Effect of Different Organic Amendments on Fungi Degrading Hydrocarbons from an Artisanal Crude Oil Refining Polluted Soil in Ngia Kiri, Degema, Rivers State.

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Abstract
The pollution of soil with different types and classes of hydrocarbon resulting from the artisanal refining of crude oil has been the greatest cause of environmental challenges in the Niger Delta. Application of agricultural waste materials as a way of bio-stimulating the indigenous microbiota for the development of sustainable bioremediation process is important and timely. Artisanal crude oil polluted soil was obtained from Ngia Kiri, in Degema Local Government Area of Rivers State. The organic substrates used as a source of bio-stimulant includes: fish pond effluent, compost dump-site soil and spent mushroom substrate. Ten per cent (10%) of the substrate was applied to 900g of polluted soil in a microcosm experiment. Samples were taken from the experimental pots and subjected to both microbiological and physicochemical evaluations using standard methods at days 0, 14 and 28 respectively. Results obtained were subjected to ANOVA to see for a significant difference. Total heterotrophic fungal counts ranged from 9 x 10^4 – 7.0 x 10^5Cfu/g, hydrocarbon utilizing fungal counts ranged from 7.0 x 10^4 – 4.5 x 10^5cfu/g. The following fungal isolates were identified according to their morphology- Aspergillus flavus, Aspergillus fumigatus, Aspergillus lentulus, Penicillum sp, Fusarium sp, Candida sp, Cryptococcus sp, Rhodotorula rubra, Rhizopus sp, Mucor sp. Amendment using fish pond effluent had a nitrate concentration ranging from 4.03 - 6.38mg/kg which had the most significant bio-stimulatory effect on the soil microflora and biodegradation efficiency of 86.65% with a half-life of 9.64 days and a predictability coefficient of 95.13% alongside a prediction model of y=-0.0719x+10.14. Statistical analysis using ANOVA and biodegradation kinetics at p-value < 0.05 revealed that a significant difference existed in the nature and duration of the bio-stimulants applied. Hence, there is a need for increased and intense research into the commercialization of fishpond effluent and other agricultural materials as tools for recovery of impacted media and mitigation of environmental issues.

Keywords: *hydrocarbon, artisanal refinery, crude oil-polluted soil, spent mushroom, bio-stimulation.*

Introduction

The Niger Delta region is the hydrocarbon basket of Nigeria and appears to be sitting on an environmental time bomb due to degradation from widespread hydrocarbon pollution (Nwozor *et. al*., 2019). Starting from 1958 when Nigeria first started her large scale exploration of crude oil which has continuously grown to become the base of the nation's economy; this has ensued in multiple environmental pollution problems in the Niger Delta region (Okpokwasili *et al*., 2013; Douglas, 2018).The major sources of environmental degradation in the Niger Delta are oil spillage (both legal and illegal), gas flaring and

improper disposal of wastes from oil drilling operations. When crude oil is refined, diverse hydrocarbon fractions are produced which creates eco-toxicological effects on the environment when spilled. These effects ranges from; groundwater and soil contamination, changes in soil physicochemical characteristics, bioaccumulation and biomagnifications in environmental receptors, detrimental effects on microflora, decrease in biodiversity, alteration of habitat and cancer in humans (Obire and Anyanwu, 2009; Kalantary *et al*., 2014; Douglas and Cornelius, 2019). Toxicity of these products varies, which depends on the concentration, composition, the prevailing environmental conditions and the biological state of the organism when the pollution occurs (Obire and Anyanwu, 2009).

Many reclamation methods have been undertaken to clean up polluted soils. Both physical, chemical and biological approaches are being followed in the remediation of polluted soils (Okoh, 2006; Erdogan and Karaca, 2011). But, the physical and chemical methods are very cost intensive, laborious and sometimes, pollutants are not eliminated completely from the polluted site leading to unwanted ecological consequences (Adebusoye *et al*., 2007). Hence, biological method is preferred which depends on microbial enzymatic processes to help remove the pollutant from the site of pollution. (Philip et al., 2005). Bioremediation of hydrocarbon-contaminated soils, which uses the ability of microorganisms to degrade and detoxify organic contamination, has been proven as an efficient, economic, versatile, and environmentally sound treatment (Margesin and Schinner, 2001). Upon the spilling of crude oil and petroleum products into the soil ecosystem, the microbial community structure is altered and their diversity as a result of stress on the environment or alterations which gives rise to the production of dominant populations amongst the altered communities which can withstand such contamination with improved substrate utilization and physiological abilities (Atlas and Philip, 2005; Douglas and Cornelius, 2019).

Bioremediation is a means of cleaning up contaminated environments by utilizing the many metabolic capabilities of microorganisms to convert contaminants to harmless products through mineralization, production of carbon (IV) oxide and water, or by converting into microbial biomass (Baggott, 1993; Mentzer and Ebere, 1996). Microbe's bioremediate the environment by biodegrading and utilizing available pollutants to obtain carbon and energy (Dadrasnia *et al*., 2015). As bioremediation depends on microbial processes, there are two major approaches to speed up microbial activities in polluted sites, namely: biostimulation and bioaugmentation. Biostimulation involves the addition of nutrients or substrates to a polluted sample in order to stimulate the activities of autochthonous microbes. It is the most widely used bioremediation procedure whereby nutrients are added to the indigenous microorganisms as input of large quantities of carbon sources (i.e. the pollutant) tends to result in rapid breakdown of the available pools of major inorganic nutrients such as nitrogen and phosphorus (Morgan and Atlas, 1989). Previously, there has been researches on the use of different waste in the bioremediation of oil polluted sites such as; the use of cow dung and poultry droppings (Obire *et al*., 2008), sewage sludge and compost (Forjan *et al*., 2017), inorganic fertilizer (Chorom *et al*., 2010), use of sawdust as soil conditioner (Udotong *et al*., 2011) use of spent mushrooms compost (Stanley *et al*., 2018) and carrot peel waste (Hamoudi-Belarbi *et al*., 2018). But no known work has been done using fish pond effluent and old compost dumpsite soil on the bioremediation of crude oil polluted soil.

In one gram of a standard garden soil, there are billions to hundreds of billions of soil microorganisms ranging from thousands of different species of bacteria to hundreds of different species of fungi and protozoa, lots of different species of nematodes and other micro arthropods (Kolwzan *et al*., 2006; Douglas, 2018). Many of these numerous soil

microorganisms are very beneficial and essential to the life giving properties of the soil. Fungi are eukaryotic, heterotrophic, aerobic, non-chlorophyll containing organisms, which use carbon and energy to build their own cell from decomposition of organic matter (Douglas, 2018). Fungi produce and secrete higher rates of different extracellular enzymes into their peripheral environment and degrade various substrates to small molecules that can be absorbed by and metabolized in their cells (Levin *et al*., 2003).). Fungal growth rate is also reasonably fast enough for applications in bioremediation processes. The aim of this research is to evaluate the effect of three organic amendments on the degradation of hydrocarbons via fungal capability to utilize the pollutant.

Materials and Methods

Collection of the crude oil polluted soil

The crude oil polluted soil sample used for this research was collected from an island in Niger Delta (Ngia Ama) along Sombrero River. Ngia Ama is situated at Tombia (40 78'60" N, 60 89' 24" E) in Degema Local Government Area of Rivers State where illegal refining of crude oil took place.

Sample collection

The polluted soil sample was collected in composite points with the aid of a sterile hand auger, taken at 0- 30 cm depth and put in a sterile polythene bag which was taken to the laboratory immediately.

Sample Preparation

The polluted soil sample collected was processed removing all foreign matters. Five grams was removed to be used for baseline physicochemical and microbiological studies (Chikere *et al*., 2018). The remaining polluted soil sample was removed of all non- soil particles and kept in a clean bag at room temperature to be used later. The amendments (spent mushroom substrate, fish pond effluent and composite soil) were filtered using a two-millimeter (2mm) pore size sieve (Amechi, *et al*., 2016), the fish pond effluent was dried in the sun for three (3) days while the others were air dried in the laboratory.

The polluted soil sample was weighed and set up into duplicates making it a total of 8 setup stands. One thousand grams (1000g) each of the soil was placed into 2 sterile pots, this was labeled as the control. Then into each of the pots marked for the 3 amendments (FPE, SMS and CS), 900g of polluted soil each was placed in all the pots and individually,100g of each amendment was added into the appropriate pot and mixed thoroughly. Twenty millimeters (20ml) of sterile distilled water was added into each of the setups, tilled properly to generate moisture and oxygen for the microrganisms; this continued at 2 days interval throughout the 28 monitoring period. This was in accordance with (Obire and Anyanwu, 2009; Douglas, 2018) with slight modification.

Physicochemical analysis

Total petroleum hydrocarbon content was analyzed using Gas Chromatograph - Flame Ionization Detector (GC-FID) instrument. The pH was checked using a calibrated pH meter (pH tester 20 model). Total organic carbon was determined based on the method used by Singh *et al*. (1999), percentage potassium, percentage nitrate and percentage phosphate was determined following the guidelines from APHA (1998).

Microbiological Analysis

Isolation and enumeration of Total heterotrophic fungi and hydrocarbon utilizing fungi

One gram (1g) of soil sample was taken from each setup and aseptically suspended in 9.0ml of sterile normal saline. This was serially diluted into 10^{-4}, using a sterile pipette 0.1ml of 10^{-3} and 10^{-4} (from all treatment setups) dilutions were spread on sterile prepared Sabouraud

Dextrose agar in duplicates and incubated at room temperature for 72h (Agamuthu *et. al*., 2013). Afterwards, counts were recorded and used to calculate the colony forming unit then isolated colonies were sub cultured on sterile Sabouraud Dextrose agar (SDA) supplemented with amoxicillin for pure colonies (Douglas, 2018).

Oil in molten agar using mineral salt medium composition of Mills et al., 1978 as modified by Okpokwasili and Okorie, 1988 was used (Douglas, 2018). This was supplemented with 0.05g/ml of amoxicillin and 1% Bonny light crude oil (Stanley *et al*., 2015). Aliquots of 0.1ml from 10^{-3} and 10^{-4} dilutions were plated out in duplicates. The plates were incubated at room temperature for 7 days. Colonies grown were counted and subcultured on sterile SDA supplemented with amoxicillin so as to get pure isolates.

The fungal characterization and identification were done macroscopically by observing the morphology of the pure isolates and microscopically by covering smear of a pure isolate on a clean glass slide with Lactophenol blue then viewing under a light microscope using x10 and x40 objective lens (Cheesebrough, 2000; Douglas, 2018).

Result and Discussion

The polluted soil was analyzed first to ascertain the level of petroleum hydrocarbon pollution before biostimulation with the organic amendments (fish pond effluent, spent mushroom substrate and compost soil from old dumpsite) were initiated; the physicochemical parameters and fungal counts were checked to know if the soil still supported growth of microorganisms after the pollution occurred. The amount of Total petroleum hydrocarbon (TPH) analyzed was 25,350.2mg/kg which was far greater than the intervention limit of 5000mg/kg set by DPR (Akuro, 2012) hence, there was need for bioremediation. Table 1 below shows the baseline characteristics of the soil and the organic amendments:

Table 1: Baseline characteristics of polluted soil sample, Fishpond waste, spent mushroom substrate and Compost

Physiochemical Parameters	Units	Polluted soil	Fishpond effluent (FPE)	Spent mushroom substrate (SMS)	Compost soil (CS)
Texture	-	Smooth	Smooth	Rough	Grainy
Colour	-	Dark brown	Greenish	Light brown	Black
pH	-	6.18	6.33	6.20	5.96
Nitrate	Mg/kg	4.49	8.3	4.10	4.34
Phosphate	Mg/kg	37.56	31.4	31.3	30.7
Total organic carbon	%	7.58	8.15	7.93	7.86
Total petroleum hydrocarbon	Mg/kg	25350.2	ND	ND	ND
Total fungal count	Cfu/g	$9.0x10^4$	$1.4x10^5$	$1.2x10^5$	$7.0x10^4$
Hydrocarbon utilizing fungi	Cfu/g	$3.0x10^4$	ND	ND	ND

Isolation and enumeration of THF and HUF

Results of the THF and HUF counts carried out were used to access the population/ the species of fungi present and also monitor the rate at which these fungi are bioremediating the polluted soil within the period of 28 days in a laboratory condition. The presence of fungal activities were confirmed by quantification and pure isolates of total heterotrophic fungi (THF) and hydrocarbon utilizing fungi (HUF) counts in each setup from setup 1 (control),

setup 2 (PS + FPE), setup 3 (PS + SMS) and setup 4 (PS + CS) over the 28 days bioremediation period. Figures1and 2 shows the THF and HUF counts over the 28 days period. The total heterotrophic fungal counts ranged from 9.0 x10^4 CFU/g to 7.0 x10^5 CFU/g and hydrocarbon utilizing fungi ranged from7.0 x 10^4 CFU/g to 5.1 x 10^5 CFU/g across the treatment setups. In fig 1 at day 0 to day 7, there was rapid increase in fungal population in FPE and SMS treatment setups but growth reduced drastically between days 14 to 28 in the SMS; FPE treatment setup encountered reduction between days 14 and 21 but increased again at day 28. The CS treatment setup had reduction in growth between days 7 to 14 with slight increase afterwards; control setup had little increase in population between days 7 to 14 but decreased further at towards the end of the bioremediating days. The FPE treatment setup supported the growth of heterotrophic and hydrocarbon utilizing fungi more than any other treatment setup, this was followed by SMS treatment setup with an initial increase and this corresponds with the work done by Udotong *et al*. (2011) where they encountered an initial increase in microbial growth in their treatment with sawdust. In fig 2, setup amended with FPE had the highest growth of hydrocarbon utilizing fungi followed by that amended with SMS then CS amended setup. The control which had no amendment had the least growth though it had slight increase in population between days 0 to 21 but reduced towards day 28.
Some fungal species that were identified in the polluted soil alone (control setup) from the baseline up till day 0 to day 14 were *Aspergillus flavus, Aspergillus lentulus, Penicillium* sp and *Mucor* sp; from day21 to day28, there was no more growth of *Mucor* sp. but there was presence of a yeast *Candida* sp. Treatment with the different organic amendments gave rise to nine (9) fungal species of which seven were hydrocarbon utilizers namely; *Penicillium* sp., *Aspergillus fumigatus*, *Aspergillus lentulus*, *Aspergillus flavus*, *Fusarium* sp., *Rhizopus* sp., *Mucor* sp., *Cryptococcus* sp. and *Rhodotorula* sp.

Polluted soil samples from each treatment setups (control, FPE, SMS, CS) were subjected to Gas Chromatograph - Flame Ionization Detector (GC-FID) instrument to monitor the level of hydrocarbon degradation in all the bioremediation monitoring period. The level of hydrocarbons in the polluted soil in each microcosm setup reduced drastically over the monitoring days as seen in fig 3 showing the biodegradation potential and half-life of TPH. The TPH (comprising of C_8 -C_{35}) at the beginning of the bioremediation (day 0) was 25350.24mg/kg but this was reduced after 28 days to 23813.44mg/kg for control, 6203.11mg/kg for CS, 2972.74mg/kg for FPE and 4532.34mg/kg for SMS.

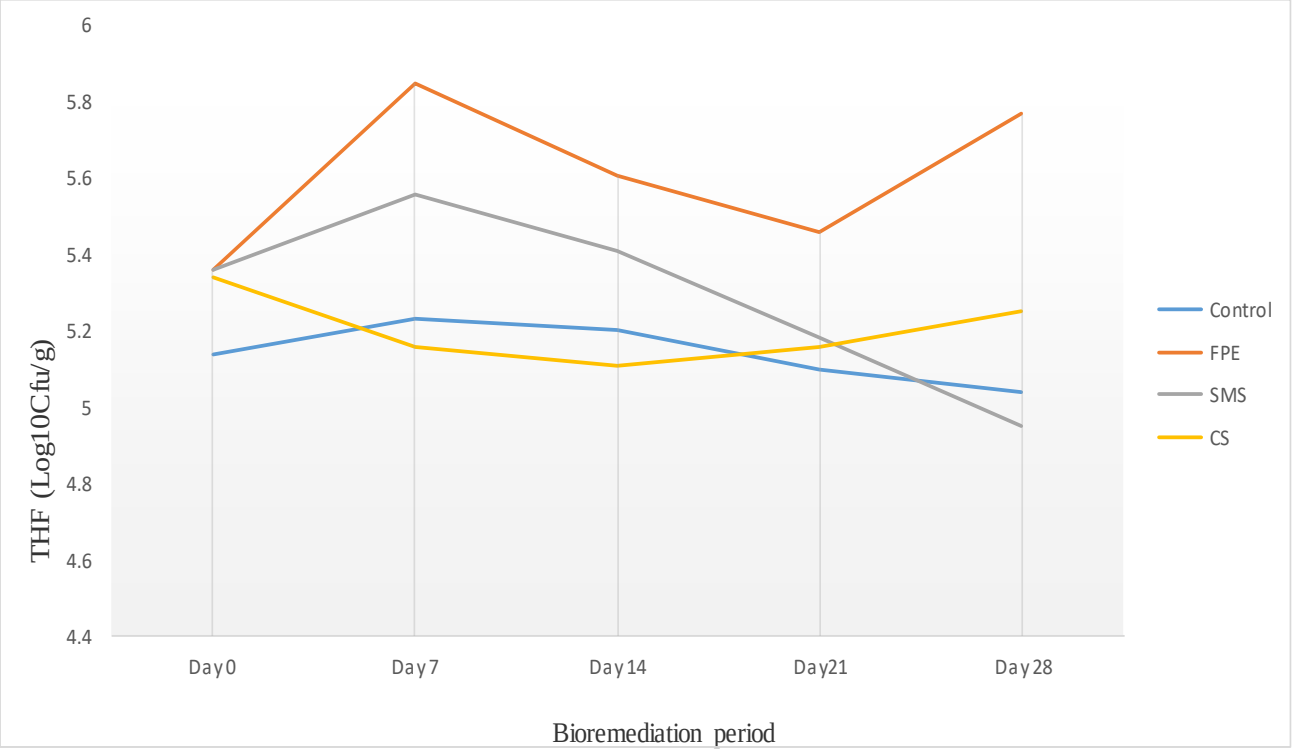

Fig 1: Growth response of Total Heterotrophic Fungal Count over the 28 days monitoring period

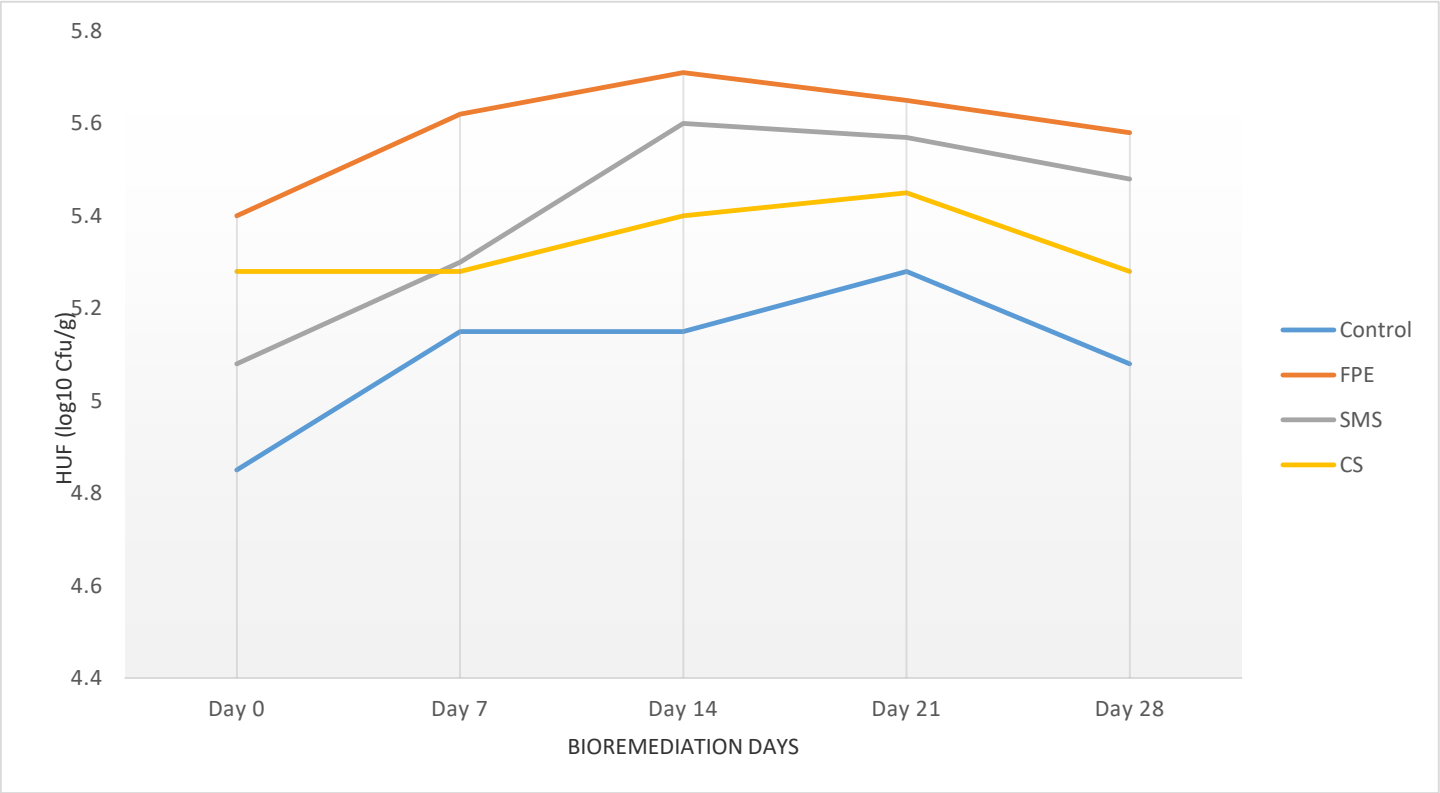

Fig 2: Growth response of Hydrocarbon Utilizing Fungal Count over the 28 days monitoring period

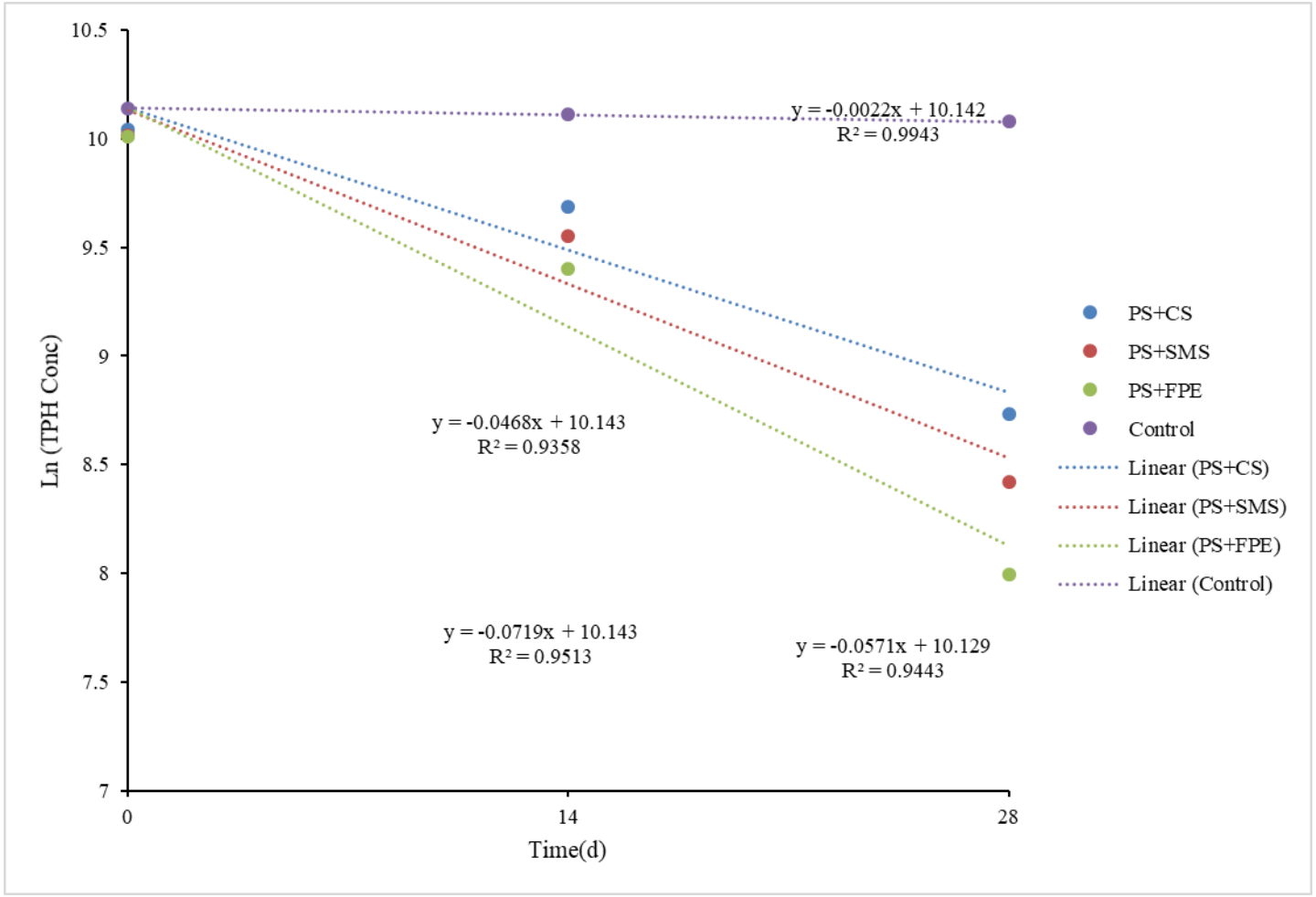

Fig 3: Rate of TPH degradation in all setups for day 0- 28

From the results above, the soil physicochemical characteristics varied and were sufficient to allow the adaptability of fungi though the high concentration of the petroleum hydrocarbon pollutants led to reduction in the fungal diversity. The TPH concentration were far above the DPR standard which is 5000mg/kg (Akuro, 2012) so there's need for intervention as these hydrocarbons are recalcitrant in nature and chemically toxicogenic, hence needs to be eliminated or reduced to harmless level to resuscitate stressed ecosystem. The polluted soil had a low acidic pH of 6.18 (close to neutral) which was optimal for fungal growth and metabolism (Chikere *et al*., 2016), the light carbon chain of petroleum hydrocarbons present in the polluted soil sample may have been the reason for the moderate counts of total culturable heterotrophic fungal counts (10^4 CFU/g) resulting from reduced toxic effect on the adapted fungal specie. The fungal species isolated from the polluted soil prior to amendment showed the presence of three species namely; *Aspergillus* sp, *Penicillium* sp and *Mucor* sp. After biostimulating with the each organic amendment, the diversity and population of fungal species increased rapidly especially in amendment with FPE resulting in accelerated increase in rate of biodegradation of the hydrocarbons more than the control which had no amendment. The presence of mycofloras present in the organic amendments enhanced by high levels of the required macro nutrient (NPK) resulted in the desired degradation of the pollutants. The nine (9) fungal specie identified which included *Penicillium* sp., *Aspergillus fumigatus*, *Aspergillus lentulus*, *Aspergillus flavus*, *Fusarium* sp., *Rhizopus* sp., *Mucor* sp., *Cryptococcus* sp. and *Rhodotorula* sp. corresponds with a research carried out by Douglas and Green (2015) & Douglas (2018) where presence of different fungal species were evaluated in soil polluted with crude oil residue such *as Aspergillus* sp. (*A. niger* and *flavus* by Douglas, 2018), *Penicillium* sp., *Fusarium* sp., *Rhizopus* sp., *Cladosporilum* sp., *Candida*, *Mucor*, *Saccharomyces* sp. and *Geotrichum* but in this research, *Aspergillus niger*, *Cladosporilum*, *Saccharomyces* and *Geotrichum* were not isolated. Also more findings by Obire *et al*. (2008) also revealed the activities of most of these fungal genera in the bioremediation of hydrocarbons, these includes; *Aspergillus* sp, *Fusarium*, *Geotrichum*, *Cephalosporium*, *Penicillium*, *Trichoderma* and some of which corresponds with the isolates identified in this study. Complex mixtures of crude oil constituents are found in the petroleum hydrocarbon pollutants and microbial degradation varies in their susceptibility to each constituents (Obire *et al*., 2008). They also reported that naturally occurring heterogeneous populations degrade hydrocarbons better than individual isolates from the multiple populations. This was evident in the effect of each biostimulating agent in the speed and duration of bioremediation whereby FPE treatment setup had a degradative efficiency of 86.7% and half-life of 9.6days implying it a very high rate of degradation with the shortest possible time to degrade half the quantity of total petroleum hydrocarbons present. This was followed by SMS treatment setup with a degradative efficiency of 79.8% and a half-life of 12.2 days; then CS treatment setup with degradative efficiency of 73% and half-life of 14.8days. The control which was non-amended (NA) had the least TPH degradative efficiency of 6.1% and a longer half-life of 315.7 days. This indicates that degradation occurred slowly even in the absence of any stimulant showing that the indigenous microbial population present had developed the capability to breakdown these hydrocarbons though it would take many days to degrade half the quantity of hydrocarbons present naturally. All counts in the 4 microcosm setups were statistically significant ($p<0.05$) implying each treatment could also be effective for degradation of hydrocarbons. Although the control (natural attenuation) will take longest to remove or reduce the hydrocarbons to an acceptable limit as the mycoflora surviving the effects of the pollution adapts and utilize the hydrocarbons as their main source of carbon based on their metabolic pathways (Eman and Andrew, 2017).

Conclusion

The effects of crude oil pollutions on the environment are very detrimental and require immediate attention and action. Since fungi are distributed everywhere in the environment, they are readily isolated from various wastes, soil and crude oil polluted soils. Crude oil spillage in this area as a result of artisanal refining resulted in severe hydrocarbon pollution leading to reduced fungal diversity and population in the polluted soil; nevertheless, biostimulation with different wastes increased fungal diversity and population hence increased biodegradation rate. Different organic wastes used as biostimulating agents had significant biostimulatory effects on the TPH degradation especially with FPE having the greatest effect on the pollutant as when compared to the unamended (control) setup. Therefore, I recommend that fish pond effluent should be investigated further and considered for use in bioremediation of crude oil polluted environment.

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