BACTERIOLOGICAL AND PHYSICOCHEMICAL ANALYSIS OF A CRUDE OIL-POLLUTED SOIL UNDERGOING LABORATORY-SCALE BIOREMEDIATION

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ABSTRACT

The bacteriological and physicochemical quality of crude oil-polluted soil undergoing laboratory-scale bioremediation was evaluated. Soil samples were collected from an abandoned oil field in the Gio community, Ogoni land, Nigeria. Four different bioremediations experimental setups comprised of the polluted soil only (A), polluted soil with bacterial consortium (B), polluted soil with NPK fertilizer (C) and polluted soil with cow dung (D) were employed in the degradation of crude oil-derived hydrocarbons present in the soil and monitored for 40 days. During treatment, there was an increase in total culturable heterotrophic bacterial count (TCHBC) and total culturable hydrocarbon utilising bacterial count (TCHUBC), especially in the test treatment (B) which experienced about 22.2% increase in TCHUBC. The total petroleum hydrocarbon (TPH) concentration reduced over treatment time by 33.8, 62.3, 61.49, and 40.25% respectively, for Samples A, B, C and D. Overall, pH reduced from highly acidic tending towards neutral pH. There was also an overall reduction in the percentage of moisture and phosphate concentration. There was an initial increase in nitrate concentration at day 20 for all samples but a decline in concentration at day 40. There was a 71% reduction in TOC in Sample A, 83% reduction in Sample B, and 79 and 24% reduction in samples C and D. This study has demonstrated the effectiveness of each treatment approach in the degradation of hydrocarbons, especially the use of indigenous bacterial consortium from polluted soil. The role of bacterial consortium in bioremediation proves their ability to clean hydrocarbon-impacted systems for environmental restoration.

Keywords: Bioremediation, Bacteria, Hydrocarbons, Soil, Petroleum, Degradation

INTRODUCTION

Crude oil drilling and other related activities like oil export by oil industries in Nigeria have resulted in continuous experiences of pollution, especially as a result of spillage of crude oil. This has been a global worry, leading to research in recent times on methods and ways to curb the menace that emanates from pollution due to crude oil spillage. A lot of impact experienced from crude oil spillage and contamination is of critical environmental and health importance hence the need to develop innovative technology that can be employed for bioremediation purposes (Maduwuba, 2023).

The pollution from crude oil spills is traced to oil exploration and exploitation facilities. Crude oil pollution could also come from oil transportation and storage, corrosion of over-aged facilities, usage and the vandalization of oil facilities and the effects sure lead to contamination of soil water bodies, aquifers and air. The spills from crude affect ecosystems, reducing soil fertility and microbial diversity. Also, the toxicity of crude oil is dependent on the chemical and physical complexities of the crude oil involved and also on the quality of the contaminated water or soil (Okafor, 2023; Maduwuba, 2023).

The negative impact of crude oil pollution may linger for as long as possible if not controlled

and remediation processes initiated as and when due no one does remediation faster and at a cheaper cost than our darling microbes hence the increase in the design of remediation process using specialized microbes (Ogidi and Njoku, 2017).

Microbes can degrade crude oil because they can utilize these hydrocarbons, which are then broken down into dissolvable and more reactive compounds which are further broken down into simpler components such as carbon dioxide and water (Bada *et al.,* 2019).

Bioremediation is a systematic and technological approach that involves the use of living organisms most times of microbial origin to eliminate pollutants from contaminated sites (Orhorhoro *et al.,* 2018). Bioremediation is also a very friendly and economical strategy that is effective for the removal of contaminants (Orhorhoro *et al.,* 2018). In remediating crude oil-polluted sites, naturally occurring hydrocarbon-degrading microbes could be used through natural attenuation. The efficacy of the remedial processes can be increased and become more effective through processes like biostimulation and bioaugmentation (Marinescu *et al.,* 2009).

The physicochemical properties and microbial community of the contaminated environment are usually altered resulting in deleterious effects on agriculture, plant growth, humans and other living organisms in the environment (Ofoegbu *et al.,* 2015). The optimal potential of the soil is reduced due to hardening or change in the structure and composition of the soil which is a result of crude oil lying undisturbed in the soil (Ezeonu, 2010). Humans are not spared from the adverse effects of crude oil contamination as various health challenges can be induced by the carcinogenic, mutagenic and teratogenic properties of crude oil (Maduwuba, 2022).

In response to this challenge, laboratory-scale bioremediation has emerged as a promising and sustainable approach, harnessing the natural capabilities of microorganisms to degrade and detoxify petroleum hydrocarbons (Maduwuba, 2023). This research will investigate and monitor the relationship between bacterial communities and the physicochemical characteristics of crude oil-polluted soil that was subjected to laboratory-scale bioremediation with the intention and aim of providing comprehensive insight into the biodegradation processes, microbial diversity, and changes in soil properties during the remediation of crude oil-contaminated environments.

The physicochemical evaluation will investigate soil parameters such as pH, organic matter content, and nutrient levels, to provide insights into the response of the soil to the remediation process while the bacteriological assessment will investigate the composition, abundance, and activity of bacterial communities involved in the degradation of hydrocarbons.

This research will add to the already existing body of knowledge and provide practical insights for the optimization of laboratory-scale bioremediation techniques which will in turn lead to the development of more efficient and sustainable strategies for remediating crude oil-polluted soils, thereby mitigating the environmental impact of hydrocarbon contamination.

MATERIALS AND METHODS

Soil Sampling and Processing: Crude oil-polluted soils were collected from an abandoned oil field in Gio community. Ogoni land, Rivers State, Nigeria while maintaining strict sterile conditions. Soil samples were taken at 0 – 100 cm depth using a soil auger from different points of the site and labelled accordingly. The soil samples were stored in dark polyethene bags and moved to the laboratory at 4^0C in an ice chest. The samples were processed using a slightly modified method of Suja *et al.* (2014). They were dried in the oven at 40^0C for 2 hours and then sieved with a 2 mm mesh sieve to get rid of undesired particles.

Sources of Nutrient Supplements: Cow dung was collected from a commercial livestock farm, while the inorganic fertilizer (NPK) produced by NOTORE Fertilizer Company was collected from the Ministry of Agriculture, Owerri, Imo State.

Enumeration of Culturable Bacterial Population: The total culturable heterotrophic bacterial count (TCHBC) was carried out using nutrient agar (Accumedia, Sweden). The media was prepared following the manufacturer's

instructions. A volume of 100 µL each of 10^{-3} – 10^{-6} dilutions of the sample was spread on the surface of the medium. The inoculated plates were then incubated at 30^0C for 24 hours after which agar plates with discrete colonies ranging from 30 – 300 were selected (APHA, 2005) and the total viable cells were estimated in Cfu/g. Similarly, counts for hydrocarbon-utilizing bacteria (HUB) were carried out using Bushnell Haas Agar (with 1% v/v Bonny light crude oil) amended with 0.01% w/v nystatin for HUB. Total viable cells were also estimated in Cfu/g.

Isolation and Enrichment of Hydrocarbon Utilizing Bacteria (HUB): The isolation of hydrocarbon-utilizing bacteria in the samples was carried out using the method of Mittal and Singh (2009) and Mnif *et al.* (2009) with slight adjustments. 10 g of the soil sample was mixed with 100 ml of normal saline in a 250 mL Erlenmeyer flask (Pyrex, USA). The mixture was vortexed for 2 minutes at high speed, and then allowed to settle for about 5 minutes. 5 ml of the supernatant was inoculated into a separate conical flask that contained 100 ml Bushnell Haas Broth (BHB) (containing in g/L: 0.42$MgSO_4.7H_20$, 0.02$CaCl_2.2H_20$; 1KH_2PO_4; 1K2HPO_4; 1 NH_4NO_3; 0.05$FeCl_3$; 15 g agar powder, 0.1 g nystatin) supplemented with 1% v/v Bonny light crude oil. Tween 80 (0.05% v/v) was introduced to improve hydrocarbon degradation. The process was replicated thrice for each Sample C and then incubated in a shaker for 7 days at 150 rpm. The resulting colonies were sub-cultured into a fresh BHB medium supplemented with 1% v/v crude oil. The sub-culturing enhanced the successful isolation of only hydrocarbon-tolerant and hydrocarbon-utilizing bacteria (Wedulo *et al.,* 2014).

At the end of the sub-culturing stage, 1 ml of the broth was diluted appropriately to make 10^{-1} – 10^{-6} dilutions. Aliquots of the dilutions were inoculated on Bushnell Haas agar plates supplemented with 1% (v/v) crude oil and incubated for 96 hours at 30^0C.

Degradation Screening Test: Hydrocarbon utilization by the bacterial isolates was screened using the turbidometric method. A UV-5200 spectrophotometer (Shanghai Metash, China) was used to measure turbidity. A volume of 50 mL Bushnell Hass broth supplemented with crude oil 1% (v/v) and 0.05% Tween 80 was prepared and dispensed into Erlenmeyer's flasks and sterilized in an autoclave at 121 ^{0}C (15 psi) for 15 minutes. After sterilization, the broth was inoculated with 5% (v/v) of the standardized inoculum of the test bacterial isolate. The inoculated flask containing the test bacterial isolates was incubated for 15 days in a shaker incubator at 150 rpm. The turbidity of each broth was measured using a UV-5200 spectrophotometer (Shanghai Metash, China) every five days throughout the incubation period. Biodegradation was scored every three days based on the turbidity of the broth and oil emulsification in the medium. The isolates were also screened for straight-chain hydrocarbon utilization, biosurfactant production and emulsification index using the methods of Morikawa *et al.* (2000) and Lima *et al.* (2019). The bacterial consortium was then prepared using the isolates in Bushnell Haas broth.

Bioremediation Experimental Design: The experimental setup consists of four experimental units in triplicate. The setups were monitored for 40 days. Qualitative data that emerged in the treatments during the study were collected and compared for each unit. The details of the unit are stated in Table 1.

Table 1: Experimental setup for bacteriological and physicochemical analysis of a crude oil-polluted soil undergoing laboratory-scale bioremediation

Treatments
A: 1000 g polluted soil only (negative control)
B: 1000 g polluted soil + 100 ml bacterial consortium (test treatment)
C: 1000 g polluted soil + 100 g NPK (positive control I)
D: 1000 g polluted soil + 100 g cow dung (positive control II)

Physicochemical Analysis: pH was determined according to the procedure of Gorski and Ritzert (1977). Moisture content was estimated by the gravimetric method of Singh

(1980). Nitrate and phosphate were measured using the method of APHA (1992) using a colourimeter (TP: HACH DR980, USA). The method as described by Brown (1998) was adopted to determine the total organic carbon (TOC). Total petroleum hydrocarbons (TPH) and residues were extracted from the samples and measured with the gas chromatography-flame ionization detector (GC-FID) system (HP5890 Series II, USA) (Cortes *et al.,* 2012).

RESULTS

The total culturable heterotrophic bacterial counts of the different treatment samples revealed that for the negative control (Sample A), TCHBC was 1.60 ± 0.15 × 10^8 Cfu/g, 4.50 ± 0.40 × 10^6 Cfu/g and 2.10 ± 0.24 × 10^3 Cfu/g at day 1, 20, and 40 respectively. For the test (Sample B), TCHBC was 2.02 ± 0.73 × 10^9 Cfu/g, 1.2 ± 0.25 × 10^8 Cfu/g and 1.56 ± 0.86 × 10^6 Cfu/g at days 1, 20, and 40 respectively. For the positive control I (Sample C), TCHBC was 1.72 ± 0.26 × 10^9 Cfu/g, 3.72 ± 0.48× 10^6 Cfu/g and 3.03 ± 0.15 × 10^8 Cfu/g at day 1, 20, and 40 respectively, while for the positive control II (Sample D), TCHBC was 1.48 ± 0.50 × 10^6 Cfu/g, 1.03 ± 0.18 ×10^7 Cfu/g and 5.80 ± 0.10 × 10^5 Cfu/g at day 1, 20, and 40 respectively. Sample B had the highest TCHBC on days 1, and 20, while Sample C had the highest TCHBC on day 40 as represented in Figure 1.

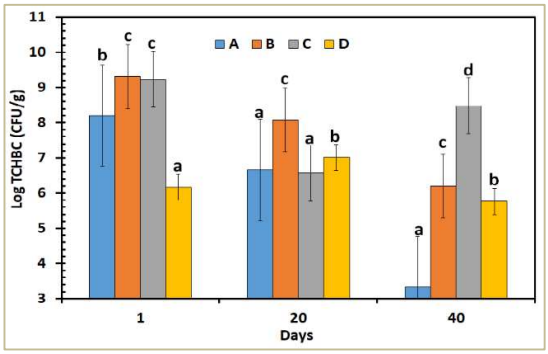

Figure 1: Changes in total culturable heterotrophic bacterial counts of samples during treatments. ***Key:*** *a-d different letters on bars per day are significantly different (p<0.05)*

The total culturable hydrocarbon utilizing bacterial counts (TCHUBC) obtained from the samples showed that the negative control (Sample A) had TCHUBC of 0.36 ± 0.08 × 10^5 Cfu/g, 0.46 ± 0.10 × 10^3 Cfu/g and 0.37 ± 0.05 × 10^2 Cfu/g at day 1, 20, and 40 respectively. Sample B had TCHUBC of 3.20 ± 0.19 × 10^5 Cfu/g, 2.72 ± 0.18 × 10^4 Cfu/g and 1.00 ± 0.02 × 10^4 Cfu/g at day 1, 20, and 40 respectively. Sample C, had TCHBC of 1.05 ± 0.08 × 10^5 Cfu/g, 2.24 ± 0.16× 10^3 Cfu/g and 1.38 ± 0.53 × 10^3 Cfu/g at day 1, 20, and 40 respectively, while Sample D, had TCHBC of 0.72 ± 0.08 × 10^5 Cfu/g, 0.62 ± 0.04 × 10^3 Cfu/g and 0.77 ± 0.12 × 10^2 Cfu/g at day 1, 20, and 40 respectively. Sample B showed the highest TCHUBC on days 1, and 20, while Sample A had the highest TCHUBC throughout the treatment period as represented in Figure 2.

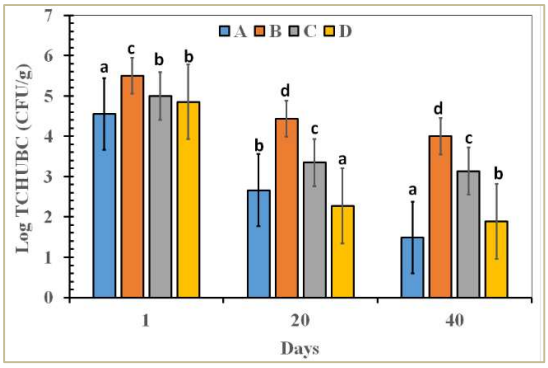

Figure 2: Changes in total culturable hydrocarbon utilization bacterial counts during treatment. ***Key:*** *a-d different letters on bars per day are significantly different (p<0.05)*

The gas chromatographic analysis carried out showed an overall reduction in TPH concentration throughout the treatment period (Figure 3).

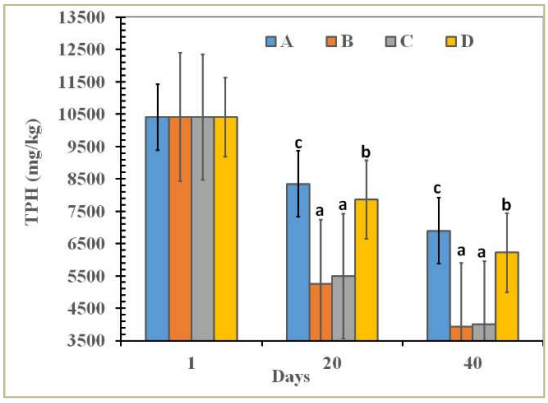

Figure 3: Changes in total petroleum hydrocarbon concentration during treatments. ***Key:*** *a-c different letters on bars per day are significantly different (p<0.05)*

For Sample A, TPH concentration reduced from 10410.50 ± 0.16 mg/kg to 8345.20 ± 0.22 mg/kg, and 6890.73 ± 0.06 mg/kg indicating a percentage loss of 19.84 and 33.81% at days 20, and 40 respectively. For Sample B, TPH concentration reduced from 10410.50 ± 0.16 mg/kg to 5261.90 ± 0.29 mg/kg, and 3924.71 ± 0.08 mg/kg indicating a percentage reduction of 50.54 and 62.30% at days 20, and 40 respectively. Sample C's TPH concentration reduced from 10410.50 ± 0.16 mg/kg to 5490.70 ± 0.59 mg/kg, then to 4009.50 ± 0.57 mg/kg indicating a percentage reduction of 47.26 and 61.49% at days 20, and 40 respectively, while for Sample D, TPH concentration reduced from 10410.50 ± 0.16 mg/kg at day 1 to 7860.30 ± 0.29 mg/kg at 20, then 6220.02 ± 0.04 mg/kg at 40 indicating percentage reduction of 24.50 and 40.25% respectively. Sample B showed the highest TPH concentration percentage reduction, while Sample A showed the lowest percentage of TPH reduction.

The pH value of the different treatment setups increased over time from 3.4 ± 0.0216 (Sample A) to 6.6 ± 0.02 and 6.78 ± 0.12 on days 1, 20 and 40 respectively. Also, for Sample B, the pH value increased from 5.0 ± 0.03 to 6.0 ± 0.04 then reduced to 5.94 ± 0.03 at day 1, 20 and 40 respectively. Sample C, pH value was 5.20 ± 0.16 on day 1, 6.91 ± 0.08 at 20 and 6.51 ± 0.12 on day 40. Sample D showed an increase in pH value from 4.5 ± 0.08 to 5.2 ± 0.36 and 5.63 ± 0.04 at day 1, 20 and 40 respectively. Sample C had the highest pH value on day 20, while Sample A had the lowest pH value on day 1. All the treatment setups had an acidic pH which increased over time tending towards neutrality as shown in Figure 4.

The treatment setups were monitored for utilization of available moisture by organisms indicating that there were changes in moisture content as treatment progressed (Figure 5). For Sample A, moisture content changed from 16.24 ± 0.15% to 28.23 ± 0.04% then to 20.42 ± 0.01% at day 1, 20, and 40 respectively. Sample B changed from 18.35 ± 0.04% to 20.41 ± 0.02%, then to 19.12 ± 0.02% on days 1, 20, and 40 respectively. For Sample C, there were changes in moisture content from 19.21 ± 0.02% to 18.91 ± 0.01%, then to 17.24 ± 0.20% on days 1, 20, and 40 respectively. Also, Sample D changed from 20.48 ± 0.02% at day 1, 20.35 ± 0.02% at day 20 then 19.12 ± 0.02% at day 40. Overall, there was a reduction in moisture content.

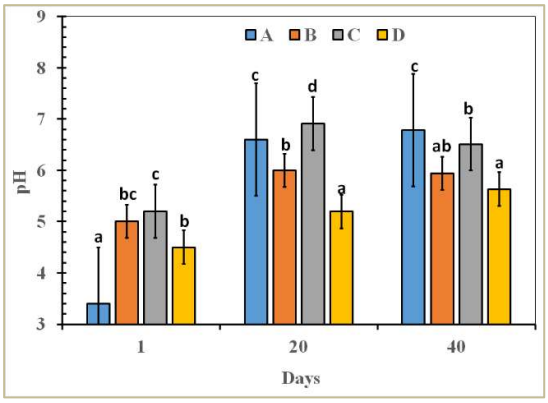

Figure 4: Changes in the hydrogen ion concentration (pH) during the treatments. ***Key:*** *a-d different letters on bars per day are significantly different (p<0.05)*

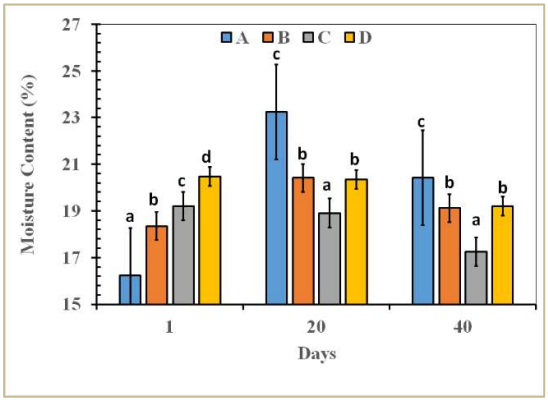

Figure 5: Changes in the moisture contents during the treatments. ***Key:*** *a-d different letters on bars per day are significantly different (p<0.05)*

There were significant changes in the phosphate concentration of the individual setup. Sample A ad a phosphate concentration of 0.02 ± 0.01 mg/kg, 0.02 ± 0.01 mg/kg, and 0.02 ± 0.01 mg/kg on days 1, 20, and 40 respectively. Sample B had 0.17 ± 0.17 mg/kg, 0.02 ± 0.00 mg/kg, and 0.01 ± 0.00 mg/kg on days 1, 20, and 40 respectively. Sample C had 1.10 ± 0.17 mg/kg, 0.10 ± 0.00 mg/kg, and 0.08 ± 0.00 mg/kg on day 1, 20, and 40 respectively, while Sample D had phosphate concentrations of 1.25 ± 0.02 mg/kg, 0.18 ± 0.01 mg/kg and 0.12 ± 0.01 mg/kg at day 1, 20 and 40 respectively.

Sample D had the highest phosphate concentration at day 1 while Sample A had the lowest concentration throughout the treatment period as represented in Figure 6.

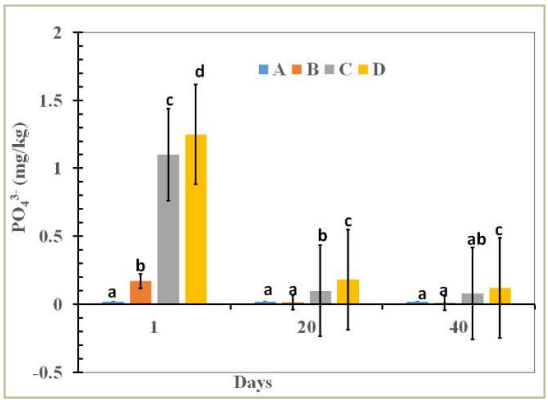

Figure 6: Changes in the phosphate concentrations during the treatments. ***Key:*** *a-d different letters on bars per day are significantly different (p<0.05)*

The different treatment setups were monitored for changes in nitrate concentration within the treatment period (Figure 7).

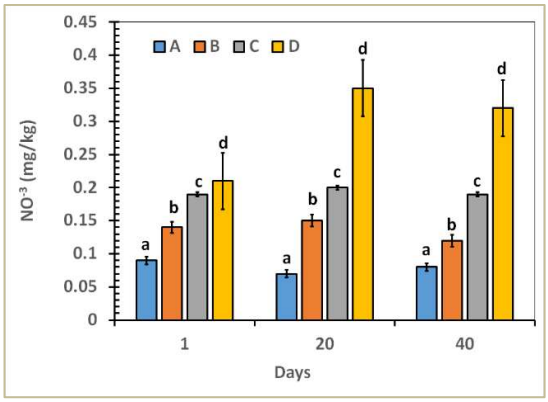

Figure 7: Changes in the nitrate concentrations during the treatments. ***Key:*** *a-d different letters on bars per day are significantly different (p<0.05)*

Results showed that Sample A was 0.09 ± 0.02 mg/kg, 0.07 ± 0.01 mg/kg and 0.08 ± 0.00 mg/kg on days 1, 20, and 40 respectively. Sample B, 0.14 ± 0.01 mg/kg, 0.15 ± 0.00 mg/kg and 0.12 ± 0.01 mg/kg at day 1, 20, and 40 respectively. Sample C, 0.19 ± 0.01 mg/kg, 0.20 ± 0.02 mg/kg, and 0.19 ± 0.01 mg/kg at day 1, 20, and 40 respectively, while Sample D had nitrate concentrations of 0.21 ± 0.01 mg/kg, 0.35 ± 0.02 mg/kg, and 0.32 ± 0.01 mg/kg at day 1, 20, and 40 respectively. Sample D had the highest nitrate concentration at day 20, while Sample A had the overall lowest nitrate concentration.

Total organic carbon (TOC) was also monitored over the treatment period in the different setups. Sample A had a TOC of 1.72 ± 0.15%, 1.54 ± 0.02%, and 1.22 ± 0.01% on days 1, 20, and 40 respectively. Sample B, 1.21 ± 0.01%, 1.21 ± 0.01%, and 1.01 ± 0.01% at day 1, 20, and 40 respectively. Sample C, 1.11 ± 0.01%, 1.25 ± 0.03%, and 0.88 ± 0.01% at day 1, 20, and 40 respectively. Sample D had 0.90 ± 0.02%, 1.39 ± 0.03%, and 1.17 ± 0.02% TOC at day 1, 20, and 40 respectively as represented in Figure 8. Sample A had the highest TOC.

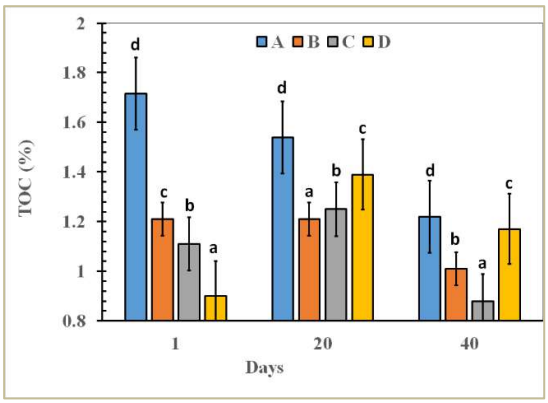

Figure 8: Changes in the total organic carbon concentrations during the treatments. ***Key:*** *a-d different letters on bars per day are significantly different (p<0.05)*

DISCUSSION

The presence of bacterial activity in each sample was monitored by enumerating the total culturable heterotrophic bacterial counts (TCHBC) and total culturable hydrocarbon utilizing bacterial counts (TCHUBC). The TCHBC were higher than the TCHUBC in all the samples. This implies that apart from the hydrocarbon utilizers, other bacterial genera were able to strive and survive in the polluted environment due to prolonged exposure and adaptation to their environment. The highest TCHBC and TCHUB were recorded in Sample B due to the addition of bacterial consortium. The ability of these bacteria to survive in the polluted soil proves the versatile nature of the soil habitat in providing support for microbial abundance and

diversity. The reduction in TCHBC and TCHUBC after day 20 during treatment contradicts the findings of the studies conducted by Chikere *et al*. (2012; 2016) and Uba *et al*. (2019) where an increase in the bacterial population was recorded following the amendment of hydrocarbon polluted soil, but in agreement with the report of Orji *et al*. (2012) and Ezekoye *et al*. (2015) where a decrease in the bacterial population was observed during the treatment of hydrocarbon polluted mangrove soil using inorganic and organic wastes. This be linked to nutrient depletion or other abiotic factors which are capable of influencing soil bacterial population as a result of stress effects and nutrient competition which causes an imbalance in the carbon, nitrogen and phosphorus proportion (Jiao *et al*., 2016).

There was an overall reduction in TPH concentration of the different treatments within the 40 days with Sample A experiencing the lowest TPH loss of 19.84 and 33.81% at 20, and 40 respectively because it was not amended, while Sample B recorded the highest TPH loss of 50.54% at day 20, and 62.30% at day 40 possibly because of the presence of bacterial augmentation. This proves that the bacterial consortium used for the amendment contained a metabolically active bacterial community with the ability to metabolize hydrocarbons as their source of energy (Chikere *et al*., 2016; Ezekoye *et al*., 2017; 2018). The presence of inorganic (NPK) and organic (cow dung) nutrient supplements in samples C and D acted as bio-stimulants to aid the indigenous microbial population in the degradation process. This factor explains the reduction in their TPH concentrations and this agreed with the finding of Uba *et al*. (2019) during the biodegradation of diesel-contaminated soil amended with organic and inorganic nutrients.

The pH of the crude oil-polluted soil was low (highly acidic). During the bioremediation treatment, pH ranged from 3.40 ± 0.22 to 6.91 ± 0.08 indicating a pH change from acidic to neutral hydrogen ion concentration. This pH range corresponds to the findings of Chikere *et al*. (2009) and Ukaegbu-Obi and Omeh (2014) which revealed that most polluted sites have highly acidic to slightly acidic pH. This be attributed to the time interval between the time of spill and sampling time (Chikere *et al*., 2016; Ezekoye *et al*., 2018). The acidity further suggests that the acidic components of the crude oil were still present in the environment. The increase in pH over treatment time in the different setups is an indication of hydrocarbon utilization. This is in agreement with the findings of Maduwuba (2022).

The low nitrate concentration corresponds to the report of Lehtomäki and Niemelä (1975) where they reported low concentrations of nitrate in crude oil-polluted soils. During treatment, there was an overall reduction in nitrate concentration which agreed with the findings of Uba *et al*. (2019) who reported a reduction in nitrate during the degradation of diesel-contaminated soil using organic and inorganic nutrients. This reduction may be attributed to the growth and degradation process of the indigenous microbial flora in the treatment samples.

Low phosphate concentration was recorded in all samples suggesting that most crude oil-impacted sites usually experience low phosphate concentration as a result of an imbalance in the carbon, nitrogen and phosphorus ratio which is usually caused by high total petroleum hydrocarbon. This TPH influx is an indication of high carbon concentration which results in nutritional struggle within the ecological habitat (Margesin *et al*., 2003; Jiao *et al*., 2016).

The cumulative extractable carbon present in the samples was measured as a percentage of total organic carbon (%TOC). The reduction in %TOC during treatment may be due to carbon assimilation by the microbial population present in the samples as their sole energy source thus improving the rate of biodegradation (Benson *et al.,* 2016).

Conclusion: This study has demonstrated the ability of indigenous bacterial community to enhance the biodegradation of crude oil-derived hydrocarbons through the efficient uptake mechanism and the possible bacterial community population shift which may easily be experienced during the application of different bioremediation treatment options. Therefore, there is a need to

harness these organisms and improve them for the effective recovery of crude oil-impacted sites.

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