gram negative. Biostimulation with compost enhanced a higher biodiversity (H^i) and dynamics (*Dy*) of the bacterial communities involved in the biodegradation of the sludge. Though the NPK treated soils enhanced the biodegradation of the sludge most, degradation started declining by the 9th week while that of compost continued to rise steadily till the 12th week. Results obtained indicate that compost is as good as NPK in the biodegradation of petroleum sludge especially at 1% sludge contamination, since there was no statistical difference between the % TPH degraded and the use of compost is environmentally friendly and economically sustainable.

Keywords: Petroleum refinery sludge; biostimulation; bacterial biodiversity; bacterial dynamics (Rate of change).

1. INTRODUCTION

Petroleum sludge are oily and viscous residues, which are formed during production, transportation, refining of petroleum and storage and are composed of basically oil, water and solids [1]. Due to their characteristics, such as varied composition, their neutralization become difficult and confer on them high recalcitrance. Furthermore, the marked stability of the multiphase system is due to adsorption of oil on solid particles, producing a highly protective layer [2]. In the same vein, the polar fractions promote charge repulsion, impairing the formation of a homogenous phase [3]. This recalcitrance can be ascribed to the presence of aromatics, polycyclic aromatic hydrocarbons (PAHs) and complex compounds such as asphaltenes. Some of these compounds act as solvents of microbial membranes and could impair biodegradation [4].

The oil industry is responsible for the generation of high amounts of oily sludge as waste by product. However; one of the problems faced by the oil industry is the safe disposal of the oily waste generated. It is estimated that approximately 1% of the total oil processed in a refinery is discarded as oily sludge [1]. These oily wastes are expensive to store or destroy and previously contaminated areas have required expensive remediation processes to minimize contaminant dispersion. Improper disposal leads to environmental pollution, particularly soil contamination and poses a serious threat to groundwater. Many of the constituents are carcinogenic and immunotoxicants [5]. The PAHs have also been known to impair chemoreceptors functions in aquatic lives and hence lead to extinction of some species. They have also being known to bioaccumulate up the food chain, resulting in cancers and other genetic malfunctioning in man and other higher animals [6].

Several disposal options for petroleum refinery sludge disposal include thermal treatment (incineration) [1], landfills [7] and biotreatment using the following methods: composting, land farming and biopile [8]. Other conventional biological treatment methods include activated sludge and anaerobic digestion [9]. An interesting alternative to circumvent these problems is the use of a bioreactor, since optimum process conditions can be easily controlled, allowing higher quality final effluent in shorter times. However they might have high costs [10]. The Department of Petroleum Resources (DPR) an agency under the Federal Ministry of Environment (FMENV), which regulates activities in both the upstream and downstream petroleum sector, recommends that petroleum sludge should be treated and disposed by methods that shall not endanger human life and living organisms [11]. Such approved methods include recycling (resource recovery), incineration, solidification, land farming (bioremediation) and land filling.

Bioremediation of hydrocarbon-contaminated soils, which exploits the ability of microorganisms to degrade and/or detoxify organic contamination, has been established as an efficient, economic, versatile, and environmentally sound treatment [12]. It employs the biodegradative potentials of organisms or their attributes, is an effective technology that can be used to accomplish both effective detoxification and volume reduction. It is useful in the recovery of sites contaminated with oil and hazardous wastes [13]. Some common factors limiting the degradation rate of hydrocarbons include its composition, pH, water potential, physical state, weathering, presence of oxidant, temperature, mineral nutrients and presence of microorganisms with hydrocarbon degrading potentials [14].

In the assessment of bioremediation of petroleum sludge contaminated soils, it has been well established that at elevated concentrations of total petroleum hydrocarbons (TPH) in a soil, the rate of TPH degradation can be best monitored by microbial respiration and concentration [15]. However to implement bioremediation in the field, the microbiological contribution to the bioremediation process and its impact on the ecosystem need to be clarified by the analysis of the microbial community profiles that take part in the bioremediation process [16]. Approaches that have been utilized for the assessment of the genetic diversity and structure of soil microbial communities include fatty acid analysis and molecular approaches. The Phospholipids Fatty Acid Analysis (PLFA) is a typical fatty acid analysis technique [17], while molecular approaches include; Denaturing/ Temperature Gradient Gel Electrophoresis (DGGE/TGGE), Amplified Ribosomal DNA extraction Analysis (ARDRA), Terminal-Restriction Fragment Length Polymorphism (T-RFLP), Ribosomal Intergenic Spacer Analysis (RISA) and Random Amplified Polymorphic DNA (RAPD) [18].

The aim of the research is to ascertain biotreatability of the sludge using bioremediation landfarming degradation techniques and to monitor the effect of the sludge on the biodiversity and dynamics (rate of change) of the bacterial community functional in the degradation of the sludge using the molecular biology techniques, denaturing gradient gel electrophoresis (DGGE).Results obtained would be useful in determining the most sustainable biostimulation substrate to be used in the treatment of refinery sludge contaminated soils.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Petroleum sludge from Warri Refinery and Petrochemical Company (WRPC) petroleum storage tank at Ekpan, Delta State in Southern Nigeria, was used for this study. It was characterized for its total petroleum hydrocarbon (TPH) content, polyaromatic hydrocarbons ((PAH), total nitrogen and carbon and heavy metal constituents (copper, lead, chromium, nickel, cadmium, barium and iron). GC-MS was used to analyze the TPH and PAH [19]. The heterotrophic bacteria counts were determined using nutrient agar, while the hydrocarbon-degrading bacteria and fungi counts were determined using mineral salts medium of Mills et al. [20]. A soil sample from an unpolluted site was used for this treatment process. Moisture content, particle size, total nitrogen and carbon and nutrient levels and the heterotrophic and petroleum sludge utilizing bacteria population were determined using standard methods.

2.2 Experimental Set-up

1) Petroleum sludge only (control)
2) Petroleum sludge and NPK fertilizer (30:10:10)
3) Petroleum sludge and compost from agricultural wastes

4) Petroleum sludge, NPK fertilizer (30:10:10) and surfactant (Triton-X 100)

One kilogram (1kg) of soil sample was weighed in triplicate for each treatment. Two concentrations {10000 mg/kg (1%) and 50000 mg/kg (5%)} of petroleum sludge were incorporated into the soil for each treatment. The compost used for the test was added to a set of test soils in a 2: 1 ratio (50%). 2 g/kg of 30:10:10 NPK fertilizer was added to another set of test soils. The concentration of NPK (2 g/kg) used for the test was informed by recommendations of Walworth et al. [21], who observed from similar studies that concentrations of NPK between 1800 and 2500 mg/kg resulted in optimal degradation of hydrocarbons. 50%/kg of compost was used for the tests as recommended by Namkoog et al., in similar studies [22]. 0.5% surfactant (Triton-X 100) was used as recommended by the manufacturers as the safe concentration for enzyme reactions (Sigma, Triton-X 100, Safety Information Sheet) and in similar studies by [23], who obtained optimal bioremediation of hydrocarbon contaminated soils on using 0.5%(w/v) Triton-X 100.Moisture content was maintained at 60%. The soil samples were tilled with a wooden spatula and watered at 3 days interval to maintain proper aeration and moisture content.

2.3 Determination of Percentage (%) TPH Degraded in Sludge Contaminated Soils

To assess the percentage of TPH degraded, samples were collected at time zero and triweekly for 12 weeks, the wet soil samples were used for quantitative and qualitative TPH analyses using GC-MS. The residual TPH was extracted from the sludge contaminated soil samples using a modified standard protocol of determining hydrocarbon content in soil according to International Standard Organisation (ISO/DIS 16703 GC-method), [24]. TPH concentration in soil was monitored throughout the experiment using GC (gas chromatography) performed on a Varian 3800 gas chromatograph equipped with a Varian 8200 Autosampler, FID (flame ionisation detector), and splitless injector valve. The capillary column used was an Alltech EC-5 (30 m×0.25 mm with 0.25 µm film thickness), with helium as a carrier gas flowing at a rate of 2 ml/min in a constant flow mode.

Percentage TPH degradation was computed using the formula below;

% TPH Degradation = TPHi – TPH r / TPHi x 100

TPHi: Inititial TPH concentration on day O

TPHr: Residual TPH concentration on day 14

2.4 Determination of Bacterial Load as Gene Copies by Quantitative real Time PCR (qRt-PCR)

Bacteria cells counts were enumerated triweekly for 12 weeks as gene copies using the nonculture molecular biology method, quantitative real-time PCR (qRt-PCR). Analyses were done using standard methods.

The primer pair selected to quantify the heterotrophic bacteria gene copies on day 0 and triweekly were 314F and 518 R [25]. These were included in reactions containing: 12.5 µl iQ5 Sybr Green supermix (iQ5 SybrGreen Supermix, Biorad), 8 µl distilled MilliQ water, 1.25 µl forward primer (10 pmol/µl), 1.25 µl reverse primer (10 pmol/µl) and 2 µl DNA template. RT-PCR amplification was carried out on iQ5 real-time PCR Detection system (Biorad, Hercules, CA) according to the manufacturer's instructions. RT-PCR amplification was initiated by denaturation at 95°C for 2 min and was followed by 40 cycles of denaturation for 10 s, annealing at 55°C for 30 s and then by extension at 72°C for 30s. A final melting curve analysis was carried out by continuously monitoring fluorescence between 55 and 95°C with 0.5°C increments every 10 s. Threshold cycles (Ct) were calculated automatically by iQ5 software using PCR baseline subtracted curve fit data method (v2.0148.60623). For quantification analysis, the threshold cycle (Ct) values (or the PCR cycle number where fluorescence is first detected) were determined for each dilution. Thereafter these values were used for obtaining log [C]/Ct plots, which were almost linear. A linear regression of threshold cycle (Ct) for each dilution verses the log dilution allowed calculation of PCR efficiency (E) according to the following equation [26]. $E = 10^{-1/slope}$; the PCR efficiency is a number showing the increase (in times) in the amount of the amplified DNA fragments taking place during one cycle. Considering the PCR efficiency (E) to be largely constant throughout the process, the amount of DNA after cycle 'n' can be expressed as follows [26]. $Nn = N_0 \times E^n$ (where $1 < E < 2$); where 'n' is the number of the reaction cycle; N_0 is the amount of DNA present initially (before the PCR); Nn is the amount of the

reaction products after the completion of the cycle n; and E is the PCR efficiency. Gene copies (cell counts) were computed from the ct values and the efficiency of the standard curve ($Nn = No \times E^n$).

Nn = final gene copies,
No = Starting quantity of genes or cells,
E = Efficiency, ($E = 10^{-1/slope}$)
$n = c^t$ (threshold cycle)

2.5 Assessing the Effect of Refinery Sludge Contamination and Bio-stimulation Treatments on the Bacterial Community Biodiversity and Dynamics (Rate of Change) in the Sludge Contaminated Soils

The effect of petroleum sludge contamination and the biostimulation treatments of the contaminated soils on the bacterial community biodiversity and rate of change was monitored using the following steps.

2.5.1 DNA extraction of bacteria community in the contaminated soils

DNA was extracted from the soil samples (0.25 g) using a MoBio Power soil DNA extraction kit according to the manufacturer's instructions (MoBio Laboratories Inc, Carlsbad, CA, USA on day 0 and triweekly for 12 weeks. PCR was used for the amplification of the bacterial community by using the community DNA which is a mixture of DNA extracted from the sludge contaminated soils as template.

2.5.2 PCR of Bacterial 16S ribosomal DNA amplification

The variable V3 region of 16S rDNA was enzymatically amplified in the PCR with primers to conserved regions of the 16S rRNA genes [27]. The forward primer 314F has an incorporation of a 40-bp GC-rich clamp in the 5' end to alter melting properties and increase resolution of DNA fragments by DGGE [28]. Reverse primer used was 518R. PCR reactions consisted of 2 µl of purified DNA (1:10 diluted) extract added to 48 µl of PCR mastermix. PCR mastermix (final volume 50 µl) contained 10 µl, 5x PCR buffer (Promega, Madison, WI, USA), 5 µl of 25 mM $MgCl_2$ solution, 1 µl of 10 mM dNTPs, 2 µl of 10 pmol/µl of each, forward (314FGC) and reverse (518R) primers, 0.25 µl of 5 U/µl of Taq polymerase, 1 ml of 500 ng/ml of bovine serum albumin to achieve maximum efficiency [29,30] and 26.75 µl of sterilised nuclease free water. PCR was carried out on a Corbett machine (Adelab Scientific, Thebarton, Adelaide). To increase the specificity of amplification and reduce the formation of spurious by-products, a "touchdown" PCR [31,32], in which the annealing temperature was set to 65°C, which is 10°C above the expected annealing temperature, and decreased by 1°C every cycle until a touchdown of 55°C, at which 10 additional cycles are carried out was performed. Touchdown PCR carried out included a 5 minute initial denaturation at 95°C, which was then followed by 10 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute. The annealing temperature (65°C) was reduced by 1°C for every cycle until it reached 55°C. The next cycle that followed was, 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute and it was repeated 20 times with a final extension at 72°C for 10 minutes. A bacterial strain known to amplify was used as positive control and a negative control with no template DNA was also included to check for contamination.

2.5.3 Generation of bacterial community profile using the DGGE

Bacterial PCR amplicons were analysed on a Universal Mutation Detection System D-code apparatus (Biorad, CA, USA) using the DGGE procedure to generate the community profiles. For bacterial analysis, the desired denaturant gradient was achieved using 45% acrylamide for lower concentration and 60% acrylamide for higher concentration, made from 0% and 70% denaturants. The DGGE gels were then stained using silver staining. After staining the DGGE gels were scanned using EPSON Expression 1600 V.2.65 E software and saved as tiff files for further analysis.

2.5.4 DNA quantification, sequencing and bacteria identification

The DNA of the bacteria in the petroleum sludge were extracted using the Mobio Powersoil™ DNA Isolation kit and amplified with bacterial universal primers 341F and 581R [27], with PCR cycling conditions (section 2.5.2). The quantity of the DNA amplified was read with Nanodrop 1000 spectrophotometer, cleaned with Promega Wizard Plus SV Minipreps DNA purification kit, sequenced with the Muyzer primers 341F and 518R at Australian Genome Research Facility (AGRF) and blasted with NBCI programme using Sequencer 4.9 software for identity.

2.5.5 Statistical analysis of DGGE banding patterns

DGGE banding patterns were analysed using total Lab-120 (TL-120) V2006F Image Software [33]. Shannon Weaver index (*H'*) was used to calculate bacteria diversity in the DGGE profiles as described by [34]. Using the formula $H' = -\sum p$ LN n*i*. The community dynamics (dy) was determined by computing the rate of change over time, using the Moving window analysis as described by [35].

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Physicochemical characteristics of the petroleum sludge

As recorded in an earlier study using the same petroleum sludge [19], the petroleum refinery sludge was acidic with a pH value of 5.81 and had a high TPH content of 340,000 mg/kg made mainly of between 10-40 carbon unit compounds (Fig. 1). The physicochemical and microbial qualities of Nigerian petroleum refinery oily sludge indicates the sludge has Polyaromatic hydrocarbon (PAH) content of 0.075 ± 0.02 mg/kg and high values of 26.04 ±1.02 mg/kg and 21.65 ± 1.21 mg/kg for nitrate and ammonium respectively. The heterotrophic bacteria and fungi counts were 5.86E + 05 and 4.72E + 05 cfu/g, respectively. Hydrocarbon degrading bacteria and fungi counts were 2.85E + 02 and 2.75E + 02 cfu/g, respectively. Zinc recorded the highest concentration of 100.65 ±2.30 mg/kg for the metals analyzed.

3.1.2 Percentage TPH Biodegraded in Petroleum Sludge contaminated and biostimulated soils

Contaminated soils with 1% and 5% sludge that were left untreated and used as control recorded 76% and 33.33% at the end of 12 weeks respectively. For soils contaminated with 1% sludge, % TPH degradation at the end of 12 weeks was; 78.25%, 50.88% and 36%, for biostimulation with NPK, Compost and NPK with Triton-X 100 surfactant respectively. In the 5% sludge contaminated soils, % TPH degraded was; 46.55%, 40.17% and 37.72% for biostimulation with NPK and Triton-X 100 surfactant, Compost and NPK respectively (Fig. 2). In the soils with 1% sludge contamination, the treatment with NPK gave the highest % TPH degradation (78.25%) while the treatment with NPK and Triton-X 100 had the highest TPH degradation (46.55%) for the 5% sludge contaminated soils (Fig. 2). There was insignificant difference in the % sludge degradation between the control and other treatments at $P > 0.05$ and F = 4.07 for the 1% sludge treated soils, while for the soils treated with 5% sludge there was significant difference between the control and other treatments at $P < 0.05$ and F= 4.07.

3.1.3 Bacterial community counts as gene copies using quantitative real-time PCR (qRT-PCR)

In the soils treated with 1% sludge, biostimulation with NPK recorded the highest bacterial count of 2.12 X 10^{12}cells/g by the end of 12 weeks, while biostimulation with NPK and surfactant (Triton-X100), recorded the lowest count of 5.88 x 10^{11} cells/g. The count recorded for the treatments with 5% sludge was highest in the treatment with NPK and Surfactant (1.30 x 10^{13} cells/g) and lowest in the control (2.77 x 10^{10} cells/g) at the end of 12 weeks (Table 1). Standard curve for the qRT-PCR obtained a percentage efficiency of 108.4 and R^2 of 0.993.

3.1.4 Effects of petroleum sludge contaminated and biostimulated soil on bacterial community biodiversity and dynamics (Rate of change)

The effect of the petroleum sludge contamination on the bacterial community biodiversity and dynamics involved in the sludge degradation was successfully monitored at three (3) weeks interval for 12 weeks using culture independent molecular biology techniques, involving PCR and DGGE (Plates 1 and 2). Interpretations of the molecular fingerprinting pattern of the bacterial community profile on the DGGE gel using the Total lab Phoretix software coupled with statistical analysis such as Moving Windows analysis (MWA) and Shannon-Weaver diversity index were used to monitor the dynamics (rate of change) and biodiversity of the microbial community profile involved in the biodegradation of the petroleum sludge respectively.

Moving Windows Analysis (MWA) was used to indicate the rate of change or dynamics, Dy (i.e the number of organisms that on average come to significant dominance) of the microbial communities that degraded the petroleum sludge over time. Treatments with MWA value of 0-10 indicates a low Dy (very close community

restricting the dominance of other species); MWA of 11-20% indicates a medium Dy (new species are able to enter the community, but without interfering with the functionality of the system); MWA of ≥ 25% indicates a high Dy (a lot of species come into dominance and leave the microbial community resulting in broad dynamics, probably causing loss of coherence)]. The Dy of the bacterial communities in biostimulation treatments with 1% sludge were, 40.00 ± 3.81%, 86.75 ± 1.90%, 33.75 ± 3.20% and 86.25 ± 2.22% for sludge only(control), compost, NPK and NPK and surfactant respectively. It was highest in the biostimulation treatment with compost (86.75 ± 1.90%), (Fig. 3) and lowest with NPK treatment (33.75 ± 3.20%). All the treatment had values that fell within the high Dy range, indicating that a lot of species come into dominance and leave the microbial community resulting in broad dynamics. The Dy of the bacteria communities in the treatments with 5% sludge were 18.75 ± 8.54%, 87 ± 2.02%, 32.50 ± 4.68% and 81.25 ± 3.02% for Sludge only (control), treatment with compost, NPK and NPK with surfactant respectively. It was highest in the treatment with compost (87.0 ± 2.02%), (Fig. 4) and lower for NPK (32.50 ± 4.68%).

The biodiversity of the bacterial community in the sludge contaminated soils and the biostimulation treatments were determined by the Shannon-Weaver diversity Index (H^i). In the soils contaminated with 1% sludge, biodiversity values of 2.39 ± 0.08, 2.76 ± 0.02, 2.67 ± 0.03 and 2.29 ± 0.13 were recorded for the biostimulation treatments with sludge only, compost, NPK and NPK with surfactant respectively. It was highest in the treatment with compost (2.76 ± 0.02), (Fig. 5). The biodiversity values of the bacterial community in the soils contaminated with 5% petroleum sludge recorded, 2.76 ± 0.07, 2.63± 0.06, 2.38 ± 0.06, 2.12 ± 0.02 for biostimulation treatments with NPK and Surfactant, NPK, Compost and Sludge only respectively. It was highest for the treatment with NPK and surfactant (2.76 ± 0.07) (Fig. 6).

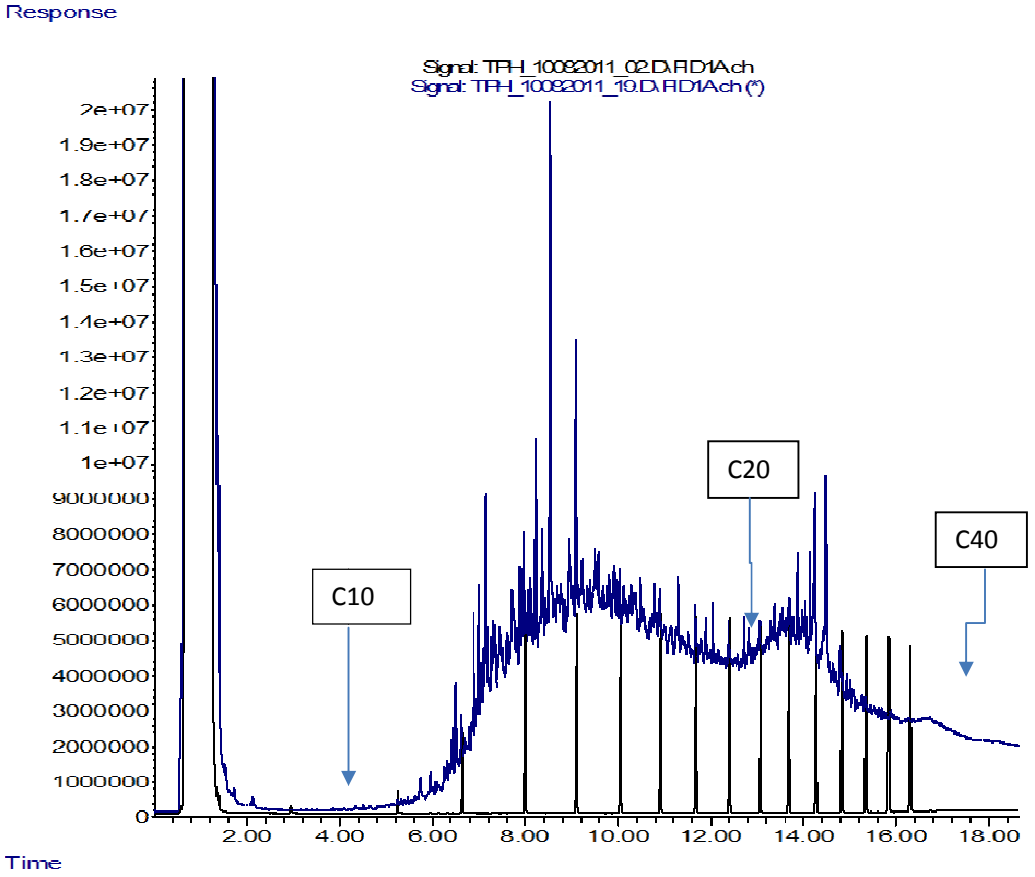


Fig. 1. Overlay of Nigerian petroleum sludge and a chromatogram of a mixture of even numbered n-alkanes between C10 and C40

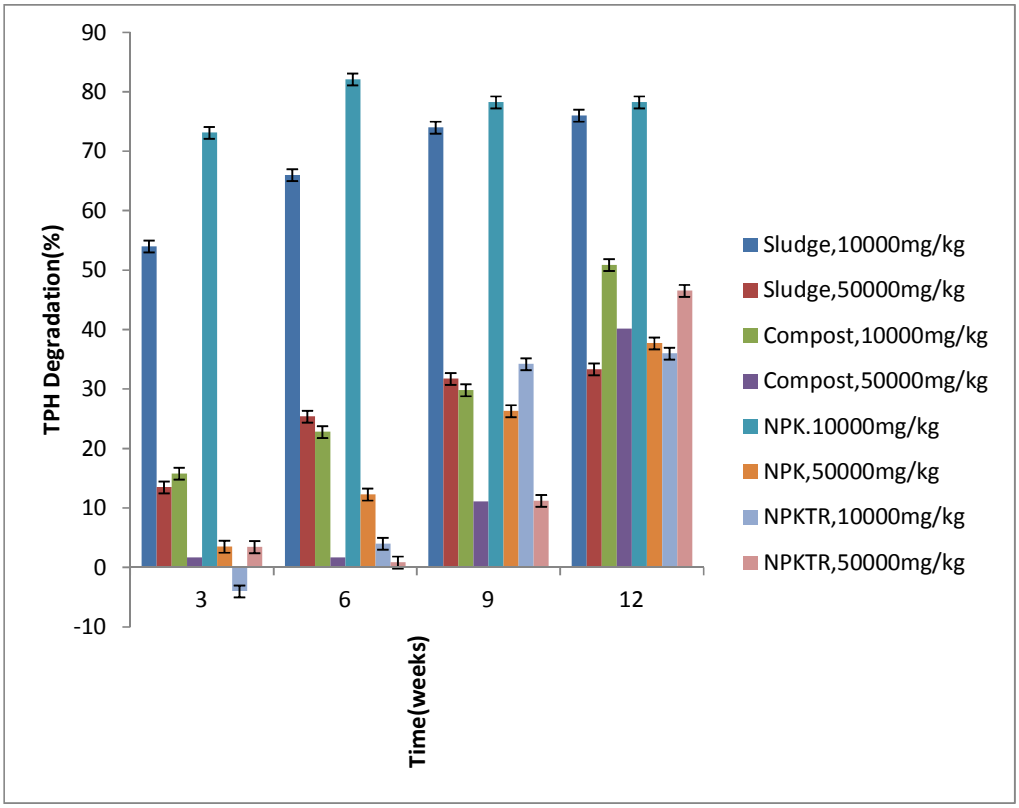


Fig. 2. Percentage (%) TPH degraded in petroleum sludge bioremediated soils

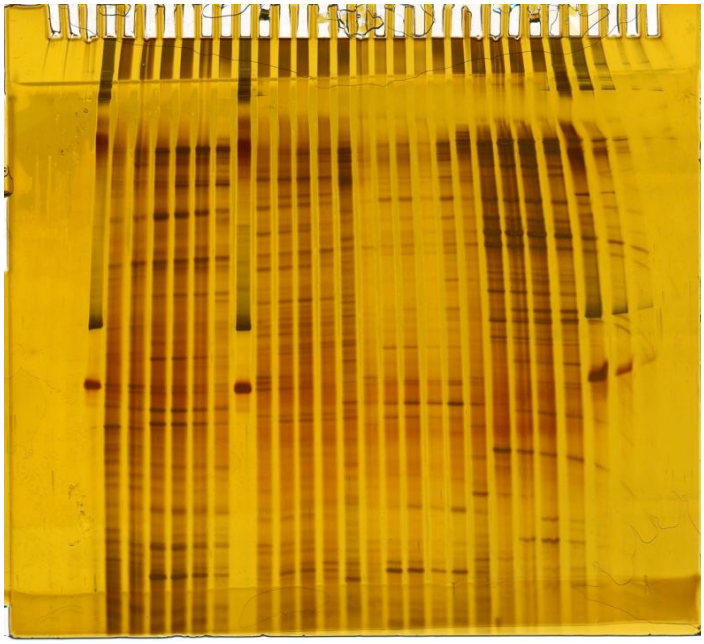


Plate 1. DGGE Profile of 16S rDNA gene sequences of bacteria community amplified from soils contaminated with 10000 mg/kg (1%) petroleum sludge and bioremedated with compost, NPK only, NPK and surfactant for 12 weeks

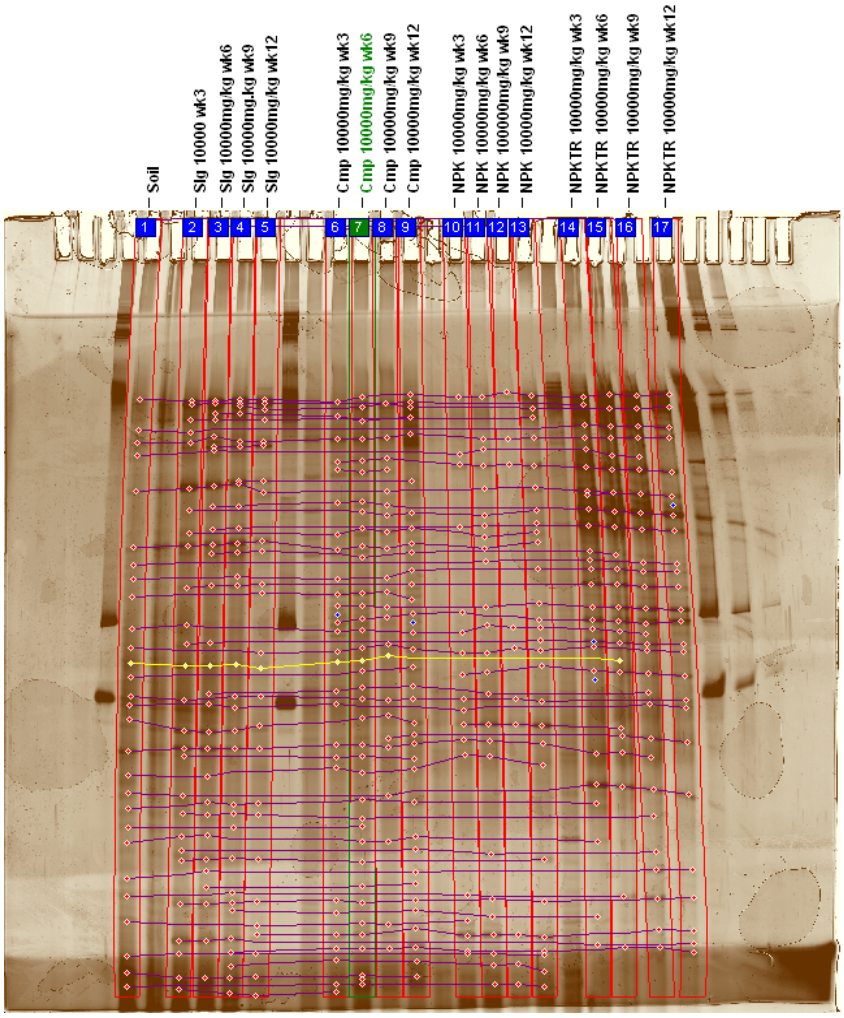
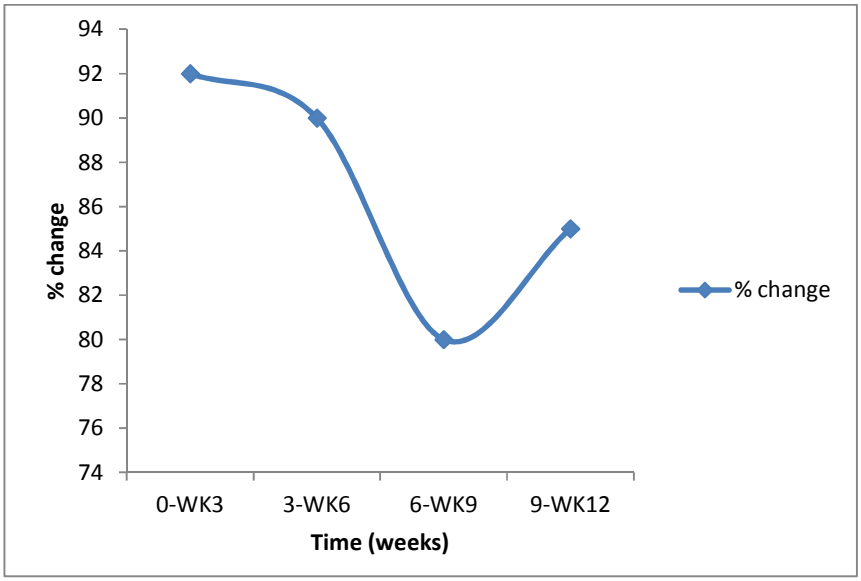


Plate 2. Band matching and lane marking of DGGE profile of 16S rDNA gene sequences of bacteria community amplified from soils contaminated with 10000 mg/kg (1%) petroleum sludge and bioremedated with compost, NPK only, NPK and Surfactant for 12 weeks

Table 1. Bacteria cell counts (gene copies) in bioremediated petroleum sludge contaminated soils

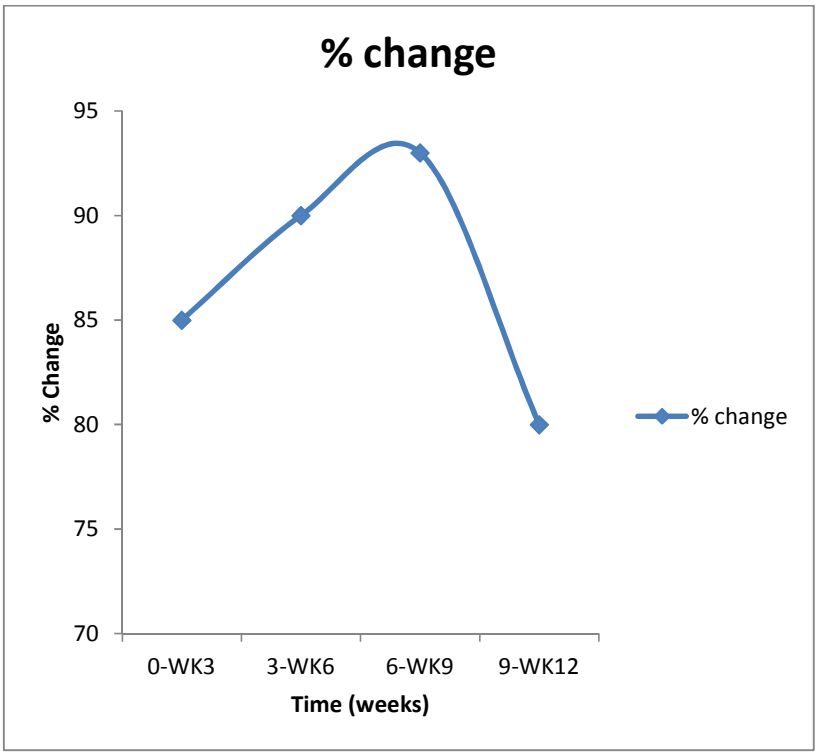
Treatments	Bacteria count, cells/g				
	Day 0	Week 3	Week 6	Week 9	Week 12
Sludge10000 mg/kg (Control)	1.27×10^{15}	1.60×10^{19}	1.70×10^{19}	1.69×10^{17}	1.08×10^{12}
Sludge 10000 mg/kg + Compost	1.09×10^{19}	1.68×10^{19}	$1.70 \times ^{17}$	1.72×10^{15}	7.50×10^{11}
Sludge 10000 mg/kg + NPK	2.29×10^{17}	1.72×10^{15}	1.69×10^{17}	5.12×10^{15}	2.12×10^{12}
Sludge 10000 mg/kg + NPK + Surfactant	2.37×10^{17}	1.69×10^{17}	1.70×10^{15}	1.72×10^{15}	5.88×10^{12}
Sludge 50000 mg/kg (control)	7.23×10^{18}	1.81×10^{15}	1.73×10^{15}	1.71×10^{15}	2.77×10^{10}
Sludge 50000 mg/kg + compost	1.08×10^{19}	1.72×10^{15}	1.73×10^{15}	5.12×10^{13}	7.79×10^{11}
Sludge 50000 mg/kg + NPK	2.53×10^{15}	2.56×10^{17}	1.73×10^{15}	1.72×10^{13}	5.78×10^{11}
Sludge 50000 mg/kg + NPK + Surfactant	2.37×10^{17}	1.72×10^{15}	1.73×10^{15}	1.72×10^{15}	1.30×10^{13}

The bacteria species isolated from the sludge *Pseudomonas* sp., *Flavobacterium* sp., were identified as; *Pseudomonas* sp. *ITRI77*, *Bacillaceae bacterium, Uncultured soil Uncultured Thauera* sp., *Uncultured bacterium, Clostridium* sp.



% Change= 86.75 ±1.90

Fig. 3. Moving window analysis (MWA) of bacterial community dynamics (rate of change) in 1% sludge contaminated soils biostimulated with compost



% Change= 87 ± 2.02

Fig. 4. Moving Window Analysis (MWA) of bacterial community dynamics (rate of change) in 5% sludge contaminated soils biostimulated with compost

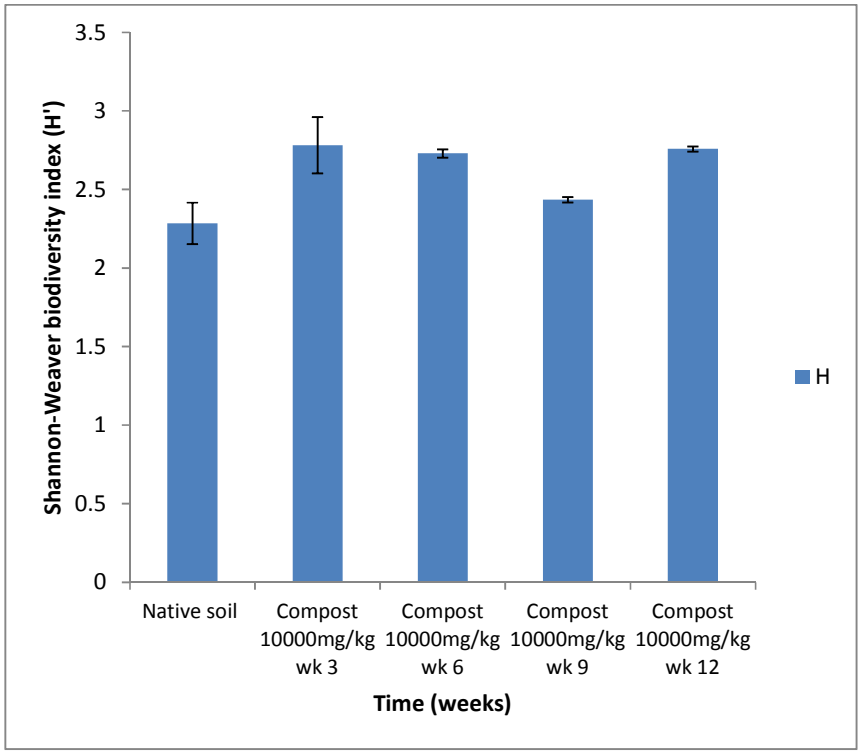


Fig. 5. Shannon-weaver diversity index of bacterial community in 1% sludge contaminated soils biostimulated with compost

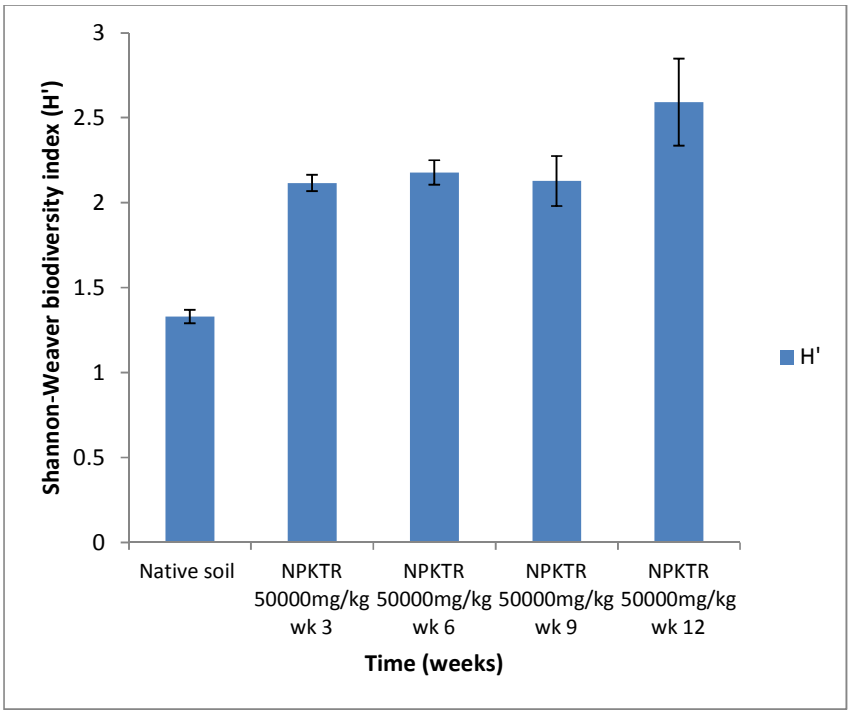


Fig. 6. Shannon-weaver diversity index of bacterial community in 5% sludge contaminated soils biostimulated with NPK and Surfactant (Triton-X100)

3.2 Discussion

In the soils treated with 1% sludge contamination, biostimulation with NPK gave the highest % TPH degradation (78.25%) followed by the control (sludge only, 76%), Compost (50.88%) and lastly the treatment with NPK and Triton-X 100(36%). NPK and Triton-X 100 had the highest TPH degradation of 46.55% followed by treatment with 50% compost (40.17%), NPK (37.72%) and lastly the control (36.33%), for the soils contaminated with 5% sludge. The results obtained for the treatments with 1% sludge biostimulated with NPK, are similar to those obtained in previous studies [36]. It was observed that NPK fertilizer stimulates higher bacterial growth for effective hydrocarbon degradation.The observed % TPH degradation in the control, could be in line with observations by several workers that hydrocarbon degraders are ubiquitous in nature [37,38] and low concentration hydrocarbon contaminated soils could be bioremediated by natural attenuation since the C:N ratio threshold is not exceeded [39]. The observed lower % TPH degradation in the treatment with compost compared to NPK, could be attributed to the low levels of its nutrient concentration as observation by several workers that compost is made up of over 90% by weight of carbon and oxygen and some amount of hydrogen, nitrogen and sulphur and the nutrients are released slowly in relation to NPK [40].

The total bacteria count (expressed as gene copies) at the end of 12 weeks using the quantitative real time PCR (qRT-PCR) showed that, in the soils treated with 1% sludge, the treatment with NPK recorded the highest bacterial count of 2.12 X 10^{12}cells/g. This corroborates with the high % of sludge degradation recorded with NPK biostimulation. The observed bacteria counts in the treatment with 1% sludge and NPK and the high percentage TPH degradation observed are similar to findings by [41], who showed that treating petroleum-contaminated soils with nitrogen can increase cell growth, decrease the lag phase, help maintain microbial populations at high activity levels and increase the rate of hydrocarbon degradation. The counts recorded for the treatments with 5% sludge were highest in the treatment with NPK and Surfactant (1.30 x 10^{13} cells/g) at the end of 12 weeks (Table 1). The low count observed for the treatment with NPK and surfactant and low percentage TPH degradation for the 1% sludge contamination, could be due to the following; inhibition by the surfactant as a result of toxicity by high concentration of surfactant or soluble hydrocarbon, preferential metabolism of the surfactant itself; interference with the membrane uptake process; or reduced bioavailability of miceller hydrocarbons [42,43]. In the treatments with 5% sludge, the high bacteria count (1.30 x 10^{13} cells/g) and high percentage TPH degraded (44.65%) in NPK and Surfactant microcosm, could be attributed to dilution of the surfactant to a concentration below its CMC by the high concentration of sludge added and emulsification of the high hydrocarbon concentration by the surfactant. These findings are similar to that of Lai et al., [44] who observed a higher percentage degradation of hydrocarbon in soils with higher hydrocarbon contamination in amendments with surfactant. This could be attributed to the explanation of Nguyen et al. [45], that mobilization mechanism occurs at concentrations below the surfactant CMC. At such concentrations, surfactants reduce the surface and interfacial tension between soil/water systems. Due to the reduction of the interfacial force, contact of surfactants with soil/oil system increases the contact angle and reduces the capillary force holding oil and soil together. By reducing surface and interfacial tensions, surfactants increase the surface areas of insoluble compounds leading to increased mobility and bioavailability of hydrocarbons to microorganisms and subsequently enhanced hydrocarbon degradation.

Results of Moving Windows Analysis (MWA) used to assess the rate of change or dynamics, Dy of the bacteria communities that degraded the petroleum sludge over time,. recorded the treatment with NPK had a lower rate of change, 33.75 ± 3.20% and 32.50 ± 4.68% at 12 weeks for the 1% and 5% sludge contamination respectively.This could be attributed to the homogeneous nature of the NPK. This indicates a more stable community in the NPK treated soils and may have accounted for the high % TPH degradation as reported by [46]. The rate of change recorded for the treatments with compost and NPK with surfactant were high and could also have contributed to the low % TPH degradation, in similar studies by Wittebolle et al., [46], who observed that a lot of species come into dominance and leave the microbial community resulting in broad dynamics, probably causing loss of coherence.

In relation to the control, the biodiversity of the bacteria community which was enhanced most

by compost treatment in both the 1% and 5% sludge treatments as indicated by the Shannon weaver index, was similar with observations by [47], who stated that compost which is made up of litter material stimulates the abundance of hydrocarbon harbouring communities in soil as well induces changes in diversity. Similarly, compost, can facilitate degradation of organic contaminants because they play a role in supplementing nutrients, providing a carbon source, aerating a contaminated soil, and retaining moisture content [22].

Some of the bacteria sequenced and identified from the petroleum sludge used for the research include; *Pseudomonas sp. ITRI77*, Uncultured *Thauera sp., Uncultured Pseudomonas sp.*, *Flavobacterium sp., Bacillaceae bacterium, Uncultured soil bacterium, Clostridium sp.* These are similar to findings by [47] and [48].

4. CONCLUSIONS

Physicochemical and microbiological analysis of the Nigerian petroleum sludge used for this research indicated that the sludge was slightly acidic with a high TPH content made up mainly of between 10-40 carbon unit compounds of phytane, pristine and hopane. Some Bacteria species identified in the sludge by molecular biology techniques include, *Pseudomonas sp. ITRI77*, Uncultured *Thauera sp., Uncultured Pseudomonas sp.*, *Flavobacterium sp., Bacillaceae bacterium, Uncultured soil bacterium, Clostridium sp.*

Biotreatability of Nigerian petroleum sludge by bioremediation in aerobic microcosm indicated that sludge contamination at lower concentration could be treated with natural attenuation since there was no significant difference between the control (natural attenuation), biostimulation and addition of surfactant when 1% sludge was used. There was however a significant difference in % TPH degradation between the natural attenuation and biostimulated sludge contaminated soils at higher (5%) sludge concentration. The addition of NPK enhanced the sludge degradation most at lower sludge concentration, but the treatment with NPK and surfactant followed by compost enhanced the sludge degradation most at higher sludge concentration.

Monitoring of the microbial communities functional in the bioremediation of the sludge using molecular biology technique (DGGE) indicated that biostimulation with compost enhanced a higher biodiversity of the bacterial communities than other treatments, especially at low concentrations. This could be due to the fact that compost play a role in supplementing nutrients, providing a carbon source, aerating a contaminated soil, retaining moisture content and contain an abundance of nitrogen and organic matter [22]. Compost also slowly releases nutrients similar to animal manures [40]. This could have accounted for the sustained degradation of the sludge steadily upto the 12th week observed in the compost treated microcosms.

The Bacterial community dynamics (rate of change), was however affected least with NPK and more by compost. This could be due to the heterogeneous nature of compost, compared to NPK. This could have enhanced the high % degradation in the treatment with NPK, since high dynamics have been shown to lead to noncoherence of the microbial community [46].

Oil exploration and production activities been the major source of revenue for the Nigerian economy, pollution of the environment by hydrocarbons would continue to be a recurring decimal in the Niger Delta ecological zone. Biostimulation as reported in this study enhanced biodegradation of hydrocarbon compounds more than natural attenuation at higher concentration, how it mostly occurs in cases of major oil spills or disposals.

The application of NPK for bioremediation of sludge contaminated soils has to be done continuously to sustain the nutrient level and complete biodegradation of the sludge since degradation of hydrocarbon started dropping on rapid depletion of nutrient, as observed in this study. This would mean application of NPK which will be another source of drain on the country's resources. The use of surfactant have also been questioned in many quarters where its argued that it only emulsifies the oil and transfers it from the surface into the water column and sediment causing harm to other pelagic and benthic organisms with its attendant adverse effects.

However, biodegradation of TPH rose steadily in a single application of compost till the end of the study due to its slow release of nutrients and improvement of soil texture, aeration and moisture content as mention earlier. Also, compost is cheap, readily available and locally produced. Findings in this research showed that it enhanced the biodiversity of the bacteria

community, and there was no significant difference between the % of hydrocarbons degraded by compost and NPK.

It is therefore recommended from the findings in this research, that the petroleum sludge could be properly treated by biostimulation using locally produced compost. This would protect the sensitive and fragile biota of the aquatic and terrestrial environments from the effects of the sludge, as well as protect human health by extension since they constitute major sources of our diet. Information gathered as regards the ideal concentration of the sludge for bioremediation could be used by environmental regulators to update the guidelines for management of petroleum sludge.

However, more research needs to be done in the field of biotechnology to exploit and enhance the potentials of indigenous microorganisms to degrade hydrocarbon wastes and similar recalcitrant organic compounds anaerobically to produce biogas.This will offer potential energy saving and is a more stable process for medium and high strength organic wastes. Apart from treating the wastes, the methane gas produced can be recovered and there is reduction of green house gases. The substitution of oil and coal with bioenergy will result in saving the global environment by reducing the use of fossil fuels and in the long run reduce the global warming and climate change effects which currently is a major global environmental challenge.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

ACKNOWLEDGEMENT

I wish to thank Professor Andrew Ball for offering his expertise and giving me the privilege to use the Biology laboratory of Flinders University, Adelaide, South Australia to conduct the molecular aspect of this research. I also thank Dr. Eric Adetutu for his guidance and assistance during the research. My thanks also goes to the Postgraduate students in the laboratory for their assistance. I thank Professor Ernest Atuayan, my home supervisor, for his motivation and encouragement.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Ururahy AFP, Marins MDM, Vital RL, Therezinha I, Pereira N. Jr. Effect of aeration on biodegradation of petroleum wastes. Reviews in. Microbiology. 1998; 29: 4.
2. Hann WJ, Loehr RC. Biological treatment of petroleum oily sludges. SPE –Society of Petroleum Engineers –Periwan Basin oil and Gas Recovering conference, Texas. 1992;519-530.
3. Louvisse AM, Freire T, Teixeira NO. Metodologia para caracteizacao Borras de petroleol Mesa Rendonda Sobre Quimica Analitica Ambiental, Curitiba; 1994.
4. Prince M, Sambasivam Y. Bioremediation of petroleum wastes from the refining of lubricant oils. Environmental Progress. 1993; 12:5-11.
5. Prospt TL, lochmiller RI, Qualls CW, Jr. Mcbee K. *In situ* (mesocosm) assessment of immunotoxicity risks to small mammals inhabiting petrochemical waste sites. Chemosphere. 1999;38:1049-1067.
6. Atlas RM, Bartha R. Hydrocarbon biodegradation and oil spill bioremediation. K.C. Marshall (Ed). Advances in Microbial Ecology. New York: Plenum Press. 1992; 12:287–338.
7. Mishra S, Jyot J, Kuhad RC, Lal B. Evaluation of inoculum addition to stimulate in situ bioremediation of oily-sludge-contaminated soil', Applied and Environmental Microbiology. 2001;67: 1675–1681.
8. Englert CJ, Kenzie EJ, Dragun J. Bioremediation of petroleum products in soil. Principles & Practice of. Petroleum Contamination, in Soils, API. 1993;40:111-129.
9. Hazardous Waste Management System. Environmental Protection Agency. 1997; 62(29):12.
10. Oolman T, Baker RR, Renfro NL, Marshall GE. Refinery uses bioslurry process to treat RCRA wastes. Hydrocarbon Processing. 1996;71-76.

11. Department of petroleum resources. Environmental Guidelines and Standards for the Petroleum Industry in Nigeria (EGASPIN). 2002;314.
12. Norris RD. Handbook of bioremediation. CRC, Boca Raton; 1994.
13. Caplan JA. The world-wide bioremediation industry: prospects for profit. Trends Biotechnology. 1993;11:320-323.
14. Bartha R. Biotechnology of petroleum pollutant biodegradation. Microbiology and Ecology. 1986;12:155–172.
15. Diplock EE, Mardlin DP, Killham KS, Paton GI. Predicting bioremediation of hydrocarbons: Laboratory to field scale. Environmental Pollution. 2009;157:1831-1840.
16. Iwamoto T, Nasu M. Current bioremediation practice and perspective. Journal of Bioscience and Bioengineering. 2001;92:1-8.
17. Kozdroj J, Elsas J. Structural diversity of microorganisms in chemically perturbed soil assessed by molecular and cytochemical approaches. Journal of Microbiological Methods. 2001;43:197-212.
18. Ranjard L, Poly F, Nazaret S. Monitoring complex bacterial communities using culture-independent molecular techniques: application to soil environment. Research in Microbiology. 2000;167-177.
19. Tudararo-Aherobo Laurelta, Atuayan Ernest. Toxicological effects of petroleum refinery sludge on the terrestrial environment using bacteria and earthworm as bioindicators. Journal of Advances in Microbiology. 2020; 20(4):30-40.
20. Mills AL, Breuil C, Colwell RR. Enumeration of petroleum-degrading marine and estuarine microorganisms by the most probable number method. Canadian Journal of Microbiology. 1978; 24(5):522-7.
21. Walworth J, Pond A, Snape I, Rayner J, Ferguson S, Harvey P. Nitrogen requirements for maximizing petroleum bioremediation in a sub-Antarctic soil. Cold Regions Science and Technology. 2007; 48:84-91.
22. Namkoong WE, Hwang JP, Choi J. Bioremediation of diesel-contaminated soil with composting. Environmental. Pollution. 2002;119:23.
23. Zhongun M. Bioremediation of petroleum contaminated soils using indigeneous cultures. A thesis submitted to school of graduate studies, in partial fulfilment of the requirements in master's of engineering, Faculty of engineering and applied science, Memorial University of Newfoundland, Canada; 1998.
24. International Organization for Standardization. ISO 16703:2004. Soil quality-determination of content of hydrocarbon in the range C10-C40 by gas chromatography. Geneva, Switzerland: ISO; 2004.
25. Muyzer G, Smalla K. Application of denaturing gradiient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leewenhoek. 1998; 73:127-141.
26. Rebrikov DV, Trofimov, Yu D. Real-time PCR: A review of approaches to data analysis. Applied Biochemistry and Microbiology. 2006;42(5):455–463.
27. Muyzer G, Waal E, Utterlinden A. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis pf polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology. 1993;695-700.
28. Sheffield V, Cox D, Lerman L, Myers R. Attachment of a 40-base-pair G+C rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. Proceedings of the National Academy of Sciences. 1989;86: 232-236.
29. Anderson IC, Campbell CD, Prosser JI. Diversity of fungi in organic soils under a moorland-Scots pine (*Pinus sylvestris* L.) gradient. Environmental Microbiology. 2003a;5:1121-1132.
30. Aroca A, Raposo R. PCR-based strategy to detect and identify species of Phaeoacremonium causing grapevine diseases. Applied Environmental Microbiology. 2007;73:2911-2918.
31. Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS. 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Research. 1991;19.
32. Teske A, Wawer C, Muyzer G, Ramsing NB. Distribution of sulfate-reducing bacteria in a stratified fjord (Mariagerfjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified

ribosomal DNA fragments. Appl. Environ Microbiol. 1996;62:1405–141.

33. Adetutu EM. The fate of azoxystrobin in soils and its effects on soil bacterial and fungal communities, Department of Biological Sciences, University of Essex; 2005.
34. Girvan MS, Bullimore J, Pretty JN, Osborn AM, Ball AS. Oil type is the primary determinant of the composition of the total and active bacterial communities in arable soils. Applied Environmental Microbiology. 2003;69:1800-1809.
35. Marzorati M, Wittebolle L, cBoon N, Daffonchio D, Verstraete W. How to get more out of molecular fingerprints: practical tools for microbial ecology. Environmental Microbiology. 2008;10(6): 1571-81. Mills M, McDonald T, Bonner J, Simon M, Autenrieth R. Method for quantifying the fate of petroleum in the environment. Chemosphere. 1999;39: 2563-2582.
36. Nduka JK, Umeh LN, Okerulu IO, Umedum LN, Okoye HN. Utilization of different microbes in bioremediation of hydrocarbon contaminated soils stimulated with inorganic and organic fertilizers. Journal of Petroleum & Environmental Biotechnology. 2012;3:2.
DOI: 10.4172/2157-7463.100016
37. Texas Research Institute, Inc. Enhancing the microbial degradation of underground gasoline by increasing available oxygen. Report to the American Petroleum Institute. Washington, D.C; 1982a.
38. Killham K. Soil ecology. Cambridge University Press. U.K; 1994.
39. Chaudhry GR. Biological degradation and bioremediation of toxic chemicals. Dioscorides Press, Portland, Oregon; 1994.
40. McBride MB. Toxic metals in sewage sludge-amended soils: Has promotion of beneficial use discounted the risks? Advance Environmental Resources. 2003; 8:5-19.
41. Efroymson RA, Alexander M. Biodegradation by an arthrobacter species of hydrocarbons partitioned into an organic solvent. Applied and Environmental Microbiology. 1991;57:1441-1444.
42. Mulligan CN, Yond RN, Gibbs BF. Surfactant-enhanced remediation of contaminated soil: A review. Environmental Geology. 2001;60:371-380.
43. Lai CC, Huang YC, Wei YH, Chang JS. Biosurfactant-enhanced removal of total petroleum hydrocarbons from contaminated soil. Journal of. Hazardous. Materials. 2009;167:609–614.
44. Nguyen TT, Youssef NH, McInerney MJ, Sabatini DA. Rhamnolipid biosurfactant mixtures for environmental remediation. Water Resources. 2008;42:1735-1743.
45. Wittebolle L, Boon N, Vanparys B, Heylen K, De Vos P, Verstraete W. Failure of the ammonia oxidation process in two pharmaceutical wastewater treatment plants is linked to shifts in the bacterial communities. Journal of Applied Microbiology. 2005;99:997–1006.
46. Schulz S, Giebler J, Chatzinotas A, Wick LY, Fetzer I, Welzl G. Plant litter and soil type drive abundance, activity and community structure of alkB harbouring microbes in different soil compartments. ISME J. 2012;6:1763–1774.
47. Van Hamme JD, Singh A, Ward OP. Recent advances in petroleum micro-biology. Microbiology and Molecular Biology Reviews. 2003;67(4):503–549.
48. Okpokwasili GC, Oton NS. Comparative applications of bioreactor and shake flask systems in the laboratory treatment of oily sludge. International. Journal of Environment and Waste Management. 2006;1:1.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/58574