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The Ex-Situ Bioremediation Kinetics of Raw and Treated Crude Oil Polluted Soil Using *Aspergillus Niger* and *Pseudomonas Aeruginosa*

M. E. Ojewumi [1*], V. A. Ejemen [2]

[1,2]Chemical Engineering Department, Covenant University, Ogun state, Nigeria,

**For Correspondence: Email address: modupe.ojewumi@covenantuniversity.edu.ng*

[1*]Orcid: 0000-0002-9254-2450

Abstract: The study was done to investigate the kinetics of first order bioremediation. The effectiveness of remediating soils polluted with raw crude oil and treated crude oil using *Aspergillus niger* (fungi) and *Pseudomonas aeruginosa* (bacteria) were investigated. Eight systems of 500g soil sample were polluted with both raw and treated crude oil. Four systems were polluted with 40g treated crude oil while the other remaining four systems were polluted with 40g raw crude oil. Two systems with raw crude and treated crude were left as control (RCC and TCC). Raw crude samples were treated with *Aspergillus niger* only (RCA) and *Pseudomonas aeruginosa* (RCP) while treated crude samples were also treated with *same* (TCA) and (TCP) only. The last two systems were treated with both *Pseudomonas aeruginosa and Aspergillus niger* (RCAP and TCAP). The first order bioremediation kinetics and biostimulant efficiency for these systems were studied by monitoring Total Petroleum Hydrocarbon (TPH). At the end of the bioremediation period, the results obtained showed that treated crude oil polluted soil generally remediated faster and better than raw crude oil polluted soil. The highest level of bioremediation occurred in systems amended with both *Pseudomonas aeruginosa* and *Aspergillus niger* which had about 98% TPH decrease.

Keywords: bioremediation; Ex-situ; *Aspergillus niger; Pseudomonas aeruginosa;* Total Petroleum Hydrocarbon; crude oil; biostimulant efficiency; Kinetics.

1. Introduction

Since the nineteenth century, petroleum has been utilized for a very long time for power generation and lubrication. The innovation of the motor engine and its quick appropriation in all vehicle structures broadened the occupation of this natural resource. This caused the expansion of the petroleum business. One of the major concerns of oil industry today is how to improve the recovery of large percentage of oil remaining unrecovered in the old and new depleted producing fields (Ojewumi *et al*., 2017a; 2018a;2018b) The processes involved in obtaining the crude oil and converting it into more useful products usually causes contamination problems. These pollution problems can be minimized, yet not completely eliminated and hence brings on several issues for the environment (Pala *et al*., 2006; Ojewumi *et al*., 2018c). The famous bioremediation is an amazing spill clean-up technique in which the normal degrading ability of microorganisms is

harnessed for the degradation and decrease of the harmful substances that pollute the environment. Some substances in this category include petroleum subsidiaries, aliphatic and sweet-smelling hydrocarbons, mechanical solvents, pesticides and metals (Korda *et al*., 1997;Ojewumi et al., 2018d). Of all the technologies and methods that have been researched into in the recent past for cleaning up oil spills on soils, bioremediation has come out as the most desirable approach due to its low cost and ability to hinder the formation and accumulation of contaminants (El-Nawawy *et al.,* 1992). Bioremediation is a term used to describe a process that takes advantage of the natural ability of microorganism to degrade toxic waste. This technique is very effective in cleaning up petroleum hydrocarbon pollution. It is a modern technique whereby the natural degrading ability of microorganisms is used to reduce the concentration and/or toxicity of wide range chemical substances that are released into the environment. The remediation process in question works by stimulating the microbes that occur naturally in the environment to degrade organic wastes found in soil and groundwater. The microorganisms that degrade crude oil use the hydrocarbon breakdown as their source of chemical energy. Some microorganisms can naturally degrade petroleum hydrocarbons by utilizing the carbon within it to survive. The hydrocarbons that exist in crude oil serve as substrates for the microorganisms. Normally without any external enhancement or intervention, as soon as an oil spill occurs there is a rise in the population of microbes that degrade hydrocarbons within the ecosystem.

Aspergillus niger as a crude oil degrading fungi is a haploid fungi that is filamentous in nature. It is an important microorganism in the field of biotechnology. *Aspergillus niger* has found wide application in waste management and bio-transformations. The fungi is most commonly found in mesophilic environments such as decaying vegetation or soil and plants (Schuster et al., 2002). Filamentous fungi play an important role in degrading hydrocarbons by producing capable enzymes. This is as a result of their aggressive growth, greater biomass production and extensive hyphal growth in soil. Fungi offer potential for biodegradation technology (Kenneth, 1995;Ojewumi *et al*., 2018e). The fungi causes a disease called black mould on some fruits and vegetables like grapes, apricots and peanuts (Samson et al., 2001).

Pseudomonas aeruginosa as crude oil degrading bacteria is a gram-negative, rod-shaped bacterium that has an incredible nutritional versatility. *P. aeruginosa*, as well as many other *Pseudomonas*, can degrade aromatic hydrocarbons and can break down toluene, the simplest form of methylbenzene. The bacteria degrade toluene through the oxidation of the methyl group to aldehyde, alcohol, and an acid, which is then converted to catechol. Hence, *P. aeruginosa* can be used in pollution control (Johnson & Olsen, 1997). Naturally, it is found in environments such as soil, water, humans, animals, plants, sewage, and hospitals (Lederberg, 2000)**.**

The bioremediation kinetics seeks to investigate how different experimental conditions can influence the speed of the microbial degradation of the crude oil as well as the construction of mathematical models that can describe the characteristics of the process. Bioremediation kinetics is very important in microbial biodegradation studies. With this, it is possible to know and

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understand the kinetics of soil bioremediation and also to determine the quantity of crude oil left at any given time (Agarry *et al*., 2013; Agarry, 2013).

An oil spill is the discharge of a liquid petroleum hydrocarbon into the environment which may be marine areas or land. This happens as a result of various human activities and it culminates into a serious form of pollution. Oil spills may be caused by the release of crude oil from tankers, offshore platforms, drilling rigs and wells heads. Spills could also be caused by refined petroleum products (such as gasoline, diesel) and their by-products.

Lucas and Macgregor (2006) reported that pollution caused by crude oil spill happens as a result of land runoff, accidents that occur involving pipelines and oil carrying vessels, oil exploration and production engineering operations, oil shipping activities and improper effluent discharge into the environment. These spills have negative and disastrous economic, environmental, and social effects on the society. As a result, oil spill accidents have created some media interest and have brought many together in a battle regarding the response of the government to oil spill incidents and the actions that are being taken to curb the menace (Broekema, 2015)**.** The type of oil that spills, the quantity or severity of the oil spill, the environment in which the spill occurs and the prevailing weather conditions are factors that must be considered before choosing the most effective technique to clean up the spill (Choi & Cloud 1992).

Raw crude oil is basically crude coming directly from the depths of the earth through the wellhead. It may or may not have some amount of water and dissolved natural gas within it. Treated crude oil in this case is not necessarily crude oil that has gone through the refining process. Treated crude refers to crude oil that has been passed through an oil production platform. These platforms are located in central locations of major oil producing fields. Raw crude from the different wellheads is channelled to the platform. Here, the crude is stripped off dissolved natural gas, excess water which forms emulsion with the crude and condensates. Raw crude also comes with a high level of sand and this is also removed.

Before the knowledge of bioremediation came into limelight, there had been the existence of some crude oil spill clean-up techniques some of which are in use till this day. According to (Larson, 2010), these remediation techniques are:

1. The physical remediation methods
2. The chemical remediation methods
3. The thermal remediation methods

A lot of microorganisms naturally have the enzymatic or catalytic ability to degrade or simplify petroleum hydrocarbons. Some of the microbes are specific in their action. Alkanes may be degraded by certain types of micro-organisms. Other micro-organisms may degrade only aromatic compounds. It has been experimentally proven that alkane compounds that range from C_{10} to C_{26} are the most easily biodegradable. Very toxic aromatic compounds like benzene, toluene and are

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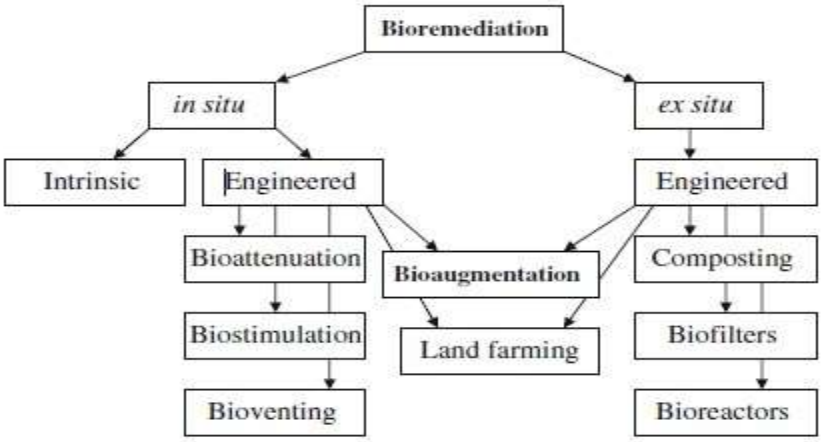
also easily biodegraded by microorganisms. More complex structures are very resistant to biodegradation. There are only few microbes that can degrade these compounds and the biodegradation rate is much lower than in pure alkanes. (Van Hamme et al., 2003). A mixed microbial population may guarantee a higher level of biodegradation. The speed and efficiency of the clean-up of a soil contaminated with petroleum and petroleum products largely depends on the presence hydrocarbon-degrading microorganisms in the soil. The factors that are necessary for population microbial growth are temperature, oxygen, pH, content of nitrogen and phosphorus. The degree and rate of biodegradation are influenced by the type of soil in which the process occurs (Rittmann & McCarty, 2001).

The main microorganisms that degrade petroleum hydrocarbons are bacteria and fungi. Using microorganisms for hydrocarbon degradation has proven to be the most environmentally friendly oil spill clean-up method (Facundo et al., 2001; Robert & Stephen, 2003). Fungi offer potential for biodegradation technology (Kenneth, 1995).

Figure 1 shows the schematic diagram for the general overview of Bioremediation process.



Bioremediation Overview (Boopathy, 2000)

3. Materials and method

3.1. Materials

Loamy soil samples used to simulate the crude oil onshore spill was obtained from Covenant University Farms, Canaan land Ota. The raw and treated crude oil samples (Escravos light) used for this study were obtained from Chevron Nigeria Limited, Delta state Nigeria.

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3.1.1. Preparation of Microorganisms

Aspergillus niger and *Pseudomonas aeruginosa* (environmental isolates), were sourced from the Applied Biology and Biotechnology Unit of the Biological Sciences, Covenant University, Ota, Ogun State, Nigeria. Both isolates are stored on Sabourad Dextrose Agar (SDA) slant. Isolates were sub-cultured on SDA sterile plate and incubated at 27 ºC for 3 to 5days. Each isolate was adjusted to 0.5 McFarland and used for the remediation. Method of Ojewumi et al., 2016a,b,c; 2018e,f,g,h were all used.

3.2. Soil Treatment

The soil used was collected from the surface layer, about 20 to 30cm below land surface. The soil sample was air dried to get rid of the excess moisture, as soil was collected during the heavy rains. The soil was homogenized and stored in a black plastic bucket at room temperature. The crude oil spill/pollution was artificially simulated in the laboratory. Before the pollution, 1kg of the soil was collected and analysed for all the parameters that were used to monitor the level of bioremediation of the polluted soil. To simulate an ideal pollution for this study, 8 transparent buckets were filled with 500g of the loamy soil. Each soil filled bucket was referred to as a system. These buckets were then randomly perforated in different areas to create needle like holes through which the soil particles would not pass through but air could pass through. This was done to ensure proper aeration of each system. Four out of the eight systems were polluted with 40g raw crude to achieve 8% w/w pollution. Four other systems were polluted with 40g treated crude. The *Aspergillus niger and the Pseudomonas aeruginosa* were respectively inoculated into the eight systems. The systems inoculated with microorganisms were replenished with nutrients once every three days to ensure the continuous survival. Samples were withdrawn from each system at intervals of 5 days or sometimes 10 days for various tests to be run.

3.3. Sample preparation

All the samples except the two control samples were inoculated. For all samples polluted with crude to be treated with *Aspergillus niger* only, (RCA and TCA), 200 ml sterile Yeast Broth was inoculated with 4 ml of the *Aspergillus niger* suspension and this was then used to dose the respective soil systems. The same procedure was repeated with the soil systems treated with *Pseudomonas aeruginosa* (RCP and TCP). In this case, 4 ml of the *Pseudomonas aeruginosa* suspension was used to inoculate 200 ml of Mueller Hinton Broth. The procedure was a little different for systems treated with both *Aspergillus niger* and *Pseudomonas aeruginosa*. In this case, for the systems polluted with crude (RCAP and TCAP), 100 ml of Mueller Hinton Broth and 100 ml of Yeast Broth were mixed in a sterile beaker and inoculated with 2 ml of *Aspergillus niger* suspension and 2 ml of *Pseudomonas aeruginosa* suspension and used to dose the respective systems. Samples were taken from all systems for testing before inoculation and the inoculated systems were left for five days before the next batch of samples were withdrawn for testing.

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3.4. Determination of Total Hydrocarbon Content [THC]

To determine the Total Hydrocarbon Content, a Jenway 6405 UV/VIS Spectrophotometer was used to read off the absorbance of the samples. 1g of soil sample from each system was dissolved in 10ml of hexane and shaken with a magnetic stirrer which was used to ensure the proper mixing and extraction of crude in hexane for 30 minutes. 1ml of this extract was measured and made up to 10ml with n-hexane and its absorbance was determined using spectrophotometer at 420nm wavelength for crude oil. The wave length was chosen after screening of several dilutions of crude oil in the spectrophotometer. Soil samples from each of the 16 systems were tested by the same way mentioned above at 0 days, 5 days, 10 days, 15 days, 20 days and 30 days period. Absorbance was converted to concentration by comparing it with standard calibration curve of hydrocarbon in hexane chart. The standard calibration curve was plotted by obtaining the absorbance of different concentrations of crude oil in hexane in mg/ml converted to mg/kg of soil. To determine the TPH at any point, the formula below was used.

$$\frac{TPHo - TPHt}{TPHo} * 100$$

Where:

TPH_o = Total Petroleum Hydrocarbon at day 0 [=] mg/kg

TPH_t = Total Petroleum Hydrocarbon at any day t [=] mg/kg

4. Results and discussion

4.1. Total Hydrocarbon Content analysis

Figures 2 – 9 shows plots for the Total Petroleum Hydrocarbon [TPH] and the natural logarithm for each system over the course of the first 30 days.

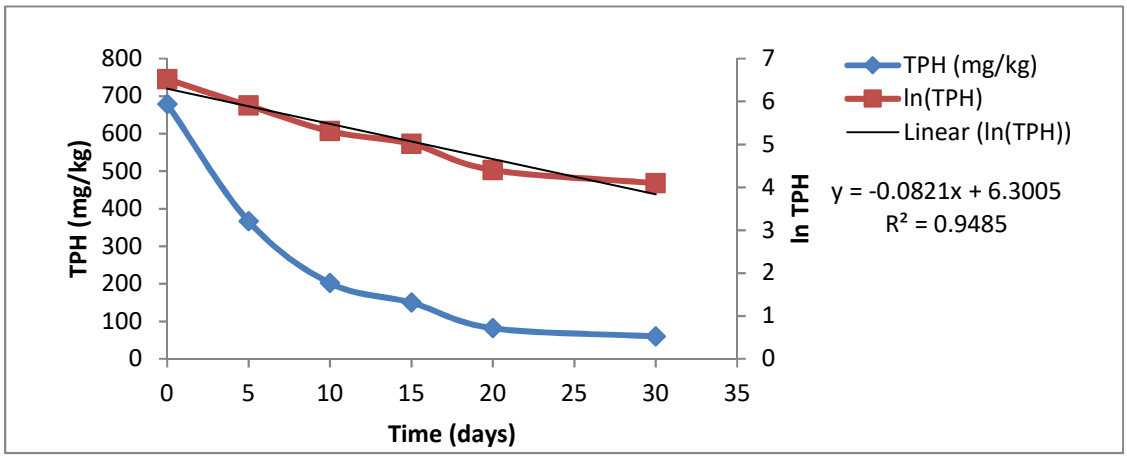

Fig. 2 TPH and LN (TPH) for RCA

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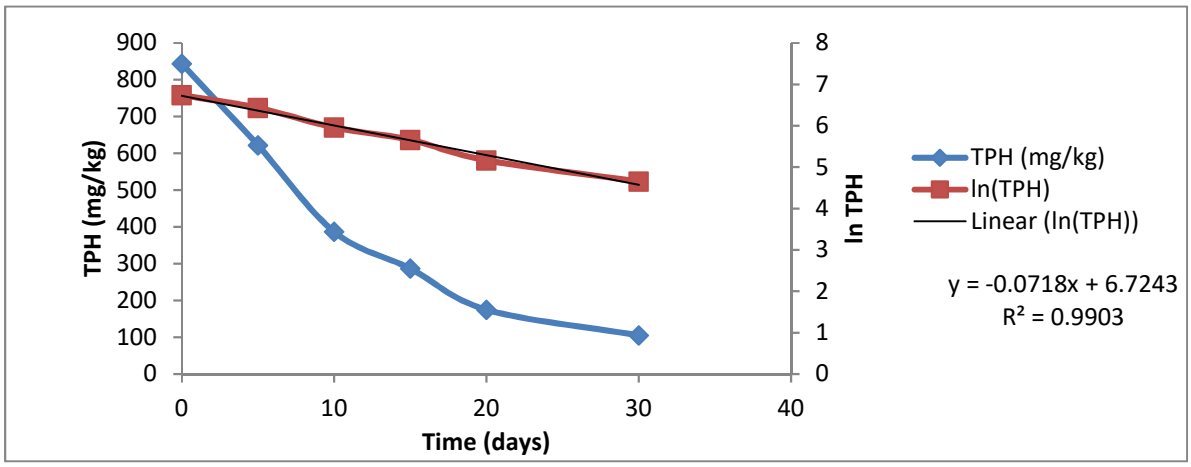

Fig. 3 TPH and LN (TPH) for TCA

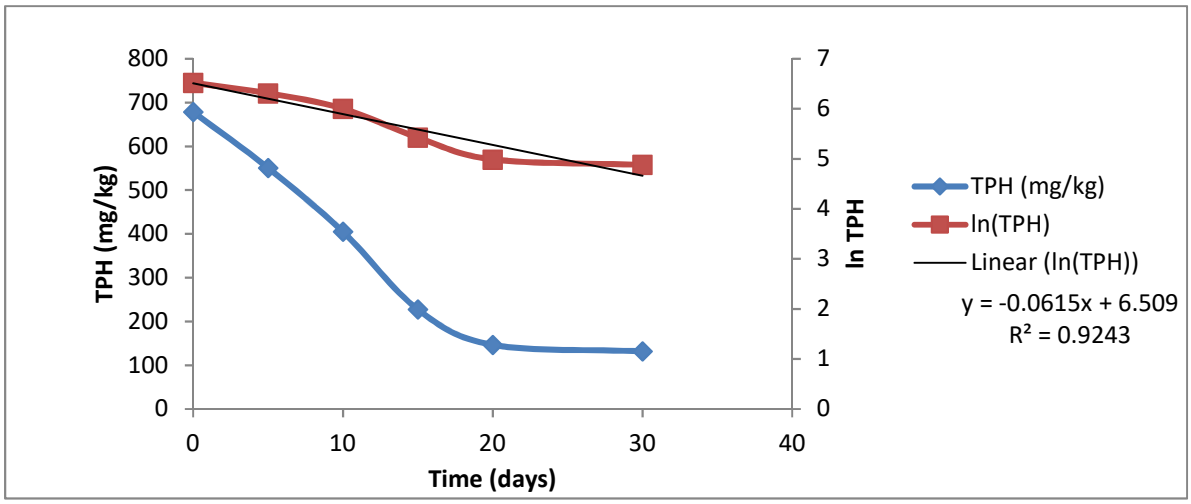

Fig. 4 TPH and LN (TPH) for RCP

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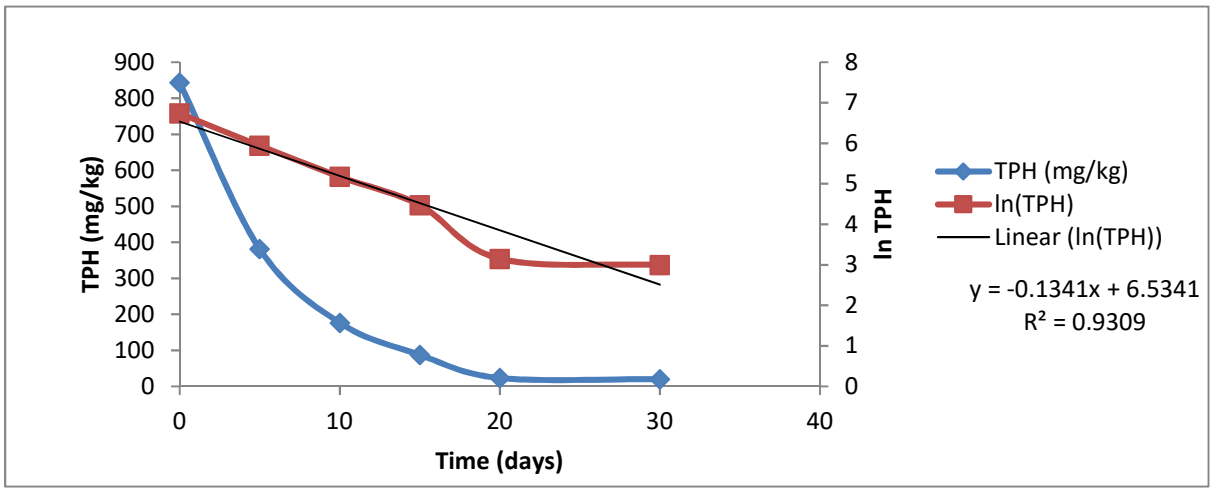

Fig. 5 TPH and LN (TPH) for TCP

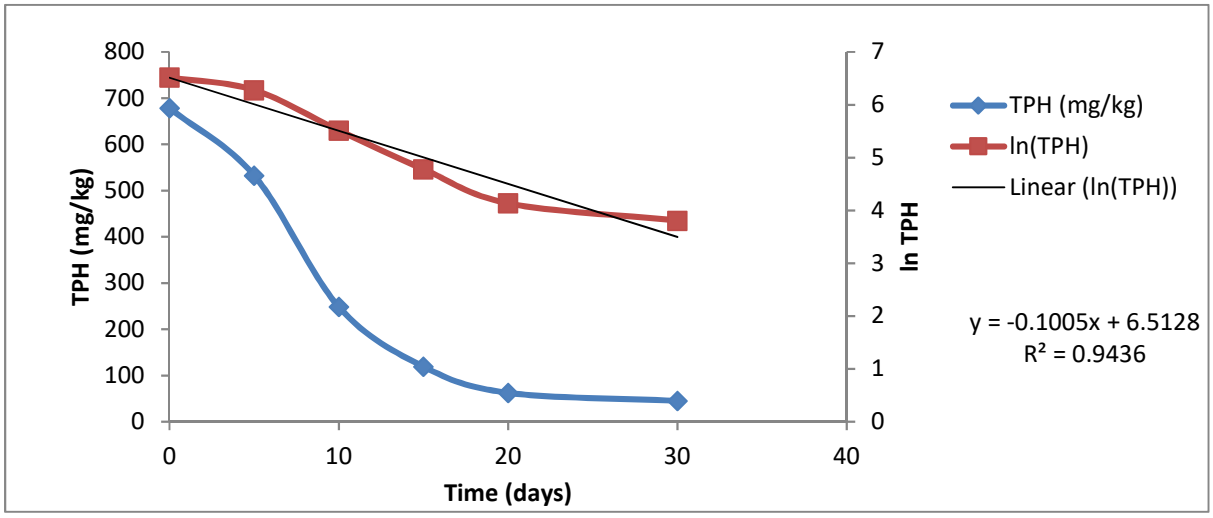

Fig. 6 TPH and LN (TPH) for RCAP

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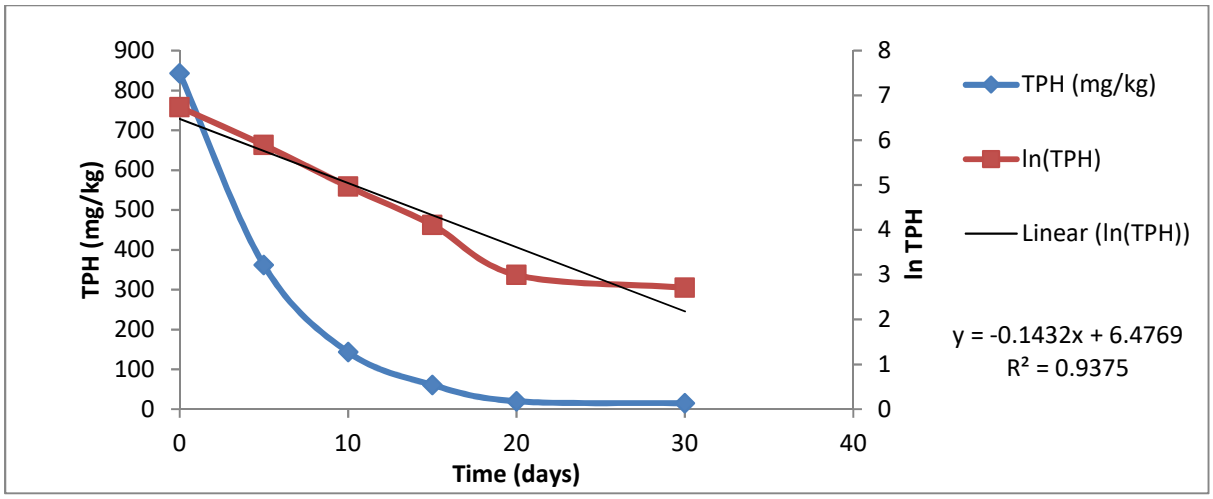

Fig. 7 TPH and LN (TPH) for TCAP

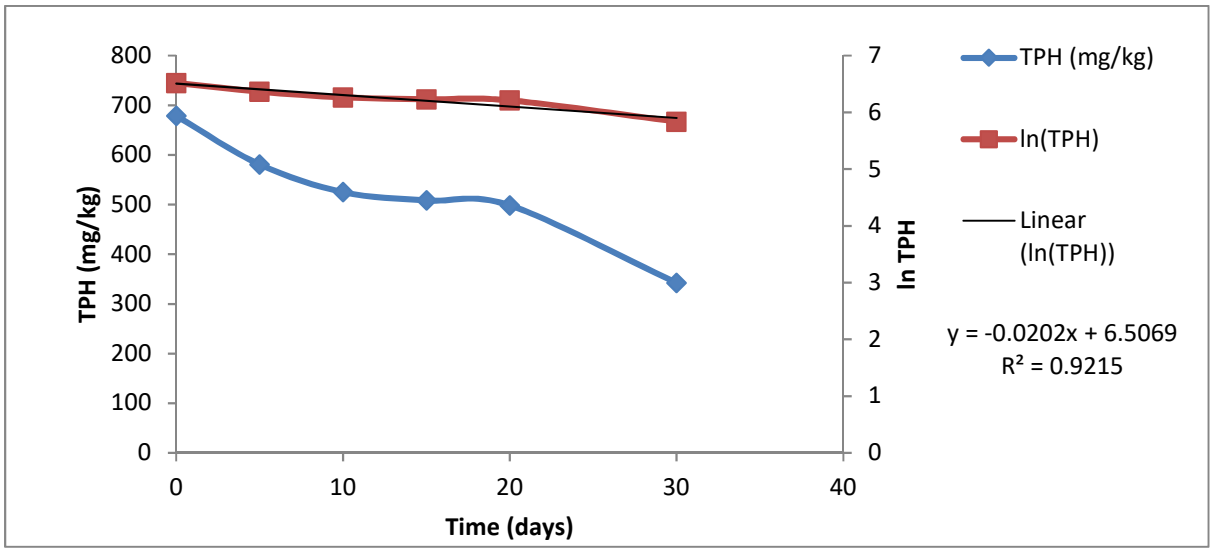

Fig. 8 TPH and LN (TPH) for RCC

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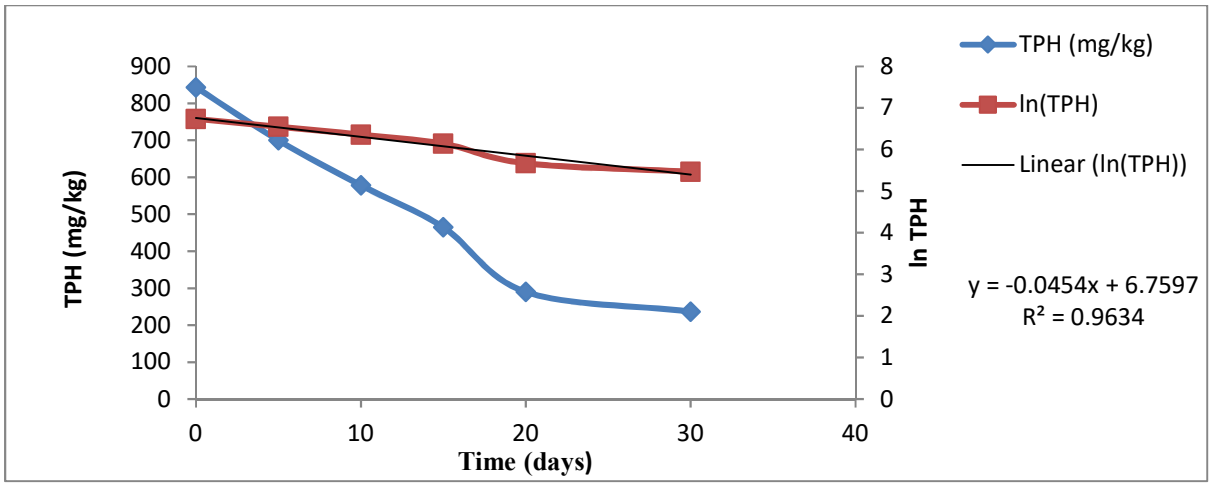

Fig. 9 TPH and LN (TPH) for TCC

4.2. First order bioremediation kinetics

The first order Bioremediation kinetics for this experiment was computed using the data obtained from the Total Petroleum Hydrocarbon computation. The kinetics was used to averagely compute the half-life of degradation of crude in each system. The computation was done using the model postulated by (Mohajeri *et al*., 2010; Agarry *et al*., 2013). The plots above (figures 2-9) shows the decreasing trend in the TPH of each system and also show how the first order kinetics was computed. The slope of each of the LN (TPH) curves gives the first order bioremediation rate constant for each system. It is this rate constant that is used to compute the half-life. The first order plots are made by plotting LN (TPH) against time (t). This is based on the computation below:

$$TPH_f = TPH_i e^{-kt}$$

$$\frac{TPHf}{TPHi} = e^{-kt}$$

$$ln\frac{TPHf}{TPHi} = -kt$$

$$\ln (TPH_f) = -kt + \ln (TPH_i)$$

Where

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TPH_i = Total Petroleum Hydrocarbon at day 0 [=] mg/kg

TPH_f = Total Petroleum Hydrocarbon at any day t [=] mg/kg

k = biodegradation rate constant [=] day^{-1}

The values of the half-life of each system were obtained from the biodegradation rate constant k.

$$\text{Half-life, } t_{½} = \frac{0.693}{k}$$

Table 1 shows the result obtained from the kinetics analysis.

Table 1. Rate Constant and Half life

Sample	k (day^{-1})	Half-life (day)
RCA	0.0821	8.443
RCP	0.0797	8.697
RCAP	0.1005	6.897
RCC	0.0202	34.314
TCA	0.0718	9.654
TCP	0.1341	5.168
TCAP	0.1432	4.840
TCC	0.0454	15.267

Key:

TCC Treated Crude Control sample

RCC Raw Crude Control sample

RCA Raw Crude Treated with *Aspergillus niger*

TCA Treated Crude Treated with *Aspergillus niger*

RCP Raw Crude Treated with *Pseudomonas aeruginosa*

TCP Treated Crude Treated with *Pseudomonas aeruginosa*

RCAP Raw Crude Treated with *Aspergillus niger* and *Pseudomonas aeruginosa*

TCAP Treated Crude Treated with *Aspergillus niger* and *Pseudomonas aeruginosa*

The Biostimulant Efficiency (BE) analysis was done to determine the efficiency of each biostimulant used to remediate the soil samples in each system. An average of the biostimulant

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efficiency for each biostimulant in the respective soil samples was done to concisely determine which of the biostimulant was most efficient. The BE is calculated from the TPH data as follows:

% BE = % Loss in TPH of soil remediated with biostimulant - % Loss in TPH of corresponding control sample remediated with no biostimulant.

From the table 1 to determine the BE of *Aspergillus niger,* all samples remediated with this biostimulant and their respective control samples are taken into consideration.

% BE of *Aspergillus niger* in RCA = % Loss in TPH of RCA - % Loss in TPH of RCC

This is done for all other system remediated with *Aspergillus niger* and the average % BE is found. The same procedure is repeated for the other biostimulants (*Pseudomonas aeruginosa* alone and *Aspergillus niger* combined with *Pseudomonas aeruginosa*).

Table 2 shows the average % BE for each biostimulant used for this research.

Table 2. Biostimulant Efficiency Summary

Soil Samples	% Loss in THC	Biostimulant Efficiency (%)
RCC	49.51	0
TCC	71.94	0
RCA	91.15	41.65
RCP	80.59	31.08
RCAP	93.37	43.86
TCA	87.55	15.61
TCP	97.63	25.69
TCAP	98.22	26.28

Table 3. Average Biostimulant Efficiency

Biostimulant	Average % BE
Aspergillus niger	28.63
Pseudomonas aeruginosa	28.39
Aspergillus niger + *Pseudomonas aeruginosa*	35.07

4.3. Total Petroleum Hydrocarbon and Bioremediation Kinetics

The results shows that all eight systems experienced a level of reduction in the Total Petroleum Hydrocarbon content over the space of 30 days. Most of the systems experienced a

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significant level of remediation within the first fifteen days. The least hydrocarbon content decrease was observed to be in the control systems (figures 8 and 9). Between the two control systems polluted with raw and treated crude, the system polluted with treated crude experienced a higher level of remediation when compared to the control system polluted with raw crude. At the end of 30 days, the hydrocarbon content in the control system RCC decreased from 678.33mg/kg to 342.5mg/kg which is about 49.50% hydrocarbon removal. The rate constant of biodegradation was obtained to be 0.0202/day as shown in figure 8. The half-life for this system is 34.3 days.

The control system TCC (figure 9) had its hydrocarbon content decrease from 843.3 mg/kg to 236.6 mg/kg an equivalent of 71.9% petroleum hydrocarbon removal, a rate constant of 0.0454/day and half-life of 15.26 days. For the samples remediated with microorganisms, general and precise inferences can be made. It was observed that on the average that figures 6 and 7, the systems remediated with *Aspergillus niger* and *Pseudomonas aeruginosa* (RCAP and TCAP) experienced the best level of remediation having an average remediation of the two samples of 95.793%. This was closely followed by the soil samples remediated with *Pseudomonas aeruginosa* alone (RCP and TCP) which had an average remediation of the two samples of 90% (figures 4 and 5).

Figures 2 and 3 showed the least remediation performance of 87% in the average of the two samples amended with *Aspergillus niger* alone (RCA and TCA).

A more critical analysis of the data showed that *Aspergillus niger* alone remediated the systems polluted with raw crude RCA (figure 1) better than the systems polluted with treated crude TCA (figure 2). This was observed from the percentage crude oil removal of each of these systems. At the end of 30 days, RCA experienced a percentage crude oil removal of 91.2% as opposed to its direct counterpart TCA that had a hydrocarbon removal of 87.5% as shown in figures 2 and 3.

The results in figures 4 and 5 also showed that *Pseudomonas aeruginosa* alone remediated the systems polluted with treated crude TCP better than the systems polluted with raw crude RCP. This was observed from the percentage crude oil removal of each of these systems. RCP experienced a percentage crude oil removal of 80.6% as opposed to its direct counterpart TCP that had a hydrocarbon removal of 98%.

Figures 6 and 7 showed the systems remediated with both *Pseudomonas aeruginosa* and *Aspergillus niger* approximately remediated at 93.3% and 98.2% for RCAP and TCAP are respectively.

The analysis of the first order bioremediation kinetics for each system showed that the system TCAP had the smallest half-life of approximately 4.8 days as shown in table 1. RCA had a half-life of 8.4 days and RCP 8.7 days. The sample with the greatest half-life was observed to be RCC (34.3 days). It can be inferred from this that on the average effective bioremediation occurred fastest in samples remediated with *Aspergillus niger* and *Pseudomonas aeruginosa.* The control samples remediated the slowest.

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For the results of the Biostimulant Efficiency, it shows on table 3 that on the average, a combination of *Aspergillus niger* and *Pseudomonas aeruginosa* had the highest efficiency of approximately 35%.

Conclusion

Using *Aspergillus niger* alone remediates raw crude oil polluted soil better than treated crude oil polluted soil. Therefore, for a spill site polluted with raw crude oil, *Aspergillus niger* would be a better choice of biostimulant compared to *Pseudomonas aeruginosa*. Both *Aspergillus niger* and *Pseudomonas aeruginosa* in combination is generally a better biostimulant compared to using either *Aspergillus niger* or *Pseudomonas aeruginosa* alone. This is observed from the biostimulant efficiency and percentage crude oil removal. The Use of *Pseudomonas aeruginosa* as a biostimulant is more effective in the remediation of soil polluted with treated crude oil. Therefore, for an oil spill site polluted with treated crude oil from a flow line or even the production platform, *Pseudomonas aeruginosa* is a better choice of biostimulant compared to *Aspergillus niger.*

Generally, treated crude oil spills remediate faster and to a larger extent than spills caused by raw crude oil. This research confirmed the previous work carry out on either in-situ or ex-situ bioremediation of crude oil polluted soil by different microorganisms.

Conflicts of interest: The authors declare that they have no conflict of interest.

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