BACTERIAL DEGRADATION OF PETROLEUM HYDROCARBONS IN CRUDE OIL POLLUTED SOIL AMENDED WITH CASSAVA PEELS

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ABSTRACT

The increase in the exploration and usage of petroleum products have resulted in wide spread contamination of the environment. This has led to concerted efforts in studying the feasibility of detoxifying oil contaminants using organic and inorganic wastes. Standard physicochemical and microbiological procedures were used to study the bacterial degradation of petroleum hydrocarbons in crude oil polluted soil amended with cassava peels (CP) (an organic waste) for eight weeks. Results showed that CP contained appreciable amounts of some biodegradation enhancing elements/nutrients such as nitrogen (2.37%), potassium (7.13 meq/100g), phosphorus (0.78 mg/kg) and organic carbon (2.37%). The soil samples used for the study were composed of 81.6% clay, 16.4 % sand and 2% silt. The pH of the amended samples during the period of study ranged from 6.54 to 8.16. The hydrocarbon degrading bacterial types and numbers were found to be lesser than their heterotrophic counterparts. Also, samples amended with CP were found to have more types and higher numbers of heterotrophic and hydrocarbon degrading bacteria than the samples without amendment (controls). The hydrocarbon degrading bacterial counts for the amended samples ranged from $3.80\pm0.01 \times 10^5$ cfu/g to $16.50\pm0.01 \times 10^5$ cfu/g with the 5% crude oil polluted sample having the highest count. For the non-amended samples the counts of hydrocarbon degrading bacteria were in the range $2.30\pm0.01 \times 10^5$ cfu/g to $4.90\pm0.01 \times 10^5$ cfu/g. The total petroleum hydrocarbon (TPH) in the samples decreased from day zero to day 56 at the various pollution levels (5%, 10%, 15%). The highest reduction in TPH was in the 5% crude oil polluted soil sample with amendment (89.82%) while the least TPH reduction was in the 15% polluted control sample (without amendment) (27.38%). These findings showed that lower

percentage of crude oil was degraded as the concentration of crude oil increased and that cassava peels which is an agro waste can enhance biodegradation of crude oil in polluted soil. Therefore cassava peels instead of being disposed of as a waste can be harnessed and used as a bioremediating agent in polluted sites.

Key words: Biodegradation; Cassava peels; amendment

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INTRODUCTION

The wide spread contamination of most of arable lands, creeks, swamps and natural source of water with petroleum and petrochemical products particularly in the Niger Delta region of Nigeria, is due to an increasing petroleum exploration, refining and other related industrial activities (Okpokwasili and Odokuma, 1990;Odokuma and Okpokwasili, 1993). An increase in the world's population has led to an upsurge in the demand for petroleum and petroleum products, which apparently has become a source of pollution to the environment (Akoachere *et al.*, 2008).

The contamination of these habitats poses major public health and socio-economic hazards which most often has developed into impetuous protestations between some of the oil companies and the surrounding communities. This has led to a concerted effort in studying the feasibility of detoxifying oil contaminants using organic and inorganic wastes (Akoachere *et al.*, 2008; Clementina and Omoanghe, 2008).

The major constituents of molecules in petroleum oil spills and refined products are biodegradable, and they will gradually diminish from the environment as microorganisms utilize

them for their metabolic activity (Prince, 1993). The biodegradation potential of petroleum compounds however, can be said to be weak when compared with most of the organic molecules concerned in the biological carbon cycle (Bertrand *et al*., 1989). Crude oil is mainly composed of hundreds of different hydrocarbon molecules, which are mainly alkanes from C_1 to C_{40} straight chain, C_6–C_8 branched-chain, cyclohexanes, aromatics and compounds containing sulphur, nitrogen and oxygen (Stafford *et al*., 1982).

Biodegradation as a means of remediation of contaminated site has drawn positive attention because of its economic viability and environmental friendliness (Walker and Crawford, 1997; Dinkla *et al*., 2001). Biostimulation or bioaugmentation technology employs various options, as a means of cleaning up of oil polluted sites and one of such options is the use of agro waste which has interestingly proven effective in pollution abatement (Daane *et al*., 2001).

In bioremediation, the contaminated site is exposed to a population of microorganisms which undergoes metabolic activity to transform or mineralize organic contaminants into less harmful, non-hazardous substances which are then integrated into natural biogeochemical cycles. However, the rate at which biodegradation occurs depend on the interactions between the number and type of microorganisms present, the chemical structure of the contaminant as well as the environment (Akoachere *et al*., 2008; Walker and Crawford, 1997; Atlas, 1981).

The availability of nutrients, especially nitrogen and phosphorus significantly control microbial activities (Margesin and Schinner, 1997), and these nutrients are necessary to enhance the biodegradation of oil pollutants (Choi *et al*., 2002). Various studies have demonstrated that effective enhancement of hydrocarbon degradation after an oil spill is linked to the provision of limiting nutrients; hence, one possible explanation may be that the addition of these limiting

nutrients, stimulated the activities of indigenous bacterial populations, sufficiently enough to enhance the degradation of the crude oil (Pitchard *et al*., 1992; Swannell *et al*., 1999).

An understanding of the microbial processes occurring in contaminated soil may suggest bioremediation strategies that could be effective in reducing hydrocarbon pollutants concentrations below toxic levels, since the proportion of hydrocarbon-utilizers implicated in crude oil degradation is naturally very low in soil and aquatic environments (Amund and Igiri 1990; Adebusoye *et al.*, 2007). Crude oil contaminant can persist in the environments undegraded for many years (Atlas 1992; Solano-Serena *et al.*, 2000) hence, bioremediation protocols involving application of agro waste as a nutrient supplement to those naturally present can improve the rate of remediation of polluted environments. Few studies have focused on solid wastes emanating from food-based industries as potential agents for bioremediation; therefore, the goal of this study was to determine if bacterial degradation of hydrocarbon in crude oil polluted soil could be accelerated through the addition of cassava peel as an amendment or nutrient enhancer.

MATERIALS AND METHODS

Sources/collection of samples

Nigerian crude oil (Bonny Light Oil) was collected from N.N.P.C Benin City, Nigeria. Cassava peels were obtained from cassava sale unit of Ekpoma main market. Top soil (0-10cm) depth was collected from a garden within Ambrose Alli University, Ekpoma, Edo State, using a ditch auger and bulked to form a composite sample.

The mineral salt medium was used as described by Mills *et al.* (1978) and modified by Okpokwasili and Amanchukwu (1988). Bacteriological agar (oxoid) was added to obtain a solid medium at a rate of 1.5 (%) when necessary. The general purpose media used included

commercial preparations of oxoid nutrient agar, nutrient broth, MacConkey agar, peptone water, urease agar and citrate agar.

Media were sterilized by autoclaving at 121 °C for 15 min. Crude oil used for biodegradation studies was filter - sterilized using sterile 0.22 µm pore size membrane (Type: MILLEX-GS Millipore Corporation, Bedford, MA01730 Rev. 9/94 12172). Glass wares were sterilized at 160 °C for one hour using hot air oven.

Experimental design

Processing of samples/experimental design

Cassava peels (Agro waste samples) used for this study were sun-dried for 5days and milled into semi fine particles using Corn Mill dx-2200, China. Two kilograms (2kg) of soil samples each were introduced into six (6) different plastic buckets (PB) labeled A to F. The plastic buckets A, B and C were polluted with 5%, 10 % and 15% crude oil respectively. Also each of the plastic buckets (A, B, C) were amended with 100g of cassava peels. Plastic buckets D, E and F served as the controls for the experimental set ups containing 2kg of soil sample polluted with 5%, 10% and 15% crude oil respectively without any amendment. Periodic sampling from each PB was carried out at 14days intervals for 56days. The samples were then analyzed for changes in pH, total petroleum hydrocarbon as well as for their bacterial types and numbers.

Bacterial enumeration and identification: The total heterotrophic bacteria count in the samples were determined by making ten-fold serial dilution of the samples on normal saline (0.85% w/v) sterile NaCl. Then 1 mL of the appropriate dilution was pour plated in duplicates on the surface of nutrient agar. The plates were then incubated for 24-48 h at a temperature of 37 °C. Thereafter emerging colonies were counted. Also, mineral salt agar medium was used for the enumeration of hydrocarbon utilizing bacteria. Sterile 9 cm Whatman (No.1) filter paper soaked in crude oil and placed in dish cover served as carbon source. Thus the hydrocarbon was supplied to the inoculums by vapour-phase transfer. The media was made selective for bacteria by adding nizoral (100mg/L). After incubation at room temperature for 1-5days, emerging colonies were counted. The phenotypic and biochemical characteristics used to characterize and identify bacterial isolates included Gram staining, colonial appearance, motility, urease, catalase, indole, oxidase, citrate, methyl red, voges proskaeur and sugar fermentation. These tests were performed

using the methods of Gerhardt (1994); Harley and Prescott (2002) and identified based on observations of Barrow and Feltham (1986) and Holt *et al.* (1994).

Determination of physicochemical properties of samples: Methods for the determination of physicochemical properties of samples (cassava peels, crude oil polluted and non polluted soil) were used as outlined by APHA (1985). The pH meter used was pocket-sized HANA pHep + HI 98108 with automatic temperature compensation. Conductivity values were determined using conductivity meter (Jenway 4010 UK. Total organic carbon was determined by dichromate wet oxidation method of Walkley and Black as modified by Dhyan *et al*., (1999). Nitrate content was determined using the macro Kjeldahl digestion method of Brady and Weil (1999) and available phosphorus was deter-mined using the method reported by Olsen and Sommers (1982). Sulphate was determined using the turbidometric method, while oil and grease were determined by the partition gravimetric method. Sodium and potassium were determined using flame photometric method, while calcium and magnesium were determined by using the method of Brady and Weil (1999). The metal contents were determined using an atomic absorption spectrophotometer (AAS) (Perkin Elmer AA Unit Model: 3100 Serial Number: 148157)

Determination of Total Petroleum Hydrocarbon (TPH)

The method of Adesodun and Mbagwu (2008) was used. Crude oil polluted soil sample (5g) was suspended in 25ml of hexane and shaken for 20 minutes using a mechanical shaker. The solution was filtered using a whatman (No.1) filter paper and the filtrate diluted by taking 1ml of the extract into 50ml of hexane. The absorbance of this solution was read at 460nm with HACH DR/2010 Spectrophotometer using n-hexane as blank. Total petroleum hydrocarbon was determined at weekly intervals for eight weeks. The actual TPH concentration (mg/kg) was deduced as follows;

$$\text{TPH} = \frac{\text{Instrument reading (Conc. obtained from calibration)} \times \text{Volume of extract (mL)}}{\text{Weight of sample (kg)}} \times \text{DF}$$

Where TPH = Total petroleum hydrocarbon

DF = Dilution factor

Conc. = Concentration

Statistical Analysis

Results were analyzed using analysis of variance (ANOVA) and means were compared for significance at $p \leq 0.05$ using Duncan's multiple range analysis.

RESULTS AND DISCUSSIONS

Bacterial Isolates and counts

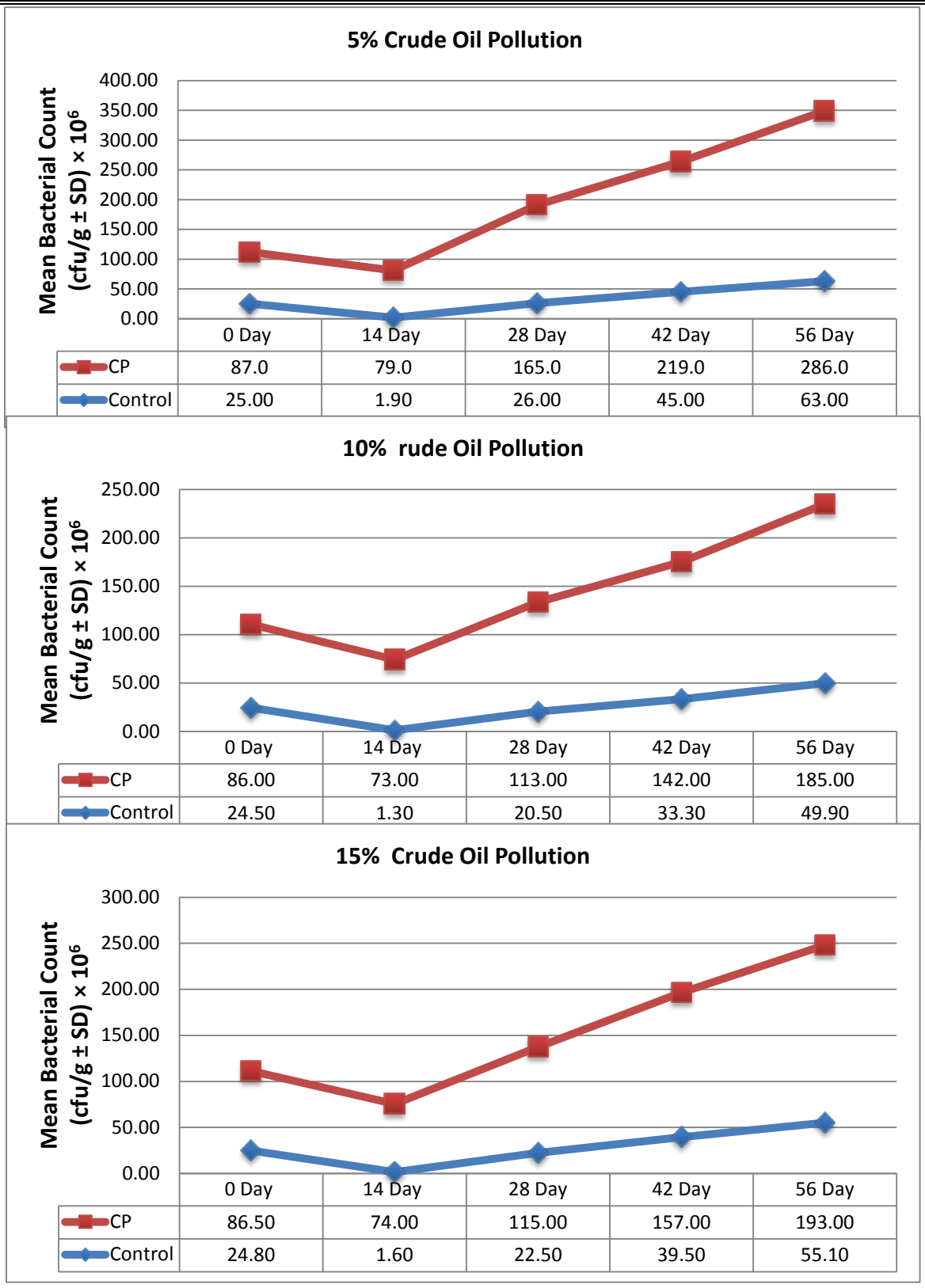
The bacterial isolates from cassava peels used in this study includes *Staphylococcus aureus, Alcaligenes faecalis, Escherichia coli, Enterobacter aerogenes, Bacillus subtilis, Proteus vulgaris, Klebsiella pneumoniae* and *Serratia marcescens.* All these isolates were hydrocarbon degrading (62.5%) except *Staphylococcus aureus, Alcaligenes faecalis* and *Enterobacter aerogenes.* The hydrocarbon utilizing isolates were predominantly Gram negative bacteria These and other bacterial species isolated from the soil sample is shown in Table 1. Similar bacteria have been reported by earlier workers in relation to hydrocarbon biodegradation (Van Hamme *et al.*, 2003; Riffaldi *et al.*, 2006; Akpe *et al.*, 2015). The higher occurrence of Gram negative over Gram positive bacteria in this study agree with the earlier reported that both Gram positive and Gram negative bacteria are encountered in the degradation of contaminants with Gram negative bacteria dominating. These findings also correlates the report of previous workers (Foght and Westlake 1987 and Esumeh *et al*., 2009) who isolated more of Gram negative organisms suggesting that they are better degraders of crude oil when compared with their Gram positive counterparts. The higher ability of Gram negative bacteria to utilize crude may not be unconnected with the possession of plasmid-borne or chromosomal genes involved in hydrocarbon degradation and porins in their cell wall which helps in the uptake of certain substances by the cell or extrusion of others which may be harmful (Vahaboglon *at al.,* 1996; Jørgensen *et al.*, 2000; Akpe *et al.*, 2013).

Counts of heterotrophic bacteria (Figure 1) during the 56 days of study were higher in the amended samples than the non-amended (control) samples and the counts of heterotrophic bacteria in the amended and non-amended (control) samples ranged from $73.00\pm0.04 \times 10^6$ cfu/g to $286.00\pm0.01 \times 10^6$ cfu/g and $1.30\pm0.01 \times 10^6$ cfu/g to $63.00\pm0.00 \times 10^6$ cfu/g respectively. There was decrease in the heterotrophic counts for all samples on the second week of study. For

the same period of study the crude oil utilizing bacterial (Figure 2) were also higher in the amended samples than in the non-amended (control) samples and ranged from 3.80±0.01 x 10^5 cfu/g to 16.50±0.01 x 10^5 cfu/g and 2.30±0.01 x 10^5 cfu/g to 4.90±0.01 x 10^5 cfu/g respectively. The heterotrophic bacterial counts were found to be higher than the crude oil utilizing bacterial counts in all the samples. This could be as a result of nutrient limitation in the enumeration media for crude oil utilizers.

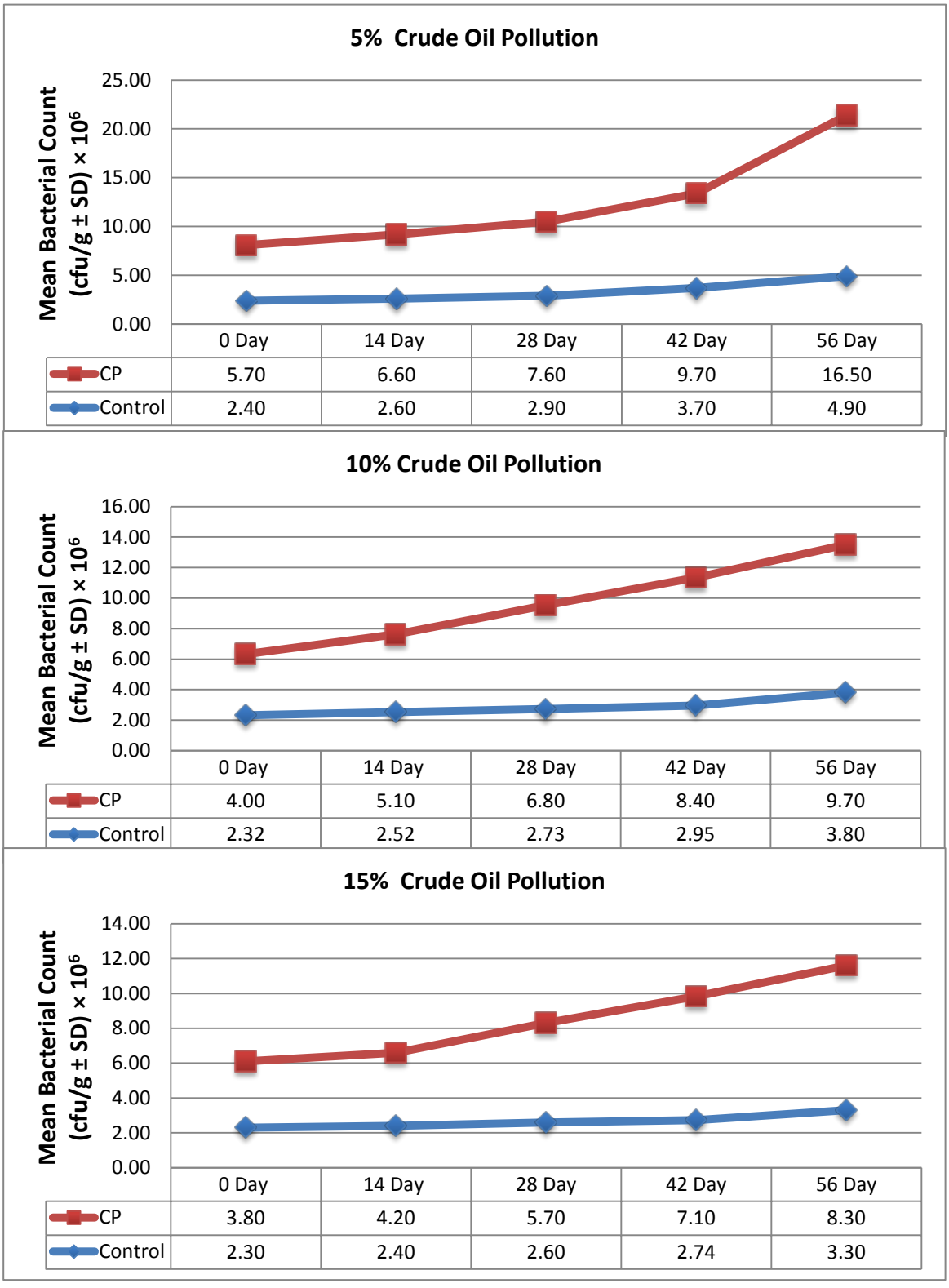
Table 1: Distribution of heterotrophic and hydrocarbon utilizing bacteria and fungi isolated in the samples

Samples	Bacterial Isolates	
	Heterotrophic	**Crude Oil Utilizing**
Cassava Peels	*Staphylococcus aureus* *Alcaligenes faecalis* *Escherichia coli* *Enterobacter aerogenes* *Bacillus subtilis* *Proteus vulgaris* *Klebsiella pneumoniae* *Serratia marcescens*	*Bacillus subtilis* *Proteus vulgaris* *Escherichia coli* *Klebsiella pneumoniae* *Serratia marcescens*
Non-Polluted Soil Sample	*Bacillus cereus*, *Streptococcus faecalis*, *Proteus vulgaris*, *Staphylococcus saprophyticus*, *Staphylococcus aureus*, *Alcaligenes faecalis* *Serratia marcescens* *Pseudomonas fluorescens*, *Bacillus subtillis*, *Pseudomonas aeruginosa*, *Corynebacterium* spp *Escherichia coli*, *Acinetobacter calcoaccticus*, *Chryseomonas luteola*,	*Pseudomonas fluorescens*, *Bacillus subtillis*, *Pseudomonas aeruginosa*, *Corynebacterium* spp, *Escherichia coli*, *Acinetobacter calcoaccticus*, *Chryseomonas luteola*, *Bacillus cereus*, *Streptococcus faecalis*, *Proteus vulgaris* *Serratia marcescens*



Legend: CP = Cassava Peels

Figure1: Viable Heterotrophic Bacteria Enumerated in 5%, 10%, and 15% Crude Oil Polluted Soil Amended with Cassava Peels(CP).



Legend: CP = Cassava Peels

Figure2: Crude Oil Utilizing Bacteria Enumerated in 5%, 10%, and 15% Crude Oil Polluted Soil Amended with Cassava Peels(CP).

Table 2: Physicochemical Analysis of Samples

Parameters		Non-Polluted soil sample	Polluted Soil Sample	Cassava Peels
Electrical Conductivity	(us/cm)	314	250	ND
Organic Carbon	(%)	1.83	4.12	2.37
Organic matter	(%)	4.61	10.03	ND
Total Nitrogen	(%)	0.09	0.149	2.03
Exchangeable Anion	(Meq/100g)	0.3	0.4	NA
Sodium	(Meq/100g)	1.07	1.50	0.67
Potassium	(Meq/100g)	0.64	0.90	7.13
Calcium	(Meq/100g)	3.45	5.04	4.23
Magnesium	(Meq/100g)	1.09	1.14	2.82
Chlorine	(Meq/100g)	15.4	16.5	ND
Phosphorus	(Mg/Kg)	9.64	3.71	19.2
Aammonical nitrogen(NH_4N)	(Mg/Kg)	6.13	8.51	ND
Nitrogen dioxide(NO_2)	(Mg/Kg)	5.20	6.18	ND
Nitrate (NO_3)	(Mg/Kg)	7.81	10.2	ND
Sulfate (SO_4)	(Mg/Kg)	7.36	8.90	ND
Fluorine	(Mg/Kg)	64.2	26.6	ND
Manganese	(Mg/Kg)	1.71	1.99	ND
Zinc	(Mg/Kg)	32.6	15.2	ND
Copper		10.7	11.3	ND
Cadmium	(Mg/Kg)	1.43	6.61	ND
Chromium	(Mg/Kg)	2.24	6.20	ND
Nickel	(Mg/Kg)	2.81	6.76	ND
Lead	(Mg/Kg)	1.51	7.03	ND
Vanadium	(Mg/Kg)	2.10	5.89	ND
Total Hydrocarbon	(Mg/Kg)	9.40	3650.17	ND
Clay	(%)	81.6	ND	NA
Sand	(%)	16.4	ND	NA
Silt	(%)	2	ND	NA

Key

Meq. = Milli Equivalent
EA = Exchangeable Acid
ND = Not Determined
NA = Not Applicable

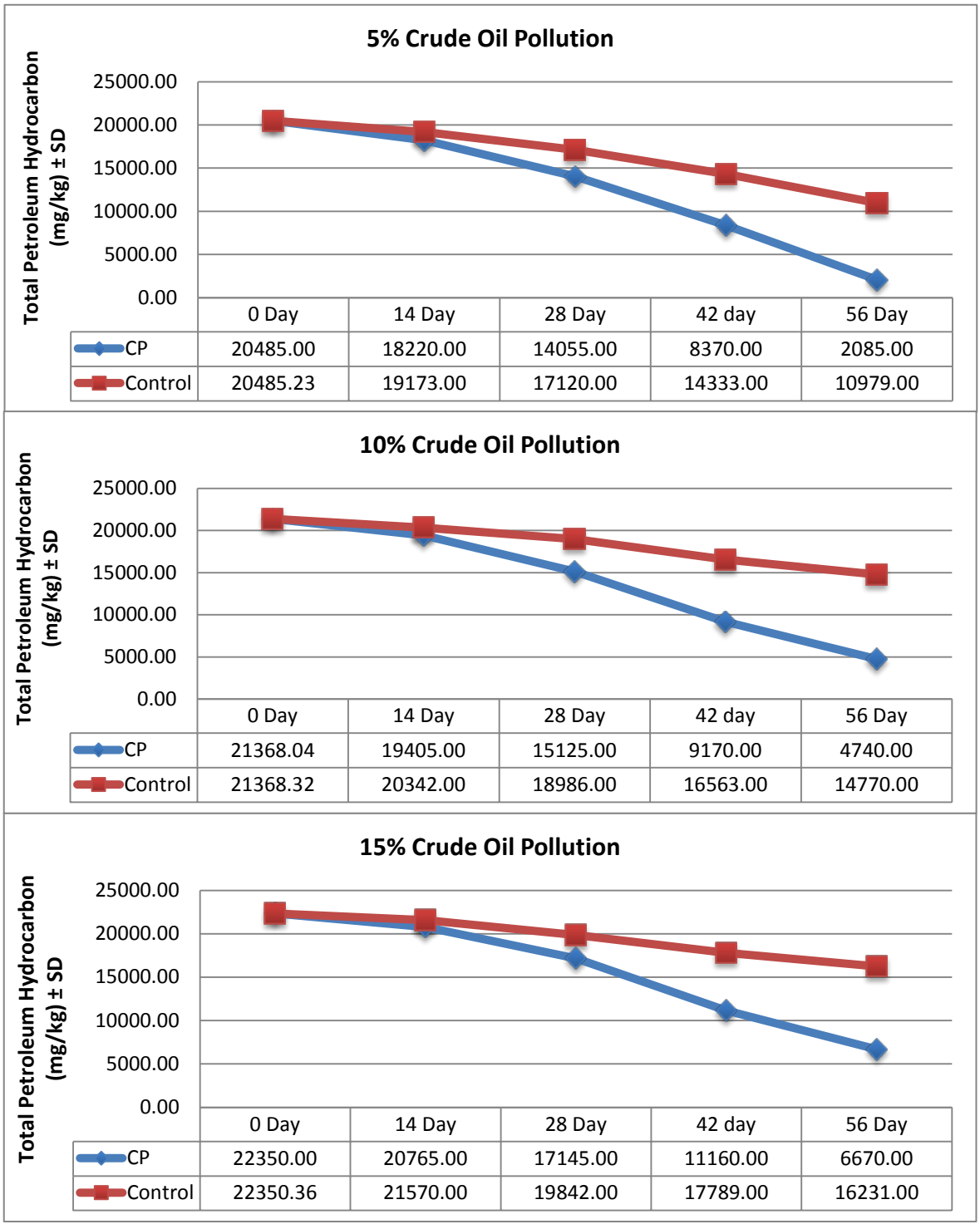
The counts of crude oil utilizing bacteria in the polluted soil samples amended with cassava peels (agro-wastes) were appreciably higher compared to those of non-amended control soil. The reason for higher counts of bacteria in amended soil might be as a result of presence of appreciable quantities of nitrogen and phosphorus in the agro-waste. These elements are necessary nutrients for bacterial biodegradative activities. The cassava peels (agro-waste) may have also served as bulking agent which helped to loosen the compactness of the soil making sufficient aeration available for the indigenous bacteria present in the soil, thereby enhancing their metabolic activities in the contaminated soil (Joo *et al.,* 2007; Abioye *et al.,* 2009; Akpe *et al.,* 2015). The 5% polluted sample amended with cassava peels recorded highest crude oil utilizing bacterial count. This justifies the fact that higher concentration of the pollutants decreases the rate of biodegradation. These findings also showed that cassava peels has the capability to neutralize the toxic effects of crude oil on microbial population by rapid improvement of the soil physicochemical properties. The initial decrease (up to the second week of study) in the heterotrophic bacterial count could be as a result of adaptation to the polluted environment as well as the toxic effect of crude oil on the microbial population. This has been asserted earlier (Akoachere *et al.,* 2008; Mbah *et al* 2009). The presence of limiting nutrients and crude oil utilizing bacteria in the amendment (Cassava peels) suggested that cassava peels played the role of biostimulation and bioaugmentation in the biodegradation process.

Table 3: pH of the samples during the period of study

Sample Period (Days)	5% CP	10% CP	15% CP	5% Control 1	10% Control 2	15% Control 3
0	6.54	7.01	7.06	7.03	7.11	7.21
14	7.12	7.09	7.22	7.11	7.21	8.04
28	7.14	7.13	6.93	7.13	8.01	8.09
42	7.22	7.51	7.13	7.11	8.00	8.10
56	7.19	7.24	7.35	7.09	8.11	8.16

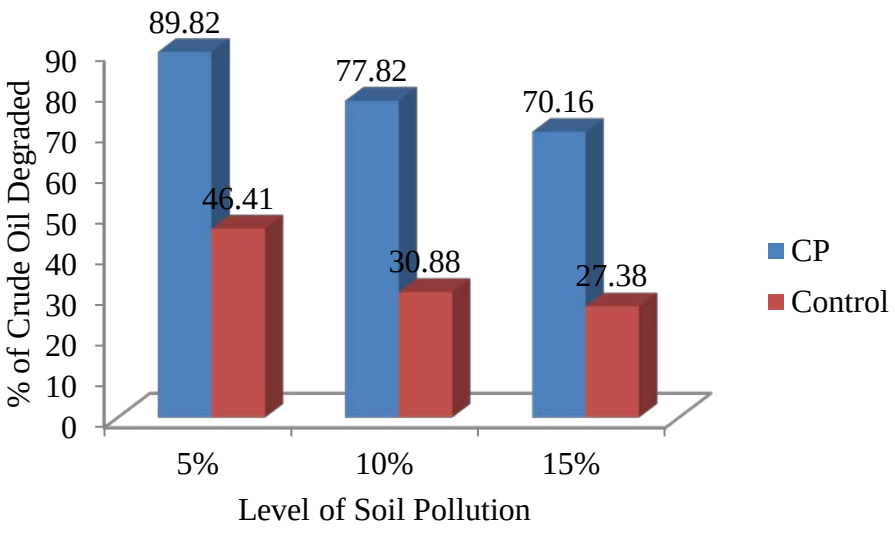
Legend:

CP = cassava peel
Controls = Soil + Crude



Legend: CP = Cassava Peels

Figure 3: Total Petroleum Hydrocarbon (TPH) Recovered from Crude Oil Polluted Soil Amended with Cassava peels.



Legend: CP = Cassava Peels

Figure 4: Percentage of crude oil degraded after 56 days of bioremediation treatment.

Physicochemical properties

The average total nitrogen content of the cassava peels samples used in this study was 2.03%, the potassium content was 7.13 Meq/ 100g while the phosphorus contents 19.2 mg/kg. The carbon, nitrogen and phosphorus content of the soil used for the bioremediation studies were 1.83%, 0.09% and 9.64mg/kg respectively. There were also appreciable levels of other trace elements. The soil samples used for the study were composed of 81.6% clay, 16.4 % sand and 2% silt (Table 2). The pH of the amended samples during the period of study ranged from 6.54 to 7.35 as shown in Table 3. The low level of carbon, nitrogen and phosphorus (C, N and P) in the garden soil samples could have been caused by leaching or erosion. The presence of these limiting nutrients (C, N and P) in the agro-waste samples analysed in this study is in consonance with previous reports (Kim *et al.*, 2005; Okoh, 2006). They noted that the addition of these limiting nutrients is a key factor in achieving effective biodegradation of hydrocarbons. The pH range of the experimental samples (6.54 to 7.35) observed in this study is within the favourable

range for biodegradation of crude oil in polluted soil. Similar observations have been documented (Agarry and Jimoda, 2013; Akpe *et al.*, 2015).

Total Petroleum Hydrocarbon (TPH)

The TPH remaining in the samples and the percentage hydrocarbon degraded is shown if Figures 3 and 4. The TPH in the samples decreased from day zero to day 56 at the various pollution levels (5%, 10%, 15%). The highest reduction in TPH was in the 5% crude oil polluted soil sample with 89.82% crude oil degraded. The least TPH reduction was in the 15% polluted control sample (27.38%). These results showed a marked significant decrease in the TPH content of the amended samples relative to the non-amended samples at the various levels of pollution. The high hydrocarbon loss in the cassava peels mended samples is in line with previous reports (Tanee and Kinako, 2008; Obasi *et al.*, 2013; Onuoha, 2013; Akpe *et al.*, 2015). They independently noted a significant loss in TPH in crude oil polluted soil amended with various organic manures. The agro-waste amendments used in this study could have enhanced biodegradation by supplying nutrient to the microbial community which was evidenced by the increased microbial count with increasing days of degradation studied. The low percentage of crude oil degraded in the control samples showed the possibility of natural degradation which occurs rather slowly. This is at variance with the work of Onuoha, (2013), whose non-amended (control) samples performed extremely well paralleling the amended samples in percentage crude oil degraded. This study also revealed that higher concentration of the pollutant (crude oil) in the soil reduced the rate of biodegradation because such high concentration could pose serious challenge to the metabolic activities of soil microorganisms. This correlates the findings of Abioye *et al.*, 2012 who observed higher percentage of crude oil loss in the 5% used motor oil polluted soil sample amended with organic waste when compared with that of 15%.

CONCLUSION

The use of cassava peels (agro-waste) significantly improved the rate of petroleum hydrocarbon biodegradation in polluted soil. The results also showed that hydrocarbon degrading bacteria were present not only in the soil but also in the cassava peels samples used for amendment. The cassava peels were thereby supplying not only nutrients but also hydrocarbon degrading bacteria

to the polluted environment. The reduction in total petroleum hydrocarbon (TPH) was highest in the 5% crude oil polluted soil sample with amendment (89.82%) while the least TPH reduction was in the 15% polluted control sample (without amendment) (27.38%). The reduction in TPH was complimentary with increase in microbial count. The higher the concentration of crude oil in the soil the lower the percentage degraded. Also cassava peels which is an agro waste can enhance biodegradation of crude oil in polluted soil. The bacterial species identified in this study when produced in large numbers, can be used for bioaugmentation in hydrocarbon - biodegradation processes. Therefore cassava peels instead of being disposed of as a waste (constituting nuisance to the environment) can be harnessed and used as a bioremediating agent in polluted sites.

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