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Microbial dynamics and physico-chemical properties of artisanal refinery polluted environment in Niger Delta

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Abstract

Artisanal refining of crude oil is a common practice in the Niger Delta region of Nigeria and this has continuously posed serious environmental and health risks. This was designed to investigate the microbial dynamics and physico-chemical properties of environmental (water, sediment and soil) matrix of impacted by artisanal refining activities. Three sampling locations (Bolo, River's state; Ekpemu, Delta state; and Twon-Brass, Bayelsa state) were studied. Finding revealed that the total heterotrophic bacterial (THB) load of the recipient water from Twon-Brass had the least count of 2.0 ±0.2 x 104cfu/ml, followed by Ekpemu (2.1 ±0.3 x 103cfu/ml), then Bolo (2.35±0.6 x 104cfu/ml). In the contrary, recipient water body in Ekpemu had the least HUB load (1.8 ±0.1 x 103cfu/ml) followed by Bolo (2.0 ±0.3 x 104cfu/ml), and Twon-Brass (2.0 ±0.8 x 104cfu/ml). The bacterial load of the un-impacted aquatic matrix revealed a higher heterotrophic bacterial load (5.8+ 0.30 x 105cfu/ml) and low HUB load (0.2+ 0.21 x 103cfu/ml). In the sediment, Bodo had the least THB load of 2.1 ±0.2 x 105cfu/g, followed by Twon-Brass (2.3 ±0.6 x 105cfu/g), and Ekpemu (2.9 ±0.3 x 105cfu/g).Bolo and Twon-Brass soil were also highly impacted with hydrocarbons with a pollution index close to 1 (HUB/THB ratio = 0.9) while Ekpemu had a pollution index of 0.7. Bacterial species associated with the samples were *Bacillus subtilis, Pseudomonas putida,* and *Pseudomonas aeruginosa* were predominant in all the sample locations. Similarly, the artisanal refining activities also altered the physico-chemical properties of the various environmental matrix studied. Based on the finding of this study, it can be predicted that bioremediation various environmental matrix by natural attenuation is ongoing as most of the organisms isolated are hydrocarbon degraders. However, it is recommended that artisanal refining activities should be discouraged.

Keywords: Microbiology; Microbes; Dynamics; Pollution; Artisanal refinery; Niger Delta

1. Introduction

Artisanal refining is the process of procuring stolen crude oil and further refining them in the so-called bush refineries with the use of local resources and skills (drawing on the indigenous technology used to distil locally made gin – ogogoro or kaikai). The basic materials typically involve rudimentary illegal stills – often metal pipes and drums welded together – in which crude oil is boiled and the resultant fumes are collected, cooled and condensed in tanks to be used locally for lighting, energy or transport (1). Oil theft or illegal bunkering as it is known in Nigeria is an organized 'theft of crude oil from product pipelines through the use of improvised conduits and direct pumping from oil well heads into barges by criminal syndicates (2).

It may appear chaotic, but there is structure and strategy to this menace. It is a well- financed and highly organized criminal phenomenon – a parallel industry with a developed supply chain and growing sophistication. It involves trained artisans who break into high pressure pipelines, using welded valves and other complex instruments to siphon oil mostly in the night.

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Figure 1 Artisanal Refinery site in the Niger Delta

Boat yards help construct and supply barges to the participants to transport crude oil around the creeks. Local women supply firewood etc. to workers at the camp. Many of the people involved in oil theft work for local markets, poorly refining products for communities desperate for affordable sources of energy (2). Although the economic impact of oil theft associated with artisanal refineries has been widely reported (3), the impact of the operations of these refineries on the highly sensitive environment of the Niger Delta is scarcely reported (4).

According to (5), it has been reported that petroleum refining contributes solid, liquid, and gaseous wastes in the environment. Some of these wastes could contain toxic components such as the polynuclear aromatic hydrocarbons (PAHs), which have been reported to be the real contaminants of oil and most abundant of the main hydrocarbons found in the crude oil mixture (6). Once introduced in the environment, PAHs could be stable for as short as 48 hours (e.g. naphthalene) or as long as 400 days (e.g. fluoranthene) in soils (7). They thus, resist degradation and, remain persistent in sediments and when in organisms, could accumulate in adipose tissues and further transferred up the trophic chain or web (8). Lyons et al., (9) opines that acute exposures to aromatic hydrocarbons, which are common constituents of oil, are known to cause respiratory symptoms and high molecular weight PAHs are of significant concern because of the mutagenicity, carcinogenicity and bioaccumulation in organic tissues due to their lipophilic character (10).

2. Material and methods

2.1. Sample Collection

Water, soil and sediment samples were collected with sterile containers from artisanal refineries impacted sites in Bolo community of Rivers State, Twon-Brass community of Bayelsa state, Ekpemu community of Delta state, all in the Niger Delta region of Nigeria.

Figure 2 Map of Niger Delta Region showing the sample states
Source: (11).

2.2. Microbiological analysis of the samples

Estimation of the total heterotrophic counts of the samples was carried out using pour plate technique (12). One (1) mg or g of the samples was serial diluted and the desired dilution was plated on Nutrient Agar (NA) to determine the total heterotrophic. All the NA plates were inverted and incubated at 25 – 28℃ for 24 hours. After the incubation, discrete colonies that emerged on the culture were counted and recorded in Colony Forming Units/ml of sample.

2.3. Determination of hydrocarbon utilizing bacteria

The hydrocarbon utilizing bacterial loads of the samples (water, sediment and soil) used in this study was determined using the modified enrichment technique. The HUB count was carried out in triplicates on mineral salt agar (MSA) of Mills as modified by (13). MSA was composed of 0.29g of KCl, 10g of NaCl, 0.42g of $MgSO_4.7H_20$, 0.42g of NH_4NO_3, 1.25g of K_2HPO_4, 0.83g of KH_2PO_4 and 15g of agar all dissolved in one litre of distilled water and autoclaved at 121℃ for 15mins. This was carried out by inoculating 0.1ml aliquot of the dilutions onto mineral salt agar plates embedded with the petroleum product (13). The bacteria colonies on the plates after 48 to 72 hours incubation at ambient temperature were counted and sub-cultured onto fresh mineral salt agar plate.

2.4. Maintenance of pure cultures of bacterial isolates

To obtain pure culture of the bacterial isolates, the primary cultures were subjected to sequential sub-culturing by streak method as described by (12). The mono-colonies of the bacterial isolates derived were sub-cultured into Mac Cartney bottles containing freshly prepared NA slants and incubated at 30 $\pm$ 2^0C for 24 hours before storage at 4^0C for future use.

2.5. Identification of isolates

Pure isolates from the corresponding agar slants were characterized and identified using morphological (cell and colonial morphology, shape, motility, and gram reaction), biochemical and physiological attributes (14, 15).

2.6. Molecular Identification of Isolates

DNA extraction: DNA extraction was carried out by using a ZR fungal/ bacterial DNA Miniprep extraction kit obtained from Inquaba, South Africa. The DNA extraction protocol was based on manufacturer's instruction. Heavy growth of the pure isolates sub cultured on MSA plates were suspended in 200µl of isotonic buffer into ZR bashing bead lysing tubes, 750µl of Lysing solution was added. They were processed at maximum speed for 5mins.The ZR Bashing bead lysing tubes were centrifuged at 10,000 xg for 1min. Four hundred (400) µl of the supernatants were transferred into ZYMO-spin IV spin filter in collection tubes and centrifuged at 7000xg for 1min. The amount of 1,200µl of fungal/bacterial DNA binding buffer was added to each filtrate in the collection tubes bringing the final volume to 1,600µl, 800µl was then transferred into ZYMO-spin11C column in a collection tube and centrifuged at 10,000xg for 1min, the flow through were

discarded from the collection tubes. The remaining volumes were transferred to the same ZYMO-spin and spun at 10,000xg for 1min. Two hundred (200) µl of the DNA pre-wash buffer were added to the ZYMO-spin 11C in new collection tubes and spun at 10,000xg for 1min followed by addition of 500µl of fungal/bacterial DNA wash buffer and centrifuged at 10,000xg for 1min. The ZYMO-spin 11C column were transferred to clean 1.5µl centrifuge tubes and 100µl of DNA elution buffer were added to the column matrix and centrifuged at 10,000xg for 30 seconds to elute the DNA. The ultra-pure DNA of each isolate properly labeled were then stored at -200C for use. After extraction, the DNA samples were quantified using NANODROP (ND1000). PCR amplification of 16S rRNA: The 16S of the rRNA genes of the isolates were amplified using the 27F and 1492R primers on a PCR System (9700 Applied Bio system thermal cycler) at a final volume of 25µl for 40 cycles. The PCR mix include: the x2 Dream tag master mix supplied by Inquaba, South Africa (tag polymerase, DNTPs, MgCl2, the primers at a concentration of 0.4M and extracted DNA as a template. The PCR conditions were as follows: initial denaturation, 95^0C for 4mins; denaturation 95^0C for 30s; Annealing, 52^0C for 3mins. Then holding temperature at 4^0C. The products were resolved on a 1% agarose gel at 120v for 15min and visualized on a UV transilluminator. The generated PCR products were dried and sent to Inquaba (South Africa) for purification and sequencing using an AB13500 genetic analyzer adopting the Bigdye Termination technique by Inquaba South Africa. Phylogenetic analysis: The sequences were edited using the bio informatics algorithm Bioedit. Similar sequences were downloaded from the National Biotechnology Information Centre (NCBI) data base using Blast N, these sequences were aligned using cluster IX. The evolutionary history of the isolates was inferred using Neighbor - Joing method in MEGA 6.0 (16). The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary of the taxa analyzed (17).

2.7. Physicochemical analysis of the water samples

The physicochemical properties of the Water samples were determined using standard analytical procedures recommended by (18). Unstable or easily changeable parameters such as pH and temperature of the water samples were measured *in situ.*

2.8. Temperature

The temperature of water samples was determined by means of a mercury thermometer calibrated in ±0.2^0C units from 0℃ to 100℃. The thermometer was dipped into the sample and left for about five minutes for equilibrium before the reading was recorded.

2.9. Hydrogen ion concentration (pH)

The pH was determined using PYE UNICAM, England PW 9418 pH meter fitted with a combined glass pH and reference electrode (18). The pH probe was calibrated with buffer 4 and 7 solution after which the electrode was rinsed with distilled water and dipped into the water sample in the beaker. The pH of the water was displayed on the pH meter screen and allowed to stabilize for 5 min before taking the reading.

2.10. Determination of Alkalinity

Alkalinity is a measure of the capacity of water to neutralize acids. Total alkalinity is measured by measuring the amount of acid (e.g., sulphuric acid) needed to bring the sample to a pH of 4.2. At this pH all the alkaline compounds in the sample are "used up." It is estimated separately by titration against standard acid using phenolphthalein and methyl orange as indicators. The result is reported as milligrams per liter of calcium carbonate (mg/L $CaCO_3$), (18).

2.11. Total Dissolved Solid (TDS)

Total dissolved solids (TDS) are a measure of the total combination of all these minerals and salts. A total dissolved solid (TDS) was determined according to (18) instrumental method using the HACH TDS meter (Mettler Toledo conductivity /TDS meter model MC 126).

2.12. Total Suspended Solids (TSS)

Total Suspended Solids (TSS) were determined by filtering a well-mixed aliquot (100ml) of the water sample through a dried and pre-weighed Millipore filter paper using vacuum filtration apparatus. The filter paper was dried at 105^0C to constant weight. The difference in weight of the filter paper represents the total suspended solids. This was reported in mg/l after calculation (18).

2.13. Dissolved Oxygen (DO)

Low levels of Dissolved Oxygen (DO) in water bodies lead to the death of fish and other oxygen-dependent organisms. A commonly used method for continuous monitoring of DO is based on membrane electrodes (voltametric and galvanometric).

2.14. Chemical Oxygen Demand (COD)

To determine the COD levels of the water sample, precisely 10 ml of water sample was measured and transferred into a glass beaker. Thereafter 0.2 g of mercuric sulphate ($HgSO_4$) was weighed and added to the sample and mixed properly before 1 ml of concentrated sulphuric acid was added and left to cool before 5 ml of 0.25N potassium dichromate ($K_2Cr_2O_7$) solution was also added. Thereafter, 14 ml of sulphuric acid with silver sulphate reagent was slowly added and swirled until the solution was thoroughly mixed. The beaker was covered with aluminium foil and heated to 100^oC for 30 minutes. The mixture was allowed to cool before diluting the acid solution to 150 ml mark with distilled water. Five drops of Ferroin indicator were added to the solution until a sharp colour change was noticed; changing from blue green to reddish brown (dark red) colour. The reading on the COD Agilent meter 4510N was taken and recorded in mg/l (18).

2.15. Determination Biochemical Oxygen Demand (BOD)

To determine BOD of the surface water sample, 1 ml of the sample suspension was transferred into 164 ml overflow flask and 0.2 ml of phosphate buffer, magnesium sulphate, calcium chloride and iron (III) chloride hexa-hydrate was added into 164 ml overflow flask. The flask was filled with thoroughly homogenized sample and transferred into a BOD bottle before 3 drops of a nitrification inhibitor was added. Thereafter 2 NaOH tablets were added in the rubber quiver and a pressure measuring head (OxiTop) was tightly screwed onto the bottle and activated to start taking measurements automatically. This was incubated at 20^oC for 5 days and stored values for the 5 days were recorded (18).

2.16. Physicochemical analysis of the soil and sediment samples

Physicochemical parameters of the soil and sediment were determined using standard analytical procedures recommended by (18).

2.17. Determination of Electrical Conductivity (EC)

EC was measured in 1:2 (sample-water) ratio suspensions with the help of conductivity meter. The EC meter was calibrated using standard KCl solution and electrical conductivity of the suspensions was determined.

2.18. Determination of sample pH

The pH of the samples was measured potentiometrically in a 1:2 (sample – water) ratio suspension. About 25g of each sample was weighed out, air-dried and sieved with a 2mm mesh. The sized sample was transferred into a 100 ml flask and 50 ml of distilled water added and shaken for one hour. The pH meter was calibrated using the pH buffer (KCl), after which the measure of pH of the suspension was taken.

2.19. Determination of Organic Matter Content

The Walkley-Black method was used to determine the organic matter content of the samples. In this the milled soil samples were passed through 0.5mm sieve. One (1) gram of sample was transferred into a 250ml Erlenmeyer flask in duplicate. Ten (10) ml of 1M$K_2Cr_2O_7$ solution was accurately added to the sample in each flask and swirled gently to disperse the soil. Thereafter 20 ml of conc. H_2SO_4 was added rapidly and immediately swirled gently until the soil and reagents were mixed, and vigorously swirled for one additional minute. The flask was allowed to stand for 30 minutes. One hundred (100) ml of distilled water was added and allowed to stand for 30 minutes. Three to four (3-4) drops of an indicator, diphenylamine, was added to the solution and 0.5N ferrous sulphate solution was titrated. A greenish cast which changes to dark green showed an approach to the end point. Continuous addition of ferrous sulphate drop by drop changes the solution colour from blue to red. The blank titration was done in the same manner but without sample (18).

Percentage Organic matter was derived from the equation thus:

$$\%Org.C = \frac{N\,(V_1 - V_2))0.3f}{w}$$

Where, % organic matter in soil = % organic C x 1.729; N = Normality of ferrous sulphate solution; V_1= ml ferrous ammonium sulphate required for the blank; V_2= ml ferrous ammonium sulphate required for the sample, W = weight of sample in gram, f = correction.

2.20. Determination of organic carbon content

The percentage organic carbon was expressed on oven-dried basis after correction for moisture in air-dry soil by multiplying with MCF, where MCF is defined as Moisture Correction Factor given as MCF = 100 + % moisture (18).

2.21. Determination of Total Nitrogen Content

Precisely 10 g of the sample was weighed, air-dried, ground and made to pass through a 0.5mm sieve into a dry 500ml micro-Kjeldahl flask and 20ml of distilled water was added. The flask was swirled for a few minutes and allowed to stand for 30 minutes. A tablet of mercury catalyst and 10 grams of $K_2SO_4$was added. Thirty (30) ml of conc. H_2SO_4 was added using automatic pipette. Low heat was applied cautiously on the stand. The heat was increased when the water has been removed and frothing ceased. The mixture was boiled for 5 hours, then, allowed to cool and 100ml water was added to the flask. The digest was transferred into another clean micro-Kjeldahl flask (750 ml capacity). Fifty (50) ml of H_3BO_3 indicator solution was added into a 500ml Erlenmeyer flask and was placed under the condenser of the distillation apparatus. The 750mlKjedahl flask was attached to the distillation apparatus and 150ml of 10M NaOH solution was poured through the distillation flask via the opened funnel stopcock and distilled. The distillation was ended after the collection of 150ml distillate. The distillate was titrated with 0.01M standard HCl to determine the NH_4-N content. Colour change from green to pink indicated the end point (18).

Percentage nitrogen was calculated as shown:

$$\%N = \frac{T\ x\ M\ x\ 14\ x\ 100}{Wt}$$

Where, T = Titre value, M= Molarity of HCl, Wt. = weight of soil used.

2.22. Exchangeable acidity

The barium chloride triethanolamine ($BaCl_2$-TEA) method was used for the determination of exchangeable acidity. To 10 grams of sample in a 125ml Erlenmeyer flask, 100ml of extracting solution was added, swirled with fitted stopper and allowed to stand overnight. The content was transferred to a Pyrex Buchner funnel, size No. 40, fitted with 4.25cm Whatman No. 42 filter paper. The flask was rinsed with the extracting solution and the sample was continuously leached with drops of the extracting solution until about 225ml of the leachate was collected. This was transferred to a 250ml volumetric flask and the volume made up with extracting solution. The leachate was poured into a 500ml Erlenmeyer flask and 5 drops of mixed indicator solution was added. This was then titrated with 0.2M HCl to get a pink end point. The volumetric flask was rinsed with the titrated solution and titration was completed. On the other hand, 250ml of the original extracting solution was titrated to precisely the same end point using the same amount of mixed indicator (18).

The exchangeable acid was calculated thus:

$$EA = (B - S)10M$$

Where, B is the volume (ml) of acid required to titrate 250ml of the extracting solution, S is the volume (ml) of acid required to titrate the soil extract and M is the molarity of the acid.

2.23. Exchangeable Cations

The batch method was used to determine the cation exchange capacity of the samples. Five (5) grams of sample and 30ml of 1MNH_4OA_c solution were added to a 50ml centrifuge tube and shaken mechanically for 2 hours. The tube was centrifuged at 2000rpm for 20 minutes and the clear supernatant decanted into a 100ml volumetric flask. Another 30ml aliquot NH_4OA_c solution was added and the mixture was shaken for 30 minutes. The mixture was centrifuged and the leachate transferred to the same volumetric flask repeatedly. The volume of the leachate was made up to 100ml with NH_4OA_c solution and kept for the analysis of cations. The residue was washed four times with 25ml aliquot of ethanol (90%) and left standing for 30 minutes each time. $NH^+_4$was replaced by extracting with 1M NaCl four times with 25ml aliquot each time. After standing for 30 minutes, the extract was collected into the same volumetric flask and the volume

made up with 1M NaCl solution (18). The concentration of NH^{+}_{4} in the extract was determined and the cation exchange capacity was calculated thus:

$$CEC\ \left(\frac{Cmol}{kg}\right) = \frac{10\ x\ C}{W}$$

Where, C = concentration of NH^{+}_{4} (Cmol/kg) in the extract; W= weight of soil used.

2.24. Determination of phosphate

A simple spectrophtometric method has been developed for the determination of phosphate dissolved in samples. The method is based on the formation of phosphomolybdate with added ammonium molybdate followed by reduction with hydrazine in acidic medium. The intensity of blue colour is proportional to amount of phosphate. If the acidity at the time of reduction is 0.5M in sulphuric acid and hydrazinium sulphate is the reductant, the resulting blue complex exhibit maximum absorption at 820 nm. The system obeys Lambert-Beer's law at 830 nm in the concentration range of 0.5-5 µg/mL of phosphate with relative standard deviation (RSD) of 0.1% and correlation coefficient of 0.99. Molar absorptivity was determined to be 2.9 x 10^4 L mol^{-1} cm^{-1} at 830 nm (18).

2.25. Determination of Nitrate (NO^{-}_{3})

The salts of nitric acid are called nitrates. Input of nitrates and nitrites to the environment can occur through industrial and domestic combustion processes. Nitrate was estimated by Wet-chemical and physical techniques. Generally, the inert nitrate was converted to more reactive nitrite by chemical or photo-induced reduction before initiating the detection sequence. NO^{-}_{3} can also be determined by reducing it to NH_3 by titanous chloride and then measuring $NH_3$and then measuring NH_3potentiometrically by gas-sensing electrode.

2.26. Determination of Sulphate (SO_4^{2-})

Sulphate was determined by gravimetric method. Sulphate was precipitated as $BaSO_4$by the addition of $BaCl_2$ to a slightly acidic solution. $BaSO_4$ is soluble in both water and dilute acids. The washed and incinerated precipitate was weighed as $BaSO_4$.

2.27. Determination of Chloride (Cl^-)

Chloride, (Cl^-) was determined using potassium chromate as an indicator in the titration of chloride with silver nitrate. Red-blue coloured silver chromate gave the end point.

3. Results and discussion

3.1. Microbial Counts of Adjoining Environment to Artisanal Refineries

The effect of artisanal refineries on the adjourning ecological metrics was measured by determining the loads of total heterotrophic (THBC) and hydrocarbon utilizing (HUB) bacteria. The analysis revealed that the mean counts of HUB were very close to the mean counts of the THBC in the adjoining water (Figure 3), sediment (Figure 4) and soil (Figure3) around artisanal refineries and unpolluted environmental matrix (Figure 6).The pollution index of all the study stations is as presented in Table 1. Comparing the bacterial loads of the sampling stations revealed that THB of the recipient water from Twon-Brass had the least count of 2.0 ±0.2 x 10^4cfu/ml, followed by Ekpemu (2.1 ±0.3 x 10^3cfu/ml), then Bolo (2.35±0.6 x 10^4cfu/ml). In the contrary, recipient water body in Ekpemu had the least HUB load (1.8 ±0.1 x 10^3cfu/ml) followed by Bolo (2.0 ±0.3 x 10^4cfu/ml), and Twon-Brass (2.0 ±0.8 x 10^4cfu/ml).The bacterial load of the un-impacted aquatic matrix revealed a higher heterotrophic bacterial load (5.8± 0.30 x 10^5cfu/ml) and low HUB load (0.2± 0.21 x 10^3cfu/ml). The pollution index (HUB/THB ratio) of 0.88 was recorded for recipient water from Bolo, followed by Ekpemu (0.9), and Twon-Brass (1.0) while the un-impacted water body had a HUB/THB ratio of 0.0003.

Analysis of the sediment matrices revealed that the sediment samples obtained from Bodo impacted environment had the least THB load of 2.1 ±0.2 x 10^5cfu/g, followed by Twon-Brass (2.3 ±0.6 x 10^5cfu/g), and Ekpemu (2.9 ±0.3 x 10^5cfu/g). The sediment sample from Bolo also recorded the least HUB load of 1.8 ±0.3 x 10^4cfu/g, followed by Twon-Brass (2.0 ±0.5 x 10^5cfu/g), and Ekpemu (2.0 ±0.9 x 10^5cfu/g). In comparison, analysis of the bacterial load of the un-impacted sediment matrix revealed a heterotrophic bacterial load of 6.2 ± 0.61 x 10^5cfu/ml and HUB load of 1.4± 0.22 x 10^3cfu/ml. A pollution index (HUB/THB ratio) of 0.7 was recorded for sediment from Ekpemu, while sediments from Bolo and Twon-Brass had a pollution index of 0.9 while the un-impacted sediment had an index of 0.002.

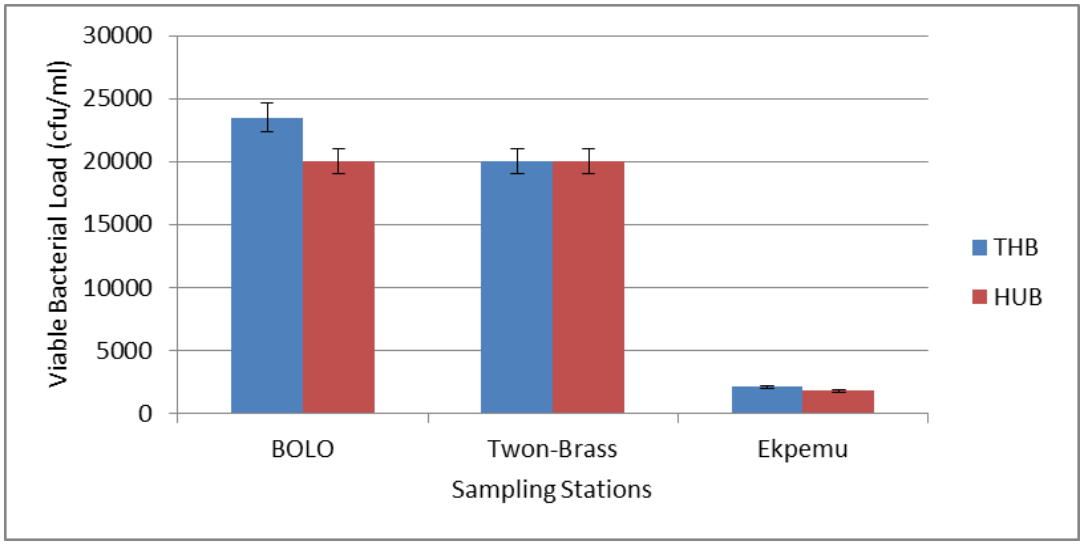

Figure 3 Bacterial loads of artisanal activities impacted aquatic matrices

Similarly, comparative analysis of the soil matrix of the adjoining communities impacted by artisanal activities revealed that soil in Bolo and Twon-Brass were highly impacted with hydrocarbons with a pollution index close to 1 (HUB/THB ratio = 0.9) while Ekpemu had a pollution index of 0.7.On the contrary, the un-impacted soil had a pollution index of 0.005. The THB load of Bolo soil was 2.1 ±0.2 x 10^5 cfu/g while that for Twon-Brass and Ekpemu was 2.3 ±0.6 x 10^5cfu/g), and 2.9 ±0.3 x 10^5cfu/g respectively. The hydrocarbon utilizing bacteria load of the soil sample from Bolo was the least with 1.8 ±0.3 x 10^4cfu/ml, followed by Twon-Brass (2.0 ±0.5 x 10^5cfu/ml), and Ekpemu (2.0 ±0.9 x 10^5cfu/ml).The bacterial load of the un-impacted soil matrix revealed a higher heterotrophic bacterial load (7.8 $\pm$ 0.21 x 10^5cfu/ml) and low HUB load (1.9 $\pm$ 0.63 x 10^3cfu/ml).

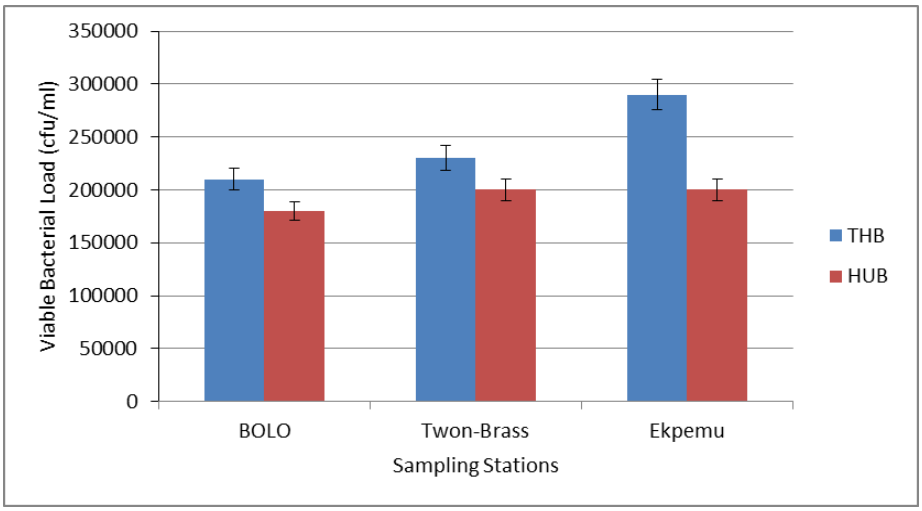

Figure 4 Bacterial loads of artisanal activities impacted sediment matrices

The findings of this study agree with several reports on the impact of hydrocarbon contamination in microbial loads. (19) in their study on the distribution of PAH-ring hydroxylating dioxygenase genes in bacteria isolated from two illegal refining sites in Niger Delta, Nigeria reported a high HUB load in the order of 10^4. According to the authors, the hydrocarbon degrading bacterial community existed in the midst of improved biodiversity. The HUBs threshold counts also indicate that a good number of the THBs had developed competence to degrade the hydrocarbons (19).

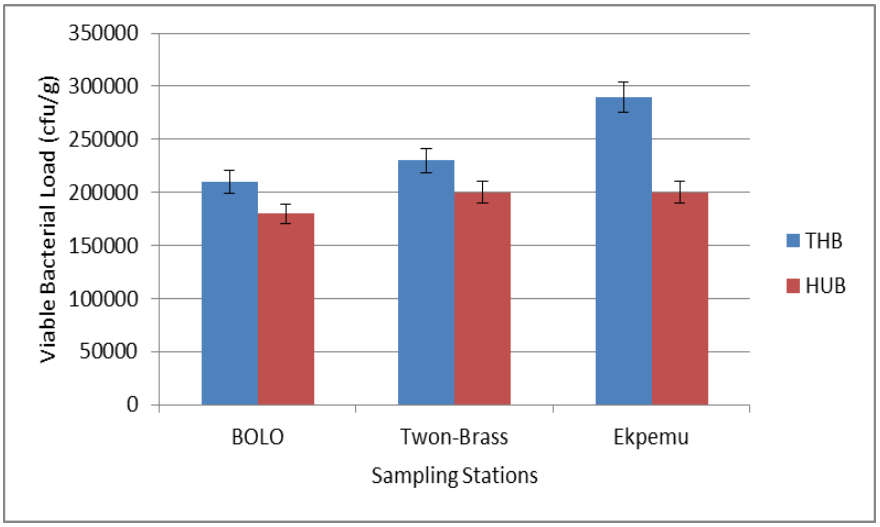

Figure 5 Bacterial loads of artisanal activities impacted soil matrices

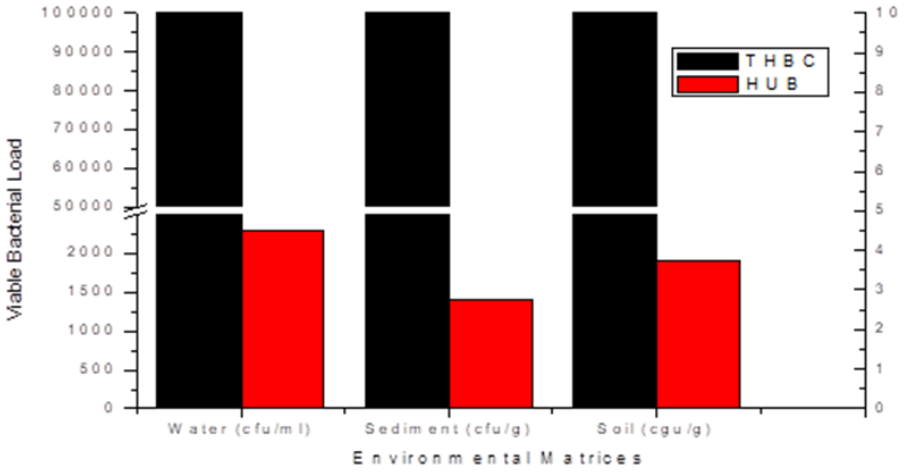

Figure 6 Bacterial loads of Un-impacted environmental matrices

Table 1 Pollution Index for the Environmental Matrices

Environmental Matrices	Bolo	Twon-Brass	Ekpemu	Un-impacted Ecosystem
Water	0.88	1.0	0.9	0.0003
Sediment	0.9	0.9	0.7	0.002
Soil	0.9	0.9	0.7	0.0005

3.2. Morphological, Biochemical and Molecular characterization of the culturable bacterial species

The morphological and biochemical attributes of the culturable bacterial species (Table 2) revealed the presence fourteen (14) bacterial isolates composed of eleven bacterial species. The bacterial species isolated include species of *Bacillus subtilis, Klebsiella* sp. *Proteus* sp., *Escherichia coli, Micrococcus* sp., *Serratia* sp., *Staphylococcus aureus, Pseudomonas aeruginosa, Chromatium* sp. and *Pseudomonas putida.* A summary of the biochemical characteristics of the bacterial isolates is as presented on Table 5. The identities of the isolates were confirmed using 16sRNA analysis. The result revealed that the isolates had a very close (95 – 100%) similarly with those in the database. The summary and the accession numbers of the isolates from the 16sRNA analysis are as presented on Table 3.

Table 2 Morphological and Biochemical characteristics of crude oil utilizing bacterial isolate

	Gram Reaction	**Shape**	**Catalase**	**Coagulase**	**Motility**	**Starch Hydrolysis**	**Citrate Test**	**Urease**	**MR**	**Voges-Proskauer**	**Spore formation**	**H_2S**	**Oxidase**	**Indole**	**Glucose**	**Lactose**	**Fructose**	**Sucrose**	**Mannitol**	**Galactose**	**Probable Organism**
W_2	+	Thick rod	+	-	-	+	+	+	-	+	+	+	-	-	A	-	A	A	-	-	*Bacillus subtilis*
W_4	-	Rod	+	-	+	-	+	+	+	-	+	+	-	-	AG	AG	-	AG	-	AG	*Klebsiella*sp
W_5	+	Thick rod	+	-	-	+	+	+	-	+	+	+	-	-	A	-	A	A	-	-	*Bacillus cereus*
W_7	-	Rod	+	-	+	+	+	+	+	-	-	+	-	-	AG	AG	AG	AG	-	AG	*Proteus* sp
W_8, W_{11}	-	Tiny rod	+	-	-	+	-	-	-	+	-	+	-	+	AG	AG	AG	AG	-	AG	*Escherichia coli*
W_1	+	Tiny cocci	+	-	-	+	-	+	+	-	-	+	+	-	A	-	A	A	-	A	*Micrococcus* sp
W_6	-	Rod	+	-	+	-	+	+	-	+	-	+	-	-	AG	AG	AG	AG	-	AG	*Serratia*sp
W_9, W_{10}	+	Cocci	+	+	-	-	-	-	-	+	-	-	-	-	A	-	AG	A	AG	A	*Staphylococcus aureus*
W_7 W_3	-	Rod	+	-	+	-	+	-	+	-	-	+	+	-	A	-	AG	AG	-	AG	*Pseudomonas aeruginosa*
W_{12}, W_{13}	-	Rod	+	-	+	-	+	+	-	+	+	+	-	-	AG	A	AG	AG	-	AG	*Chromatium*sp
W_{14}	-	Rod	+	-	+	-	-	-	+	-	-	+	+	-	A	A	A	A	A	A	*Pseudomonas putida*

Key: AG = Acid and Gas, A = Acid, - =Negative, + = Positive

Table 3 Molecular identities of the isolates

Isolates	Identity	E. value	Percent Identity	Accession number
Bacillus subtilis	*Bacillus subtilis* BAB-1684	0.0	95	KF535143.1
*Klebsiella*sp	*Klebsiella pneumonia* subsp. Pneumonia	0.0	99.4	HS11286
Bacillus sp.	*Bacillus cereus*	0.0	99	JQ579629
Proteus sp	*Proteus mirabilis*	0.0	91.60	MK802115.1
Escherichia coli	*Escherichia coli*	0.0	100	AF530554.1
Micrococcus sp	*Micrococcus luteus* SUBG006	0.0	100	JOKP00000000
*Serratia*sp	*Serratia marcescens* DB11	0.0	100	ERR486499
Staphylococcus aureus	*Staphylococcus aureus*	00	99	NC_007795.1
Pseudomonas aeruginosa	*Pseudomonas aeruginosa*H4292.1	0.0	99	CP008861.1
*Chromatium*sp	*Chromatium okenii* DSM 169	0.0	99	NR025315.1
Pseudomonas putida	*Pseudomonas putida* KT2440	0.0	100	AE015451

3.3. Distribution and Occurrence of the Isolates among the samples

Analysis of the distribution and occurrence of the bacterial isolates among the samples revealed that *Bacillus subtilis, Pseudomonas aeruginosa* and *Pseudomonas putida* had the highest occurrence (100%) as they were present in all the samples (water, sediment and soil). A summary of the distribution are as presented in the figures5.

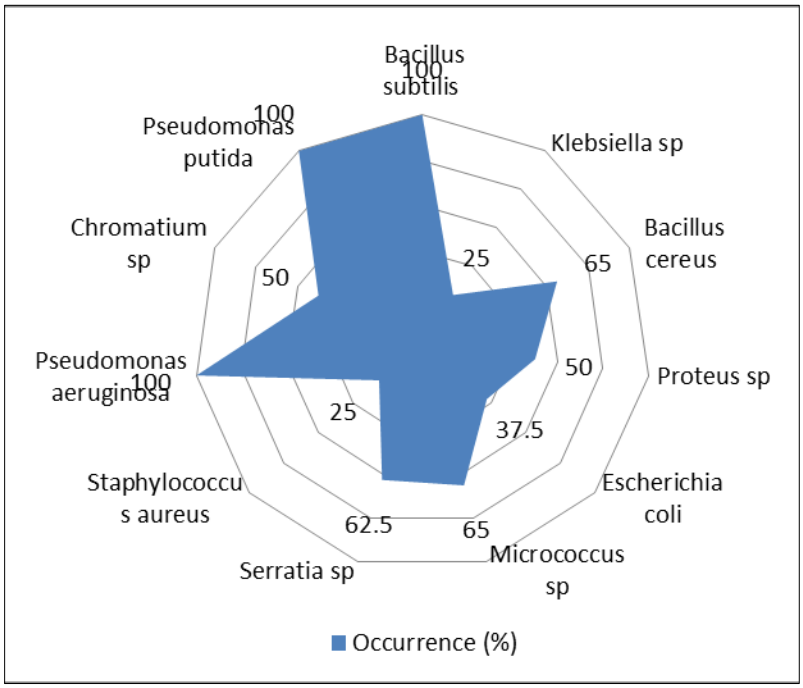

Figure 8 Percentage (%) Occurrence of Bacterial isolates from impacted recipient environment

The results from the adjoining recipient environment (Land, Sediment and Water) to the artisanal refineries recorded high counts of Hydrocarbon utilizing bacterial (HUB), indicating that the adjoining recipient environment was heavily impacted by the hydrocarbons from the artisanal refinery. The pollution index (HUB/THBC ratio) revealed a high pollution status as the values were closer to 1 indicating that most organisms in the recipient environment are hydrocarbon utilizing organism (20).The results from the adjoining recipient environment was compared with un-

impacted sample which had lower counts of hydrocarbon utilizing bacterial (HUB), and higher counts of total heterotrophic bacterial (THBC). Pollution index (ratio of HUB/THB) had values less than one (1). The result from the characterization of the bacterial community in the petroleum products revealed that the bacterial species isolated were mainly implicated in biodegradation of hydrocarbon (19, 21). This result indicates that the adjoining recipient environment has been chronically polluted by hydrocarbon.

The physicochemical properties of the water samples from the impacted adjoining recipient environment, and an unimpacted site are as presented on Table 4. The pH of the water samples ranges from 5.96± 0.3 - 7.3± 0.3. High values of total dissolved solid were recorded; ranging from 2900.39±0.38mg/L to 5063.17±0.93mg/L. Electric conductivity of the water sample from the different location also ranged from 5183.13±0.90µS/cm to 10,972.08±0.71µS/cm. The BOD values ranged from 22.32±0.63mg/L to 323.5± 0.50 mg/L, while COD ranged 50.98±0.92mg/L to 1251.70± 0.90 mg/L.

Table 4 Physico-chemical Properties of un-impacted and impacted water samples

Parameter	**Un-impacted site**	**Ogu-Bolo**	**Twon-Brass**	**Ekpemu**
pH	7.3± 0.3	6.32± 0.9	6.15± 0.1	5.96± 0.3
Appearance	Clear	Not Clear	Not Clear	Not Clear
Odour	Odourless	Unpleasant	Unpleasant	Unpleasant
Taste	Un object	Object.	Object.	Object.
Temp (^{0}C)	29.0± 0.9	27.0± 0.3	26.7± 0.6	26.3± 0.3
TSS (mg/L)	0.23± 0.30	30.29± 0.52	33.33± 0.90	37.0± 0.50
TDS (mg/L)	2900.39± 0.38	3132.43±0.51	5063.17±0.93	4438.50± 0.10
Dissolved O_2 (mg/L)	5.43± 0.70	3.10± 0.30	3.38± 0.38	3.02± 0.20
EC (µS/cm)	5183.13± 0.90	7392.43±0.55	10972.08± 0.71	8905.71± 0.30
Salinity (mg/L Cl^-)	5000± 0.30	5300± 0.51	7200± 0.86	5800± 0.30
Turbidity (NTU)	20.41± 0.41	24.35± 0.70	36.47± 0.63	25.35± 0.47
Acidity (mg/L)	1.18± 0.33	1.44± 0.42	1.54± 0.80	1.61± 0.75
Alkalinity (mg/L $CaCO_3$)	31.17± 0.44	52.46± 0.36	47.31± 0.75	45.53± 0.20
Total Hardness (mg/L)	1138.6± 0.41	1197.9± 0.90	1231.9± 0.36	1206.1± 0.77
BOD_5 (mg/L)	22.32± 0.63	322.7± 0.78	323.5± 0.50	322.8± 0.10
COD (mg/L)	58.98± 0.92	1051.47± 0.38	1251.70± 0.90	1151.17± 0.27
Cl^- (mg/L)	500.1± 0.60	572.6± 0.20	601.8± 0.18	571.4± 0.40
SO_4^{2-} (mg/L)	0.021± 0.53	0.131± 0.46	0.125± 0.50	0.132± 0.19
NO_2^- (mg/L)	130.80± 0.20	148.42± 0.1	184.70± 0.90	151.78± 0.104
NO_3^- (mg/L)	0.120± 0.109	0.134± 0.011	0.128± 0.021	0.123± 0.013
PO_4^{3-} (mg/L)	120.50± 0.090	149.49± 0.020	192.54± 0.021	151.51± 0.001

Comparison of the physicochemical properties of the impacted sediments with that of the un-impacted sediment as presented on Table 5 revealed that the activities of the artisanal refinery did not only had a negative impact on the water quality but also on the sediment physiochemistry. Moisture content in the sediments impacted sites were lower than that of the un-impacted soil. The lowest moisture contest was observed in sediment sample from Bolo (30.8± 0.4%) followed by Twon (31.5± 0.1%) and Ekpemu (34.5± 0.3%). This finding of this study is in agreement with previous reports (22, 23).

Table 5 Physico-chemical Properties of un-impacted and impacted Sediment samples

Parameters	Un-Impacted Sed.	Ogu-Bolo	Twon- Brass	Ekpemu
pH	5.1± 0.1	6.2± 0.3	6.6± 0.4	6.4± 0.3
Total Organic Carbon (g/kg)	1.02± 0.1	1.31± 0.4	1.42± 0.2	1.41± 0.3
Moisture (%)	59.2± 0.5	30.8± 0.4	31.5± 0.1	34.5± 0.3
Conductivity (ds/m)	0.05± 0.02	4.4± 0.1	3.9±1.1	5.2± 0.7
Avail Phosphorus (mg/kg)	0.45± 0.3	0.12± 0.1	0.19± 0.1	0.15± 0.2
Total Nitrogen (%)	0.41± 0.4	0.13± 0.1	0.20± 0.2	0.18± 0.7
Ca^{2+} (mg/kg)	2.18± 0.1	1.61± 0.6	1.52± 0.4	1.72± 0.2
Mg^{2+} (mg/kg)	1.21± 0.3	0.85± 0.1	0.75± 0.2	0.81± 0.4
Na^{+} (mg/kg)	0.06± 0.1	0.14± 0.2	0.16± 0.3	0.15± 0.1
K^{+} (mg/kg)	1.14± 0.6	0.18± 0.6	0.19± 0.1	0.17± 0.7
Cl^{-} (mg/kg)	3.06± 0.1	32.14±1.1	22.24±1.7	24.41±0.9
SO_4^{2-} (mg/kg)	0.361± 0.1	8.18± 0.4	8.21± 0.3	6.28± 0.4
NO_3^{-} (mg/kg)	0.036± 0.03	0.06± 0.01	0.04± 0.01	0.057± 0.01
Sand	52±2.0	68.7±1.2	69.2±1.6	65.2±1.1
Silt	20±2.1	13.1±0.5	14.4± 0.3	18.4±1.1
Clay	27±2.0	18.2±0.8	16.4±1.1	16.4±2.1

Table 6 Physico-chemical Properties of un-impacted and impacted Soil samples

Parameters	Un-impacted soil	Ogu-Bolo	Twon- Brass	Ekpemu
pH	6.39± 0.2	5.8± 0.1	6.6± 0.1	5.9± 0.3
Total Organic Carbon (g/kg)	1.59± 0.5	1.81± 0.3	1.64± 0.4	1.54± 0.6
Moisture (%)	61.03± 0.3	11.8± 0.7	11.5± 0.1	12.5± 0.2
Conductivity (ds/m)	0.0005± 0.0002	8.4±1.1	7.9±1.3	8.2± 0.9
Avail Phosphorus (mg/kg)	0.14± 0.1	0.052± 0.02	0.069± 0.04	0.059± 0.01
Total Nitrogen (%)	0.41± 0.2	0.01± 0.01	0.02± 0.03	0.02± 0.01
Ca^{2+} (mg/kg)	1.98± 0.2	1.72± 0.3	1.61± 0.2	1.72± 0.5
Mg^{2+} (mg/kg)	1.03± 0.2	0.95± 0.1	0.85± 0.3	0.91± 0.1
Na^{+} (mg/kg)	0.16± 0.1	0.15± 0.2	0.15± 0.2	0.15± 0.3
K^{+} (mg/kg)	1.14± 0.1	0.19± 0.1	0.18± 0.4	0.19± 0.3
Cl^{-} (mg/kg)	3.06± 0.2	39.99± 0.1	24.54± 0.3	34.61± 0.2
SO_4^{2-} (mg/kg)	0.261± 0.2	8.58± 0.3	9.08± 0.1	8.28± 0.5
NO_3^{-} (mg/kg)	0.026± 0.01	0.05± 0.01	0.03± 0.01	0.05± 0.01
Sand	52±2	68.7±1.1	69.2±1.7	65.2±1.5
Silt	20±1	13.1±2.1	14.4±2.0	18.4±1.8
Clay	27±1.6	18.2±1.4	16.4±1.2	16.4±1.4

The physicochemical properties of the impacted soils were studied and compared with that of a non-impacted soil (Table 6). The analysis revealed that the impacted soil samples had a higher organic carbon and conductivity. Soil sample from Bolo had the highest organic carbon content (1.81±0.3g/kg) followed by Twon (1.64± 0.4 g/kg) and Ekpemu (1.54± 0.6 g/kg) while the unimpacted soil had an organic carbon content of 1.59±0.5g/kg. Similarly other physiochemical properties such as available phosphorous, total nitrogen, moisture content, exchangeable cations (Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}) were observed to be lower in the artisanal refinery impacted soils than in the non-impacted soils.

The results of this study are in agreement with (24) who reported that contamination of soil results in a change in soil properties, viz. soil moisture levels, hydraulic conductivity, Atterberg limits, total organic carbon, total nitrogen, available phosphorus, etc. Oil contamination is a severe problem especially in the Niger Delta region as it blocks air diffusion resulting in a change of physical properties like permeability characteristics (25) and chemical properties like pH, total organic carbon, soil minerals nutrients such as sodium, potassium, sulfate, phosphate and nitrate of soil, thus indirectly affecting the growth and development of plants and microorganisms (20,26, 27).

4. Conclusion

The study revealed the presence of hydrocarbon degraders as the predominant organisms in the impacted adjoining environment of artisanal refineries in the Niger Delta region. The high pollution index reported for the impacted ecosystems indicates a negative effect of the artisanal activities in the environmental matrix. The isolates were characterization by culture dependent and molecular methods. The prevalence of hydrocarbon degraders in the environmental matrix strongly suggests that the environment have been chronically polluted by petroleum hydrocarbon. This phenomenon also gives a ray of hope, indicating that overtime; bioremediation by natural attenuation will ensue.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors have declared that no conflict of interest exists.

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