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Effect of Nitrogen-15, Phosphorus-15, and Potassium-15 Fertilizer on Indigenous Microorganisms Involved in Biodegradation of Crude Oil Contaminated Soil Collected from Shawguwolo Jeddo Metropolis, Okpe L.G.A, Delta State, Nigeria

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ABSTRACT: The increasing occurrence of oil pollution presents a significant environmental challenge, necessitating effective remediation strategies. Hence, the objective of this paper was to evaluate the effect of Nitrogen-15, Phosphorus-15, and Potassium-15 fertilizer on indigenous microorganisms involved in biodegradation of crude oil contaminated soil collected from Shawguwolo Jeddo Metropolis, Okpe L. G. A, Delta State, Nigeria using appropriate standard methods. It was observed that the hydrocarbon reduced from 5315.80 to 2276.57 within four weeks. The highest rate was observed in sample C, which showed a 95.7% reduction in the hydrocarbon contamination. Sample C also showed a 34% increase in the hydrocarbon utilizing bacteria present in the soil. Bioremediation leverages microbial activity to degrade pollutants, offering a cost-effective approach compared to other technologies, as it speeds up the process of degrading hydrocarbon by increasing the growth rate of hydrocarbon utilizing bacteria.

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Petroleum contamination in soil poses a serious threat to human health. When petroleum pollutes the soil, it can migrate to groundwater, lakes, and other water sources essential for domestic and industrial use. This issue is particularly critical in areas that depend on groundwater as their primary source of clean water (Hafiluddi, 2011). One of the most challenging oil contaminants to break down is hydrocarbon compounds. When these compounds contaminate the soil surface, they can evaporate, be washed away by rain, infiltrate the ground, and accumulate as toxic substances. While the environment has a natural capacity to degrade pollutant compounds through biological and chemical processes, the pollution load often exceeds the natural degradation rate (Mandri *et al.*, 2007). Consequently, pollutants accumulate, necessitating human intervention and the application of existing technologies to address pollution issues (Karwati, 2009). Bioremediation is an environmental recovery method that utilizes the biological activities of microbes to degrade the toxicity of various pollutant compounds. This approach is considered more cost-

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effective than other technologies for addressing environmental contamination (Romanus *et al*., 2015). The addition of nutrients in treatments such as biostimulation and bioaugmentation has been shown to enhance hydrocarbon degradation, with significant results observable within the first three weeks of the incubation process (Cappuccino *et al*., 2013). Biodegradation involves the complete mineralization of organic contaminants into carbon dioxide (CO_2), water, inorganic compounds, and cell proteins, or the transformation of complex organic contaminants into simpler organic compounds by biological agents like microorganisms (Nalinee *et al*., 2013). This process encompasses the breakdown of organic matter by microorganisms such as bacteria and fungi. Biodegradation can be divided into three stages: biodeterioration, biofragmentation, and bioassimilation. Biodeterioration is a surface-level degradation process that alters the mechanical, physical, and chemical properties of materials. This stage occurs when materials are exposed to abiotic factors in the outdoor environment, leading to further degradation by weakening their structure. Key abiotic factors influencing these initial changes include mechanical compression, light, temperature, and environmental chemicals. While biodeterioration typically represents the initial phase of biodegradation, it can occasionally occur concurrently with biofragmentation. Biofragmentation, especially in polymers, is the lytic process where bonds within the polymer are cleaved, generating oligomers and monomers. The fragmentation process varies depending on the presence of oxygen in the system (Nicholas *et al*., 2014).

Crude oil consists of a complex mixture of paraffins, alicyclic, and aromatic hydrocarbons, including benzene, toluene, ethylbenzene, and xylene, which are major components in many petroleum products. Hydrocarbons enter the environment through waste disposal, accidental spills during transportation, storage, and use. Their accumulation in the environment poses serious problems. Certain microorganisms possess enzymes capable of breaking down hydrocarbons. After degradation, these microorganisms utilize the carbon released to generate energy. In a pristine environment, microorganisms capable of degrading hydrocarbons constitute approximately 1% of the total microbial population. However, in chronically polluted environments, this number rises to about 10% as some organisms adapt to the new conditions over time (Kattshik *et al*., 2006). There are six primary factors affecting biodegradation: nutrient availability, oxygen availability, temperature, presence of oil degraders in the polluted environment, hydrocarbon type, pH of the environment, and soil salinity (Ramirez *et al*., 2017). Hence, the objective of this paper was to evaluate the effect of Nitrogen-15, Phosphorus-15, and Potassium-15 fertilizer on indigenous microorganisms involved in biodegradation of crude oil contaminated soil collected from Shawguwolo Jeddo Metropolis, Okpe L. G. A, Delta State, Nigeria.

MATERIALS AND METHOD

Demographic Description of Sample Site (Soil Sample): The garden soil sample was collected from Shawguwolo Jeddo Metropolis, Okpe Local Government Area, Delta State, at the geographical coordinates of 5.5970° N latitude and 5.7040° E longitude. The NPK fertilizer (Nitrogen, Phosphorus, and Potassium) used in this study was purchased from a commercial market in Effurun, along Warri-Sapele Road.

Experimental Procedure: Three separate samples of soil weighing 2500g each, were collected from Shawguwolo Jeddo metropolis, Okpe L.G.A. Delta state in a sterile polythene bag at a depth of 0 – 15cm, using a sterile spade and transported to the Petroleum Training Institute microbiology laboratory. Nitrogen 15, Phosphorus 15, and Potassium 15 were bought from the Effurun commercial market along Warri Sapele Road. The soil sample was sun-dried for a period of one week and was sieved through a 2mm mesh sieve. 600g of soil sample was weighed using an electronic weighing balance into four clean experimental bowls respectively labeled A B C and D; 100ml of crude oil was measured using a measuring cylinder and was poured into each sample respectively. The Nitrogen 15, phosphorus 15, and potassium 15, which was grinded and sieved using the 2mm sieve, were weighed in different variations, 50g, 75g, and 100g were then added to the crude oil-contaminated soil in the experimental bowls A, B, and C respectively. While bowl D was left as a control without amendment. The setup was left for a period of 4 weeks while the microbial analysis and the physiochemical analysis were carried out at the zero weeks and subsequently at 1-week intervals with proper supervision.

A setup of six test tubes containing 9ml of distilled water was arranged into two places in a test tube rack then 1g of poultry dung and the contaminated soil were weighed and introduced into the first beakers respectively. It was shaken for even distribution after which 1.0ml of the aliquot (the mixture) was aseptically transferred into the second test tube 10-1 (tenfold) dilution, and a further tenfold serial dilution was carried out to factor 10-6 dilution factor. After the serial dilution process, nutrient agar was prepared by measuring 7grams into 25oml of volumetric flasks and shaken very well until the agar was readily dissolved

then autoclave at 5000 reads per minute (rpm), with temperature for some minutes when the agar was fully sterilized, it was left to cool for some minutes on a sterile working bench until when it was favorably to handle. The agar was poured into sterile Petri dishes (two plates for each sample) and was blended with Nystalin (to suppress the growth of fungi growth. The plate was allowed to solidify within 24 hours and also to check if it is contaminated while pouring or not.

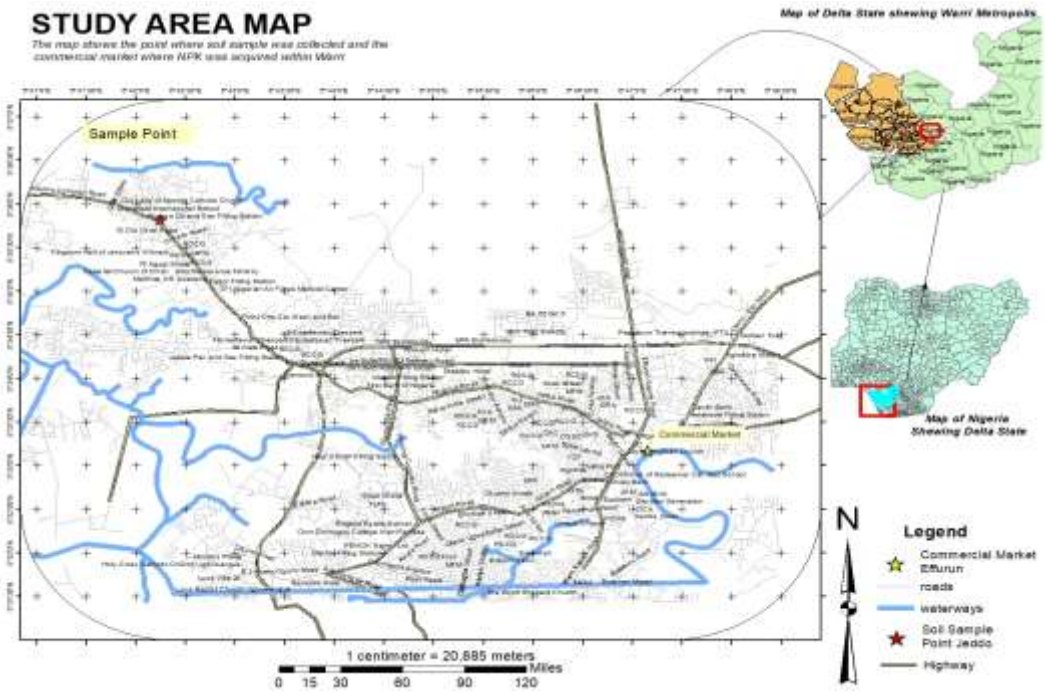

Fig 1: Shows the Warri metropolis, indicative of the site where the soil sample was collected and the fertilizer was bought.
Source: (the map was generated by one of the authors)

Meanwhile, after 24 hours, 0.1ml of the serial dilutions from both samples were measured using a micropipette from factor 10-4 and 10-6which were afterward inoculated into the poured plate and gently swirled using the fume cupboards at room temperature (280c) for 24 hours. After which bacterial colonies that grew were counted using the standard plate counting techniques.

Discrete colonies from the primary plate were picked with the help of a sterile wire loop and sub-cultured into a fresh agar plate and incubated for another 48 hours inside the fume cupboard. The morphological characteristics of the isolates were observed and identified after 48 hours of incubation, and each organism with different morphological characteristics was further plated in a slant bottle and preserved in the refrigerator at 400c for biochemical characterization.

In determining the hydrocarbon utilizing bacteria, serial dilution and pour plate techniques were adopted. Mineral salt medium (MSM) was prepared and sterilized by autoclaving at 1210c, 15psi for 15 minutes and dispensed into Petri dishes. The plates were inoculated in duplicated with 0.1ml aliquot of the sample serially diluted at 10-4 and dilution factor. The plates were incubated at 280+/-200c for 7 days and the colonies were counted from triplets and mean values were recorded in colony-forming units per gram (cfu/g).

Morphological Characteristics: The isolates were characterized and identified based on their cultural characteristics and biochemical procedures and reactions are as follows:

Gram reaction: This was carried out to differentiate gram-positive from gram-negative organisms. It involved using a sterilized wire loop in a Bunshen burner and allowed to cool then a loopful of growth was collected from the agar plate and applied on a clean grease-free slide then a drop of normal saline was added, emulsified and heat fixed by passing over a flame three times. The smear was flooded with crystal violet for 30-60 seconds and then covered with iodine (as mordant) for 30-60 seconds and then washed off; it was decolorized with acetone until no color ran off the slide and rinsed immediately. The slide was covered with Safranine dye for 1 minute and then washed off with clean water. The slide was kept

in a track to air dry after wiping the back with cotton wool. The stand smear was then examined microscopically under oil immersion at x100 objectives lens. The gram-positive bacteria appeared dark purple while the gram-negative bacteria appeared red.

Motility Test: The motility test was aimed at identifying motile bacteria. To achieve the motility test, a drop of normal saline was placed on a sterile slide, and the colony of the test organism was suspended and then covered with a cover slip. The slide was examined microscopically using x10 and x40 objective lenses. Movement in different directions gave a positive test while static position gave a negative test.

Catalase Test: This was carried out to differentiate those bacteria that produce enzyme catalase such as Staphylococcus aureus and Escherichia coli which were also used as positive and negative controls respectively. To achieve the catalase test, three milliliters (3ml) of hydrogen peroxide solution was poured into a sterile test tube. Then a sterile glass rod was used to collect several colonies of the test organisms and inoculate into the hydrogen peroxide solution. It was observed for immediate active bubbling for positive test

Oxidase Test: This was carried out to identify bacteria species that will produce the cytochrome oxidase enzyme, Pseudomonas aeruginosa and Escherichia coli were employed as positive and negative controls respectively. The procedure for the oxidase test involves placing a piece of filter paper in a clean petrol dish and adding 2-3 drops of fresh or nascent reagent. A colony of test organisms was collected using a glass rod and smeared on the filter paper and observed. Blue-purple color within few a seconds showed a positive test.

Citrate Test: This test is based on the ability of an organism to use citrates as its source of carbon. It was used to identify the Enterobacteria. Simon's citrate agar medium was prepared in a slant bijou bottle, using a sterile wire loop was used to inoculate the test organism onto the slant medium and incubated at 300C or 48 hours after which it was examined for color formation. A bright blue color in the medium gave a positive citrate test. Klebsiella pneumonia and Escherichia coli were employed as positive and negative controls respectively.

Methyl red test: This was carried out to identify Enterobacteria based on the ability to produce and maintain stable acid end product from glucose fermentation. Escherichia coli were used as positive control. The test procedure involved using glucose phosphate peptone for the inoculations of test organisms and incubating for 48 hours at 37^0c after which a few drops of methyl red solutions were added to the culture and read immediately showed a positive test.

Physiochemical Characterization: Determination of pH: The pH of the crude oil contaminated soil was determined using pH meter (Jenway 3015 UK). Ten grams of sun-dried soil (passed through 2mm sieve) was weighed into a 20ml beaker and 5ml of distilled water was added. The suspension was shaken with the use of a mechanical shaker for 25 – 30 minutes, then allowed to stand for 50 minutes and stirred occasionally with a glass rod. The electrode was rinsed with water and dried with a piece of tissue. The electrode was inserted into the partly settled suspension to be analyzed and the pH range o the solution was measured. The pH meter was calibrated at pH 7.0

Determination of Total Nitrates (NO_3): Total Nitrogen of the soil samples was determined by the macro Kjedah digestion method. 100g of potassium chloride was weighed into 1000ml volumetric flask, 800ml of deionized water was added to it and stored thoroughly until it dissolves. Distilled water was added to make up to 1000ml; the volumetric flask capped with paraffin and inverted several times to mix. 4g of the amended soil sample was weighed and placed into a conical flask, 20ml of the extraction solution will be added to the soil sample and the flask was played in a mechanical shaker box and shaken for 1 hour at a given speed. The content was filtered using a line filter paper. Filtrate was analyzed using Atomic Absorption Spectrophometer.

Determination of Phosphorus: 50ml of 0.5m Hydrochloric acid solution was added to a weighed 5g of soil sample in a conical flask. The mixture was shaken and allowed for a 50-minute digestion. A filtration apparatus was set up and the mixture was filtered. The filtrate was then collected in a beaker. 1.0g of phosphate mixture was weighed and dissolved in 100 ml of 2m sulfuric acid which was left to stand for about 20 minutes. The content in the beaker was left to stand for about 30 minutes for full-colour formation. UV-visible spectrometer was used to determine the absorbance of the phosphate concentration of the soil sample. (Abbu N., 2006).

Estimation of Total Petroleum Hydrocarbon (TPH): Using soil sample extraction method (ASTM D575^6 – 97). 10g of each of the amended and contaminated soil was weighed into an organic-free amber glass container and 10ml of the extractant (N – hexane,

dichloromethane, and acetone in a ratio of 2:1:1) was added, using a mechanical shaker the mixture was gently shaken 30minutes. The sample was extracted from the solutions using a sonicator and was filtered. The final volume of the extract was stored in a dried organic free chromic acid pre-clean vial and 1.00micro liter was withdrawn using an automated gas–tight syringe of the autosampler and analysis by direct injection into the GC – FID preset at and at various temperatures, the hydrocarbon peaks were shown on the screen and the analysis of the results were obtained. The extract remaining was refrigerated at about 4^0c for further analysis (Osuji, 2005).

RESULTS AND DISCUSSION

Below are the tables representing the initial values of the physiochemical and microbial characteristics of the crude oil-contaminated soil that was amended with Nitrogen, Phosphorus, and Potassium (15; 15; 15) at the beginning of the practical process. The impact of time on petroleum degradation is substantial, particularly when combined with nutrients that stimulate microbial activity, as supported by Abu G.O (2007). Table 1 shows the initial physiochemical analysis of the soil sample at week zero, revealing a pH of 6.73 (weakly acidic) and a temperature of 29.8°C before amendment with NPK fertilizer. The initial nitrogen, phosphorus, and potassium contents were 290.00, 72.58, and 48.06, respectively. Table 2, show that at zero week, after crude oil contamination, the total petroleum hydrocarbon (TPH) concentration was 53153.80 ppm across all samples, indicating no significant decrease due to the fact that the samples were just polluted with equal concentrations of crude oil and also the acclimatization of indigenous bacteria (Ijah et al., 2007). After four weeks, TPH levels ranged from 50013.59 ppm to 2276.57 ppm, with the highest degradation observed in the sample with 100g NPK amendment, reducing TPH to 2276.57 ppm which translates to a 95.7% reduction in the hydrocarbon present, while the control showed the least degradation at 19214.15 ppm. This confirms that hydrocarbons serve as a primary carbon source for microorganisms (Philip *et al*., 2015).

Table 1: Physiochemical analysis of Nitrogen, Phosphorus, and Potassium; pH and temperature of soil at zero week

Sample	pH	Temperature	Nitrogen	Phosphorus	Potassium
Soil	6.73	29.8	290.00	72.58	48.06

Table 2: Degradation of total petroleum hydrocarbon sample from zero – four weeks

NPK (15;15;15)	0 WEEK	WEEK 1	WEEK 2	WEEK 3	WEEK 4
CONTROL	53153.80	40013.59	44010.97	33028.53	19214.15
50 (A)	53153.80	38341.59	20010.01	4121.86	3269.19
75 (B)	53153.80	30819.34	17315.26	6419.04	2984.24
100 (C)	53153.80	28690.90	16652.19	5632.16	2276.57

Table 3: Microbial parameters (Total heterotrophic bacteria count)

Days (dilution factor)	Control	50G	75G	100G
0 (X 10^{-6} /0.1 $CFUG^{-1}$)	30.3	35.3	37.3	38.9
7 ((X 10^{-6} /0.1 $CFUG^{-1}$)	15.4	24.5	30.7	40.5
14 (X 10^{-6} /0.1 $CFUG^{-1}$)	15.2	23.1	30.0	40.0
21 (X 10^{-6} /0.1 $CFUG^{-1}$)	26.6	39.2	47.9	63.1
28 (X 10^{-6} /0.1 $CFUG^{-1}$)	30.7	39.7	49.1	65.1

Table 4: Total hydrocarbon utilizing bacterial count

Weeks (Dilution Factor)	Control	50G	75G	100G
0(X 10^{-3} /0.1 $CFUG^{-1}$)	10.3	15.5	20.1	27.5
1(X 10^{-3} /0.1 $CFUG^{-1}$)	15.6	18.5	21.5	23.5
2((X 10^{-3} /0.1 $CFUG^{-1}$)	12.7	16.3	23.3	25.4
3 (X 10^{-3} /0.1 $CFUG^{-1}$)	25.3	27.4	29.9	33.4
4 (X 10^{-3} /0.1 $CFUG^{-1}$)	26.4	29.7	31.2	35.4

Table 3 details the total heterotrophic bacterial count over the first week, showing a reduction due to environmental changes. The control sample's bacterial count ranged from 30.3 x 10^4 to 15.4 x 10^4, while sample A (50g NPK) ranged from 35.3 x 10^4 to 24.5 x 10^4, sample B from 37.5 x 10^4 to 30.7 x 10^4, and sample C from 48.9 x 10^4 to 40.5 x 10^4. This reduction is attributed to environmental factors like temperature and oxygen availability (Sebiomo *et al*., 2011). By the second week, a slight decrease in bacterial count was observed, consistent with the bacterial growth curve moving from lag to exponential phase, with steady-state conditions leading to some cell death (Tor *et al*., 2011). From week two to three, bacterial counts increased as organisms adapted to the environment, supported by added nutrients (Shukor *et al*., 2016). By the fourth week, the highest bacterial growth was in the sample with the highest NPK amendment (100g), affirming the role of NPK in microbial growth and nutrient availability (Miah *et al*, 2022).

Table 4 illustrates hydrocarbon-utilizing bacterial growth from week zero to week four, showing the highest increase in the sample with the most NPK, supporting Olivieri *et al*. (2006). The bacterial isolates from crude oil-polluted soil confirm the presence of microorganisms capable of degrading hydrocarbons, both with and without NPK amendment (Udeme *et al*., 2007). *Conclusion:* Petroleum contamination in soil poses significant health risks and environmental threats. It can pollute groundwater and other water sources, leading to infertility of the soil and health problems for people in affected areas. Hydrocarbon compounds in petroleum are particularly difficult to break down and can evaporate, wash away, infiltrate the ground, and disrupt the soil ecosystem, accumulating as toxic substances due to the slow natural degradation process. Human intervention is essential to address this pollution, with bioremediation being an effective solution. Bioremediation leverages microbial activity to degrade pollutants, offering a cost-effective approach compared to other technologies. Key factors influencing its success include moisture, pH, and temperature, along with the addition of nutrients to boost the activity of indigenous microorganisms.

Declaration of Conflict of Interest: The authors declare no conflict of interest.

Data Availability Statement: Data are available upon request from the first author or corresponding author.

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