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MICROBIAL POPULATION DYNAMICS AND MINERALIZATION RATES IN HYDROCARBON CONTAMINATED ULTISOL AMENDED WITH ORGANIC WASTES

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ABSTRACT

Microbiological analysis and analytical techniques were used to determine the population dynamics and mineralization rates of a simulated hydrocarbon-contaminated garden ultisol treated with the bacterized organic wastes. The In-Vessel System of composting (IVC) was adopted to treat organic feedstocks (Sewage Sludge and Brewer Spent Grains; BSG), while derived amendments (biosolid and BSG) were seeded with an hydrocarbonoclastic strain of *Bacillus subtilis* before land application. A total of 22 species of microorganisms were isolated from the garden Ultisol at the beginning of this study. A 2log reduction (from 10^5 to 10^3) in abundance was observed for heterotrophic bacteria, while diazotrophs and phosphate solubilizers were also adversely affected after hydrocarbon treatment. Species richness was significantly reduced from 22 to 6, representing a reduction rate of 72.7%. As a consequence of amendment, microbial abundance significantly ($p<0.05$) increased (from 10^3 to 10^6) across microbial groups, while a boom in species richness (from 6 to 17 microbial species) was also observed. Mineralization rate constants were remarkably high in the garden soil amended with organic wastes with rates of 0.007 and 0.005 respectively when compared to contaminated but amended beds. This implies that the bacterized amendments generally improved the natural attenuating potential of the hydrocarbon contaminated soil. Organic wastes can therefore serve as nutrient rich bio-stimulants that can improve the nutritional status of hydrocarbon contaminated soil and also enhance biological activities vital in nutrient transformation and recycling in soil. The organic wastes used in this study successfully ameliorated the negative effects of hydrocarbon on soil microbial communities and dynamics. It may, therefore, be appropriate to recommend their use as a suitable biostimulant during the remediation of agricultural land previously contaminated with hydrocarbon.

KEYWORDS: Organic waste, hydrocarbon contamination, Remediation, Microbial Dynamics.

INTRODUCTION

The soil is a dynamic component of the terrestrial ecosystem that serves as a reservoir of microorganisms and environmental sustainability depends largely on it. Soil quality is one of the important agro-ecosystem variables for which management efforts must improve in order to achieve sustainability. Soil quality has been defined as capacity of a certain kind of soil to function, within natural or managed ecosystem limits, to sustain animal and plant productivity, maintain or promote water and air quality, and support human wellbeing. Soil quality has been estimated with soil physical, chemical and biological parameters. Most biological indicators are known to be related to soil quality, for example microbial biomass, soil enzyme activities, macro-organisms such as earthworms and nematodes number (Savin *et al*., 2015). Furthermore, the diversity of microorganisms can directly influence plant productivity, development and nutrient uptake. Thus, microbial diversity is important in soil quality assessments. Crude oil soil contamination is known for its negative environmental impact including reduced microbial diversity and evenness, resulting in impairment of some key microbial function including nitrogen cycle (Jung *et al.* 2016; Abena *et al.* 2020). Crude oil-polluted soil may remain unsuitable for plant growth for months or years depending on the level of contamination. The adequate protection and restoration of contaminated soils require their characterization and remediation. In the last decade, efforts have been made towards the reduction of pollutants directly at the source and the establishment of new environmental guidelines for contaminated site remediation. Several techniques such physical, chemical and biological entities have been used to remediate these sites. These technologies include the source control (*in situ* and *ex situ* treatment) and the containment remedies (Nwadinigwe and Onyeidu, 2011). Generally, these approaches have limitations i.e. the high costs, the applicability to high contaminant concentrations, the applicability to mixed wastes (organics and inorganics) and the irreversible changes in soil physicochemical properties (Nwadinigwe and Onyeidu, 2011). The Nigeria economy is principally crude oil driven with proven oil reserves estimated at between 16 and 22 billion barrels (3.5×10^9 m^3). Its reserves make Nigeria the tenth most petroleum-rich nation, and by far the most affluent in Africa. The oil sector is majorly located in the Niger Delta region of Nigeria and it is where the "light and sweet" (classed because of its free sulphur content) oil is actively explored and exploited. The Niger Delta has experienced over 4000 oil spill incidences since 1960 with severe cases of contamination of farmlands and drinking water sources (Essien *et al*, 2010; Itah and Essien, 2000). The frequent crude oil spillage on agricultural soils, and the consequent fouling affect all forms of life, renders the soil (especially the biologically active surface layer) toxic and unproductive. It adversely affects the soil physical and chemical properties and more specifically hinder soil mineralizing (heterotrophic) activity as well as the activities nitrogen fixers and ammonium oxidizing bacteria (Essien *et al.*, 2013).

Agriculture is the major source of food and potentially the largest sector that can improve the Nigerian economy. However, Nigeria like other countries of the world faces challenges with respect to food security. There are over sixty million farmers with about 89 million hectares of land available for cultivation, yet food shortages are on the increase. Low agricultural productivity in Nigeria over years has been largely due to low fertilizer use. The challenges associated with shortage of fertilizer is complicated by frequent cases of oil spill in the Niger Delta region of Nigeria. To address this challenge, and improve the fertility of the hydrocarbons impacted soils, emphasis has been on the development of easy-to- use, cost effective remediation approaches.

Recently, attention has shifted to the remediation of contaminated soil using methods that are non-destructive, less disruptive to the soil and are low-cost technologies. Composting technologies have been developed to convert wastes into compost and organo-mineral fertilizers (Egbewumi, Sridhar and Azuzu, 1997; Sridhar and Adeoye, 2003). The process of composting relies upon the inter-related activities of a diverse range of micro-organisms to convert organic waste substrates into a stabilised material ('compost'), which is high in humic substances ('humus') and contains useful plant nutrients (Ros *et al*., 2006). Compost has two main effects on soils, particularly nutrient-poor soils: replenish soil organic matter and supply plant nutrients (Sanchez-Mondero *et al*., 2004; Tejada *et al*., 2009). The organo-mineral fertilizers produced from composting is a good soil conditioner and is ideal for Nigerian soil for good crop yields. Composts have several advantages compared to plant residues when applied to soils, such as reduced volume, slower mineralization rates and recycling of municipal biosolid wastes (Fatunla *et al*., 2017). This explains the use of sludge in septic systems for organic fertilization (Adegoke and Stenström, 2019).

Despite the advantages of bioremediation, its efficiency is limited majorly by the reduced bioavailability of hydrocarbons to microorganisms. This is attributed to the low solubility and strong and/or irreversible sorption to soil matrix (Essien *et al.,* 2012, 2016, Rockne *et al.,* 2002). To solve this problem, several methods have been developed to enhance the bio-availability of hydrocarbons and the Remediation by Enhanced Natural Attenuation (RENA) is one, with preference to biological sources. The protocol focuses on remediation of hydrocarbon using nutrient-rich and highly bacterized organic amendments. However, its effects on microbial community population and dynamics have not been properly elucidated and remains the focus of the present study.

MATERIALS AND METHODS

Experimental Site

The soil used for the experiment was the sandy-loamy soil of the Botany Department, University of Uyo Botanical garden located between Latitude N05º02'25.3" and Longitude 07º58'42.9". elevation level 109m 109 above sea level, in the University of Uyo Main Campus, Nwaniba, Uyo, Akwa Ibom State. Uyo has a humid, tropical climate with an average precipitation of 2432 mm per year. The annual average temperature is between 25 and 28°C. The soil on the experiment site is acidic with a pH between 4.8-5.3 and classified as ultisol according to USDA's soil taxonomy system.

Composting of Feedstocks

The In-Vessel System of composting was employed for the production of biostimulants from the feedstocks as described by Fatunla et al., 2017 and Andreoli et al. 2007.

Bacterization of Biostimulants

Strong hydrocarbon-degrading strain of *Bacillus siamensis* (GenBank accession number KY569499.1) was used for the bacterization of the treated biostimulants. The inoculum of the bacterizing agent was prepared by growing the isolate in a Yeast Extract-Mannitol Broth. The broth was then blended with the biostimulants (BSG and biosolid) to obtained bacterized biofertilizers. The number of cell of *B. siamensis* in 1ml of bacterization substrate was approximately 10^8 CFU.

Experimental Procedure

Treatments were completely randomized. Six 2m x 2m plots prepared as crop beds were contaminated with 12.05 mg/kg, 15.15 mg/kg and 21.25 mg/kg levels of hydrocarbon source and allowed to condition for two weeks. Four uncontaminated beds served as control (**Fig. 1**). Bacterized biosolid and BSG were applied as basal dressings on the beds and monitored for eight (8) weeks.

HCSA1 (12.05 mg/kg) + 10 kg bacterized biosolid	HCSA2 (15.15 mg/kg) + 10 kg bacterized biosolid	HCSA3 (21.25 mg/kg) + 10 kg bacterized biosolid	HCS (21.25 mg/kg)	GSA (Garden Soil) + 10 kg bacterized biosolid
HCSB1 (12.05mg/kg) + 10 kg bacterized BSG	HCSB2 (15.15mg/kg) + 10 kg bacterized BSG	HCSB1 (21.25mg/kg) + 10 kg bacterized BSG	GSB (Garden Soil) + 10 kg bacterized BSG	GS Garden Soil

Fig. 1: Experimental Set-up

Sample Collection and Analysis

Samples were obtained from both organic wastes and analyzed for pH, moisture content, organic matter, total organic carbon (TOC), available phosphorus, total Nitrogen (TON), nutritive salts ($-NO_2$, $-NO_3$, $-SO_4^{2-}$, $-NH_4^+$, Cl), and Electrical conductivity using methods of AOAC, 1999. Soil samples for microbiological analysis were collected with the aid of a sterile hand trowel, stored in sterile glass bottles and transported to the laboratory for immediate analysis**.** The soil samples were homogenized and the total culture-able heterotrophic bacterial (THB), Hydrocarbon Utilizing (HUB), phosphate solubilizing Bacterial (PSB), Diazotrophs and fungal load were determined using aerobic culture techniques described by Cappucino and Sherman, (2002) and Aneja, (2003). At the end of the study, the residual organic matter content was determined and used to calculate

the mineralization rate constant as described by Nwite, (2013) as follows;
K = (2.303/(t2 - t1) log (C1/C2)
where;
K = organic carbon mineralization rate constant/day
C1 = amount of organic carbon in the soil (g) at the beginning of the experiment (t1)
C2 = the residual amount of organic carbon (g) at the end of study
(t2) (with t1 – t2 expressed in days) (Mbah, 2009).

Data obtained were subjected to descriptive statistics and multivariate analysis of variance (MANOVA). The LSD and Levene's homogeneity of variance were also investigated using IBM SPSS windows version 20 package (IBM Corp, USA), and the significance of the treatment means was tested at $p \leq 0.05$.

RESULTS AND DISCUSSION

Microbiological and Chemical Properties of Organic Waste Used.

The microbiological and chemical properties of the amendment used (Biosolid and BSG) is presented in Table 1. Of the two amendments, biosolid had the highest value for nitrogen (10.05%) and sulphate (4.33 mg/kg), while BSG had the highest phosphorus (2.87%) and ammonium (11.2 mg/kg).

Table 1 Microbiological and chemical properties of organic wastes used.

Properties	Amendment Type	
	Biosolid	BSG
Heterotrophs (HET) (logCFU/g)	3.90	2.30
Hydrocarbon Degraders (HDB)(logCFU/g)	2.10	-
Fungi(logCFU/g)	3.6	2.80
pH	7.34	6.67
Organic Matter (%)	22.16	10.4
Nitrogen (%)	10.05	1.91
Phosphorus (%)	0.76	0.81
Potassium (mg/kg)	2.89	2.80
Phosphate (mg/kg)	1.13	2.87
Ammonium ion (mg/kg)	0.84	11.2
Sulphate (mg/kg)	4.33	1.29
Chlorides (mg/kg)	1.07	0.002

Properties of Potent Hydrocarbon Degrader Used for Bacterization of Organic Wastes.

The organism used for bacterization is *Bacillus siamensis* (GenBank accession number KY569499.1). *B. siamensis* is a Gram positive, rod shaped bacterium measuring 0.5-2.5µm. it is a motile aerobic bacteria producing round, cylindrical or oval spores. The number of cell of *B. siamensis* in 1ml of bacterization substrate was approximately 10^8 CFU.

Microbial Population and Dynamics in a garden ultisol contaminated with petroleum hydrocarbon and amended with Biosolid

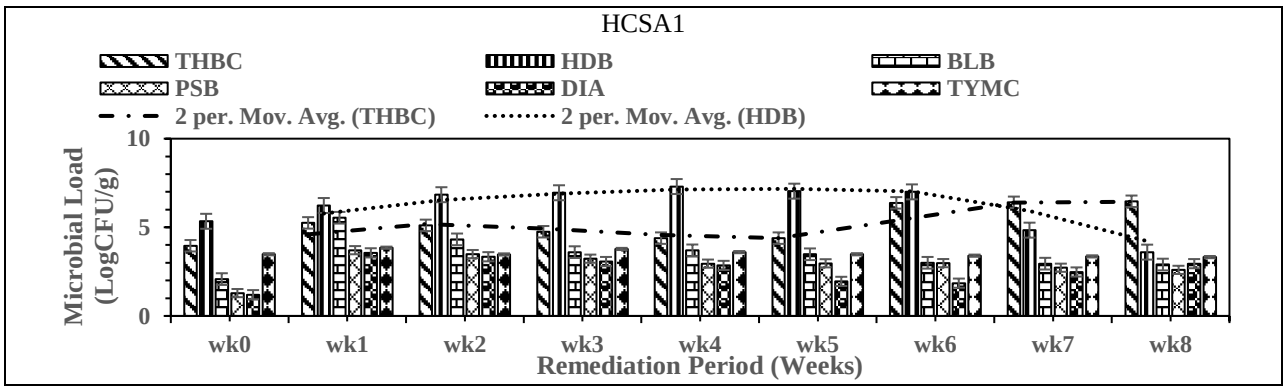
The results presented in Figures 2 and 3 revealed that the influence of the bacterized feedstocks on the activities of plant growth promoting bacteria (PGPB) and hydrocarbon degrading bacteria (HDB) varied with level of hydrocarbon in soil. A marked variation exists in the densities of the different heterotrophic microbial groups. For HCSA1, application of biosolid led to a sharp increase in the densities of all the heterotrophs in week one and peaked at week two after which a noticeable decline was observed over the next six weeks with the exception of heterotrophic bacteria that increased again between week six and eight. The hydrocarbon degrading bacteria however, peaked at week five and declined sharply over the next three weeks (Figure 2). HCSA2-A3 and bed HCSB1-B3 followed the same trend as HCSA1 although, their corresponding loads varied. The microbial loads for all the groups in GSA and GS) over the eight-week remediation course showed a sharp increase that peaked at week 3-4 respectively, followed by a steady decline between week five to eight (Figure 2 and 3). For HCS however, all the heterotrophs except hydrocarbon degraders, exhibited a sharp decline within the first three weeks and remained relatively steady over the next five weeks. Diazotrophs died off after the first three weeks. The hydrocarbon degraders showed a marked increase that peaked at week five and then steadily declined in the next three weeks (Figure 2).

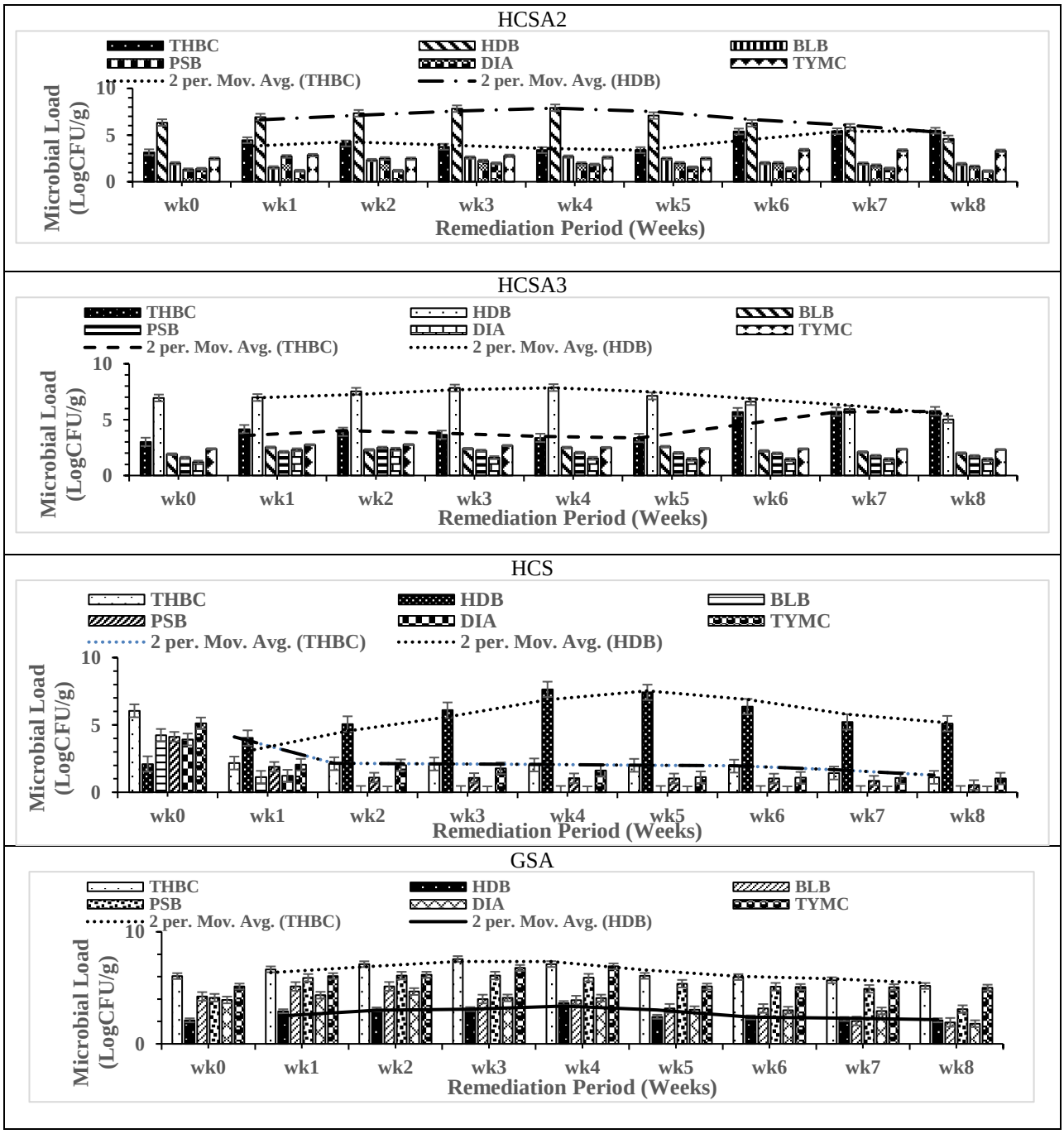
Using aerobic culture techniques, a total of 22 species of microorganisms were isolated from the garden soil sample at the beginning of this study before hydrocarbon contamination (Table 2). These included; *Bacillus*, *Corynebacterium*, *Aeromonas*, *Pseudomonas*, *Staphylococcus*, *Citrobacter*, *Penicillum*, *Aspergillus*, *Cladosporium*, *Verticillum*, *Fusarium* and *Candida*. Hydrocarbon contamination however had a profound effect on soil microbial communities. A 2log reduction (from 10^5 to 10^3) in abundance was observed for heterotrophic bacteria, while diazotrophs, phosphate solubilizers and bioluminescence bacteria were also adversely affected as hydrocarbon treatment increases. However, the increase in the number of culturable hydrocarbon degrading bacteria (HDB), demonstrates how rapidly indigenous soil organisms adapt to the influx of new substrates. The high carbon content of hydrocarbon presents a suitable substrate for microorganisms and previous studies have revealed that an increase in the number of HDB shares a positive correlation with increase in hydrocarbon concentration (Margesin *et al*., 2000; Dindar *et al*., 2017). Specie richness was also significantly affected as number of species reduced from 22 to 6 (Table 2), representing a reduction rate of 72.7%. *Bacillus* and *Pseudomonas* species represented the bulk of bacteria that survived hydrocarbon treatment, while *A. niger* was the only fungal representative. These organisms have earlier been isolated from petroleum contaminated soils and implicated in the degradation of oil (Essien, Ubom and Udosen, 1999). However, depending on the level of contamination and amount of amendment added, both biosolid and BSG improved the soil heterotrophic bacteria

properties and indirectly, the hydrocarbonoclastic bacteria activities of soil. This implies that the bacterized amendments generally improved the natural attenuating potential of the hydrocarbon contaminated soil. Amendment did not only improve the competitive saprophytic status of the soil, but also the diversity of microorganisms in the contaminated soil. As a consequence of amendment addition, microbial abundance significantly ($p<0.05$) increased (from 10^3 to 10^6) across microbial groups, while a boom in specie richness (from 6 to 17 microbial species) was also observed (Table 2). The proliferation of proven hydrocarbon degraders such as *Pseudomonas aeruginosa*, *P. putida* and *Citrobacter diversus* that were otherwise absent from uncontaminated garden soil and contaminated beds was also observed.

Similar results were reported by Yergeau *et al*., (2012) and Liang *et al*., (2011). These findings suggest that crude oil degrading microorganisms are crucial to the degradation of petroleum hydrocarbons, and that they significantly influence the transformation and fate of petroleum hydrocarbons in the environment. No single bacteria can degrade the entire petroleum hydrocarbon fractions, hence, there is always a need for a bacterial consortium. Indeed, most bacteria can only effectively degrade or utilize certain petroleum hydrocarbon components, while others are completely unavailable (Chaerun *et al*., 2004; Varjani, 2017). This can be attributed to the fact that different indigenous bacteria have different catalytic enzymes; thus, their roles in oil contaminated sites also vary widely. This also implies that the remediation of petroleum hydrocarbon contamination requires the joint action of multiple functional bacteria to achieve the best environmental purification effect (Dombrowski *et al*., 2016).

Figure 4 shows the effect of the bacterized organic wastes on the mineralization rate constants of hydrocarbon contaminated soil. Mineralization rate constants were remarkably high in GSA and GSB with rates of 0.007 and 0.005 respectively when compared to contaminated beds amended with the composted feed-stocks.Crude oil contaminated soil amended with biosolid and BSG showed low mineralization rate constant but HCS recorded the least mineralization rate of 0.000727. The low mineralization rate constants in hydrocarbon contaminated soil could be attributed to low content of nitrogen and phosphorous. The high total hydrocarbons obtained initially few days after hydrocarbon contamination could partly be explained to be as result of low microbial activity due to initial shock before adaptation to the applied crude oil. Nwite, (2013) reported that oil degradation was a natural process limited by pH, oxygen, scarcity of nutrients such as nitrogen and phosphorus. Ogbo *et al.* (2009) reported high hydrocarbon contents in the soil due to poor oil degradation as a result of lack of nutrients. While Amadi *et al. (*1996), attributed same to low N and P which are limiting to degradation of hydrocarbons. Subsequent increase in decomposition or mineralization rates of amendments 2-month post application could be due to adaptability of microorganisms as well as proliferation of other inhibited microorganisms and availability of readily utilizable substrate liberated during the initial degradation of hydrocarbon. Similar observation was made by Mbah and Mbagwu (2000), Saviozzi *et al*. (1994) and Jama- Adams (1993). Generally, decomposition was faster after the acclimatization periods (month 2-3) then subsequently rapidly declined (month 3-5). This may be as a result of increase of microbial activity due to readily available C and low C: N ratio of these materials, which provided increased surface area. Biswas and Mukherjee (2008) reported that C:N ratio was of great importance in the decomposition of organic wastes. The authors also noted that low C:N ratio between 10 to 12 encouraged faster decomposition of organic wastes. Higher C:N ratio leads to loss of carbon and immobilization of nitrogen (Biswas and Mukherjee, 2008). The faster rate of decomposition observed in oil contaminated plots amended with biosolid could be attributed to high surface area exposed for microbial activity and low C: N ratio compared to values obtained BSG amended beds (Fig. 4). This corroborates the findings of Nwite, (2013) who noted that an inverse relationship existed between C:N ratio and amount of nitrogen mineralized from organic materials.

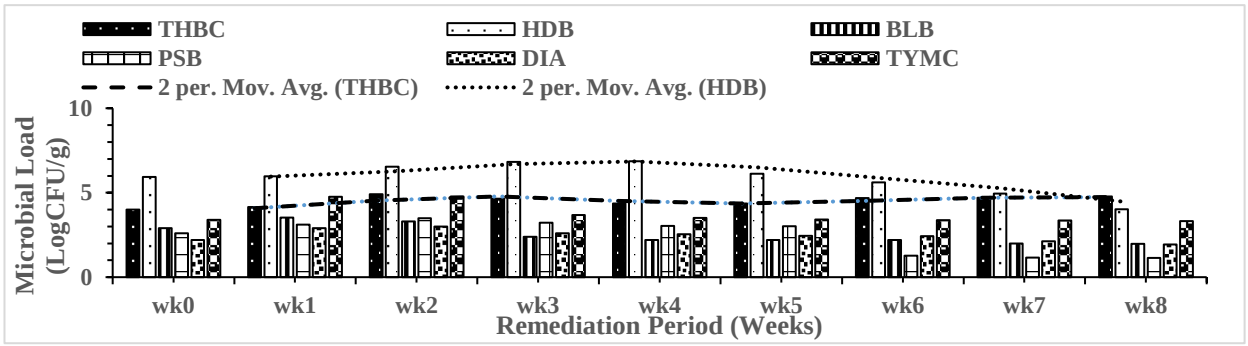




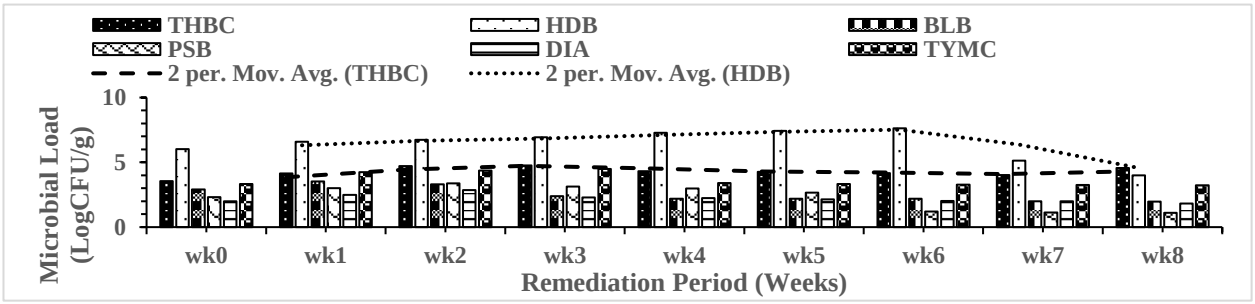
Key: HCS = Hydrocarbon Contaminated Soil (21.25mg/kg); HCSA1= Hydrocarbon Contaminated Soil (12.05mg/kg) + 10kg Biosolid; HCSA2= Hydrocarbon Contaminated Soil (15.15mg/kg) + 10kg Biosolid; HCSA3= Hydrocarbon Contaminated Soil (21.25mg/kg) + 10kg Biosolid; GSA = Garden Soil +10kg Biosolid.

Figure 1: Microbial population dynamics in a garden ultisol contaminated with petroleum hydrocarbon and amended with Biosolid

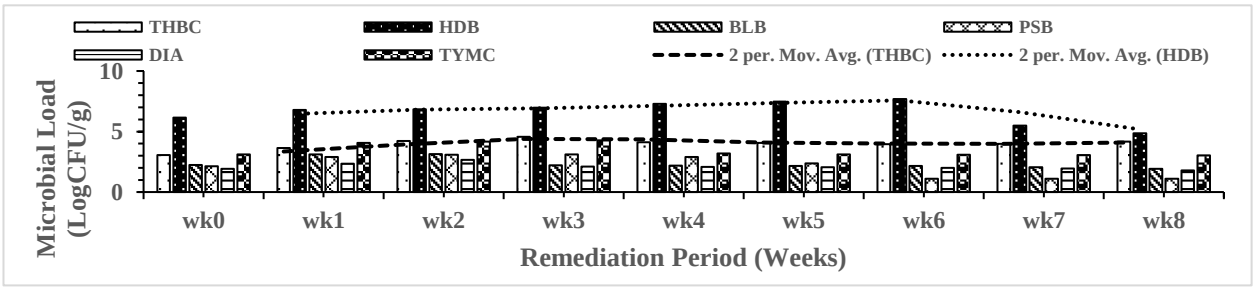
HCSB1



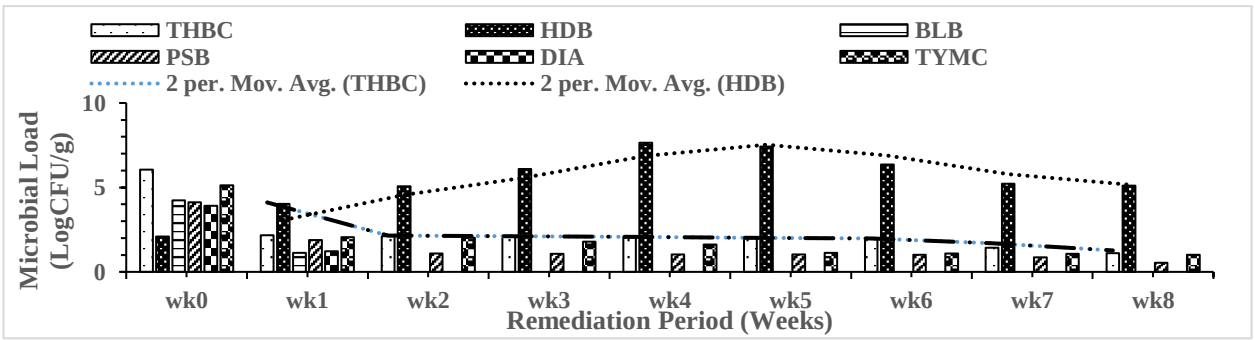
HCSB2

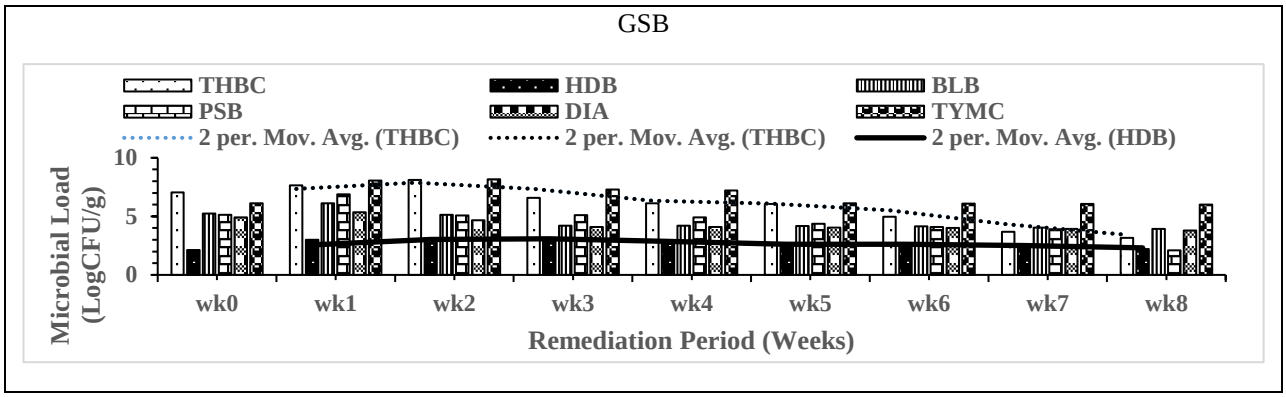


HCSB3



HCS





Key: HCS = Hydrocarbon Contaminated Soil (21.25mg/kg); HCSB1= Hydrocarbon Contaminated Soil (12.05mg/kg) + 10kg BSG; HCSB2= Hydrocarbon Contaminated Soil (15.15mg/kg) + 10kg BSG; HCSB3= Hydrocarbon Contaminated Soil (21.25mg/kg) + 10kg BSG; GSB = Garden Soil +10kg BSG.

Figure 2: Microbial population dynamics in a garden ultisol contaminated with petroleum hydrocarbon and amended with BSG

Table 2: Microbial specie richness in a garden ultisol contaminated with petroleum hydrocarbon and amended with organic wastes

Isolates	**Garden Soil**	**HCS**	**HCSA**	**HCSB**
Bacteria				
Bacillus cereus	-	-	+	+
Staphylococcus aureus	-	-	-	+
Bacillus megaterium	+	+	+	+
Enterobacter sp	-	-	-	-
Bacillus mycoides	-	-	-	-
Aeromonas hydrophila	+	-	-	+
Vibrio sp	+	-	-	-
Bacillus subtilis	+	+	+	+
Bacillus circulans	+	-	-	-
Pseudomonas aeruginosa	+	+	+	+
Pseudomonas fluorescens	+	+	-	+
Pseudomonas putida	+	-	+	-
Escherichia coli	+	-	-	-
Bacillus polymyxa	+	+	+	+
Klebsiella pneumoniae	+	-	-	-
Corynebacterium kutsceri	+	-	-	+
Citrobacter diversus	+	-	+	+
Salmonella sp	+	-	-	-
Shigella sp	+	-	-	-
Corynebacterium xerosis	+	-	+	-
Proteus mirabilis	+	-	-	-
Total	**17**	**5**	**8**	**10**
Fungi				
Cladosporium sp	+	-	+	+
Fusarium sp	+	-	-	+
Candida sp	+	-	+	-
Aspergillus niger	+	+	+	+
Penicillium frequentans	+	-	+	+
Verticillium sp	+		-	+
Total	**5**	**1**	**4**	**5**
Specie Richness	**22**	**6**	**12**	**15**

Key: HCS = Hydrocarbon Contaminated Soil (21.25mg/kg); HCSA= Hydrocarbon Contaminated Soil (21.25mg/kg) + 10kg Biosolid; HCSB= Hydrocarbon Contaminated Soil (21.25mg/kg) + 10kg BSG

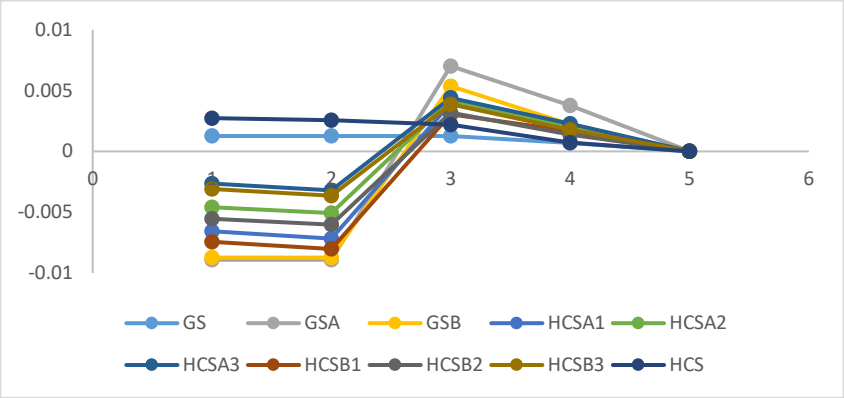


Figure 4: Mineralization rate constant per day.

CONCLUSION

Findings in this study suggest that the bacterized organic wastes can generally improve the natural attenuating potential of the hydrocarbon contaminated soil. Organic wastes can therefore serve as nutrient rich bio-stimulants that can improve the nutritional status of hydrocarbon contaminated soil and also enhance biological activities vital in nutrient transformation and recycling in soil. The organic wastes used in this study successfully ameliorated the negative effects of hydrocarbon on soil microbial communities and dynamics. It may, therefore, be appropriate to recommend their use as a suitable biostimulant during the remediation of agricultural land previously contaminated with hydrocarbon.

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