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SOIL ENZYMATIC ACTIVITY – AN INDICATION OF ECOSYSTEM STRESSED WITH HYDROCARBON

EBULUE, M.M.

Pollution Control Unit; Department of Biotechnology, Federal University of Technology, Owerri. Imo State, Nigeria.

Email : ebuluemm801f@yahoo.com

ABSTRACT

The activity of selected microbial exudates; the soil enzymes (alkaline phosphatase, dehydrogenases and catalase) as well as pH, total petroleum hydrocarbon (TPH) and microbial biomass was studied *ex-situ* in an ecosystem stressed with hydrocarbon from crude oil. This study investigated this impact using a culture-dependent approach to evaluate the total microbial counts; biochemical and physicochemical tools to determine the activities of their exudates, pH and TPH. The results indicated a significantly ($p<0.05$) stimulated activity at 1.0 – 3.5% contamination across days-zero to -28 for dehydrogenases: from 4.72 ± 0.01 to 9.78 ± 0.04 mol/min and from 3.82 ± 0.03 to 8.05 ± 0.00mol/min for alkaline phosphatase. Soil catalase suffered inhibition in activity significantly ($p<0.05$) from 0.195 ± 0.00 to 0.042 ± 0.00 mol/min. These two groups of enzymes have demonstrated that they could be used in concert as biomarkers of hydrocarbon-contaminated soil ecosystem. An initial reduction in microbial biomass from 1.36 x 10^9 ± 0.00 to 4.06 x10^8 ± 0.00 cfu/g following the impact and induction of hydrocarbon-degrading organisms, (the hydrocarbonclastics) thereafter, to 5.26 x 10^8 ± 0.10cfu/g overtime correlated with significant increase ($p<0.05$) in total petroleum hydrocarbon (TPH) from 2.60 ± 0.00 to 5.10 ± 0.00 with increase in acidity from pH 5.4 ± 0.00 to 3.1 ± 0.00. The lowered pH affected the entire soil biochemistry and slowed soil organic matter mineralization and associated nutrient re-mineralization.

Keywords: Crude oil, Microorganisms, Alkaline phosphatase, Dehydrogenases, Catalase, pH, TPH

INTRODUCTION

The soil is the major site of degradative activity with an impressive capacity to reduce intractable to harmless compounds. This capacity is vested in the catabolic activities of soil enzymes which are the microbial exudates that comprise the decomposer community. Utilization of chemical contaminants incidented on the soil by different microbial communities as sources of carbon and energy ameliorates a wide range of contaminants from oily waste such as petroleum-by-products and polyaromatic hydrocarbons.

Crude oil is a complex mixture of hydrocarbons and other organic compounds, including some organo-metallic constituents (Butler and Mason, 1997). It contain hundreds or thousands of aliphatic, branched and aromatic hydrocarbons (Prince, 1993; Wang *et al*., 1998), most of which are toxic to living organisms (ATSDR, 1995), mutagenic and carcinogenic (Mandal, *et al*., 2012). Petroleum hydrocarbon being ubiquitos and the principle source of energy is an important global environmental pollutant / threat. Therefore, the release to the environment is strictly controlled and they are classified as priority environmental pollutant by US Environmental Protection Agency, due to their adverse impaction on human health and environment (ATSDR). In spite of its accidental contamination on soil ecosystem, large amounts of oil sludge generated in refineries pose a lot of problems due to high cost of disposal (Vasudevan and Rajaram, 2001).

The most obvious effect of pollutants' exposure to microbial communities is direct toxicity which results to rapid death. The extent of loss in microbial activity/ biomass and alteration in their exudates (enzymes) can be used to assess the toxicity of that pollutant in the environment. Thus hydrocarbons increase the abundance of hydrocarbon degrading microorganisms, but on the other hand induce a limitation in microbial diversity (Ebulue *et al*., 2017).

Dehydrogenases (EC 1.1.1.1) are enzymes which catalyze the removal of hydrogen atoms from substrates (Nelson and Cox, 2000). Active dehydrogenases are considered to exist in the soil as integral part of intact cells. By their oxidative activities they degrade organic matter and hydrocarbons (Margesin *et al*., 2000) which reflects in the changes in the respiratory activity of the soil environment (Schinner *et al*., 1996).

Catalase (EC 1.11.1.6), an iron porphyrin and antioxidant enzyme catalyzes very rapid decomposition of hydrogen peroxide to water and oxygen (Nelson and Cox, 2000). The enzyme is widely present in nature, which accounts for its diverse activities in the soil. The activities of soil catalase and dehydrogenase are used as biomarkers of hydrocarbon-polluted soil ecosystem and their values have been suggested to be used as simple toxicity test (Rogers and Li, 1985) in oil pollution.

Alkaline phosphatase catalyzes the hydrolysis of ester–phosphate bonds, sodium p-nitrophenyl phosphate, leading to the release of phosphate (P), which can be taken up by plants or microorganisms (Quiquampoix and Mousain, 2005). They catalyze the hydrolysis of both esters and

anhydrides of phosphoric acid and, according to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NCIUBMB), they can be classified as phosphoric monoester hydrolases or phosphomonoesterases (EC 3.1.3.2), phosphoric diester hydrolases or phosphodiesterases (EC 3.1.4.17).

Soil pH which is an important physical property refers to acidity or alkalinity, which is a measure of the concentration of hydrogen ions $[H^+]$. It is defined by the equation: pH = – Log $[H^+]$. Soil with a large $[H^+]$ is acidic (i.e., low pH). This acidity increases the solubility of elements which increases their mobility, lability and probability of leaching into ground water (Ebulue, 2020); while when the $[H^+]$ is low, the soil is basic (i.e., high pH), and as a result, cations will be on the particle exchange sites causing lower probability of leachability (Zhang *et al*., 2007, Ebulue, 2020), thus causing increase in cation exchange capacity (CEC) (Ebulue, 2020).

Research design

This research was designed for a-forty-two-day investigation in consideration of the volatility and biodegradability of hydrocarbons: day- zero, -14, -28, -42; within which, the activities of the aforementioned parameters were determined.

Materials and methods

Determination of pH of crude oil-contaminated soil

The method of Ebulue, (2020) was used to evaluate the pH, where Bench pH Meter 3510 was used for easy read-out of pH after soil inoculation with crude oil at different degrees of contamination.

Determination of total petroleum hydrocarbon (TPH)

Principle: It is based on the estimation of the total petroleum hydrocarbon (TPH) in the soil with reference to the standard curve derived from fresh crude oil diluted with toluene using the equation y = 1.094x; where y = absorbance and x = concentration.

Procedure: Total petroleum hydrocarbon content was determined gravimetrically by the method of Odu *et al*. (1989), to provide an estimate of the available total hydrocarbon with time. In this procedure, 20ml toluene was added into six test tubes containing 10g soils contaminated with different concentrations of crude oil: 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5% w/w (oil-soil mixture). Into the 7th tube, the control, 20ml toluene was introduced into 10g of uncontaminated soil. After shaking for 30min, the liquid phase of the extract was measured spectrophotometrically at 420nm. The total petroleum hydrocarbon (TPH) in the soil was estimated with reference to the standard curve derived from crude oil diluted with toluene using the equation y = 1.094x; where y = absorbance and x = concentration.

Determination of microbial population in the crude oil-impacted soil

Sterilization of materials

The Petri dishes were washed with tap water, dried in a dryer at a temperature of 45^oC; then oven- dried at 210^oC for 2hr. The test tubes, Erlenmeyer flask, pipette tips, crucible, spatula and beakers were autoclaved at a temperature of 120^oC and fifteen pounds pressure for 15 min.

Bacterial culture

To sterile water, 10g of soil sample was aseptically introduced into test tubes, tightly capped and vortexed for 5min. Thereafter, 1ml was aseptically transferred into 9ml of sterile distilled water, and ten-fold serial dilutions were carried out. 0.1ml of the solution from the fourth dilution was evenly spread on an already prepared nutrient agar plate and the culture was incubated for a period of 24h. After the incubation period, the total viable count was determined by counting the colony forming units (cfu) and distinct colonies were isolated.

Identification of isolates

The isolates were subjected to the routine bacterial identification procedure using Bergey's Manual of Systematic Bacteriology (Baumann and Schubert, 1994).

Determination of soil catalase activity

Principle: Catalase generates oxygen in the presence of H_2O_2. The activity was determined by the method of Cohen *et al*. (1970), where decomposed hydrogen peroxide was measured by reacting it with excess potassium tetraoxomanganate (VII), ($KMnO_4$) and residual $KMnO_4$ was measured spectrophotometrically at 480nm.

Protocol: One tenth ml of the supernatant was introduced into differently labeled test tubes containing 0.5 ml of 2m Mol hydrogen peroxide and a blank containing 0.5 ml of distilled water. Enzymatic reactions were initiated by adding sequentially, at the same fixed interval, 1ml of 6N tetraoxosulphate (VI) acid, (H_2SO_4) to each of the labeled test tubes containing different concentrations of soil contaminated with crude oil: 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5% w/w (oil-soil mixture); to the blank, 7 ml of 0.1N $KMnO_4$ was added within 30s and thoroughly mixed.

Spectrophotometric standard was prepared by adding 7ml of 0.1N $KMnO_4$ to a mixture of 5.5 ml of 0.05N phosphate buffer, pH 7 and 1ml of 6N H_2SO_4. The spectrophotometer was then zeroed with distilled water before taking absorbance readings at 480nm.

The concentration of catalase was determined using the Beer-Lambert's law, A = ECL with the molar extinction coefficient of catalase at $4.02 Mol^{-1} cm^{-1}$; and the activity was determined thereafter as follows: A = ECL where C = A/EL. And Activity = mol/min.

Determination of dehydrogenase activity

Principle: The activity of dehydrogenase was determined using the method described by Tabatabai, (1982). Dehydrogenases convert 2,3,5-triphenyltetrazolium chloride (TTC) to formazan. The asbsorbance of formazan was read spectrophotometrically at 485nm.

Protocol: The protocol of Ebulue, (2021) was used where the absorbance of formazan was read spectrophotometrically at 485nm after contaminating the soil with different concentrations of crude oil. The concentration of formazan was evaluated using the molar extinction coefficient of dehydrogenase at $15433 Mol\ cm^{-1}$ Dushoff, (1965); and the activity was determined thereafter as follows: A = ECL where C = A/EL. And Activity = mol/min.

Determination of alkaline phosphatase activity

Principle: The method of Tabatabai and Bremner, (1969) was used, which involves colorimetric estimation of the p-nitrophenol released by phosphatase activity when the crude oil-contaminated soil was incubated with sodium p-nitrophenyl phosphate solution and toluene in 0.1N modified universal buffer at pH 11.

Protocol: The phosphatase activity determination method consisted of incubation of reactive mixture containing 10g soil samples: 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5% w/w (oil-soil mixture) with the substrate, 5ml of 0.1N sodium p-nitrophenyl phosphate. Soil inoculation was carried out by weighing 10g of sieved soil sample into six different test tubes. To the first tube, 0.1g of crude oil (corresponding to 1.0%), was added and mixed thoroughly with a steering rod. This procedure was repeated for 1.5, 2.0, 2.5, 3.0 and 3.5%; and into the 7th tube, the control, 20ml of distilled water was added. After one hour (1hr) incubation process at 27°C, the intense yellow color due to the released p-nitrophenol was measured spectrophotometrically at 410nm with the molar extinction coefficient of 18,000 M^{-1} cm^{-1} alkaline phosphatase and the activity was determined thereafter as follows: A = ECL where C = A/EL. And Activity = mol/min.

Statistical analysis

The results were expressed as mean ± standard deviation (SD). All results were compared with respect to the control. Comparisons between the concentrations and control were made by using Statistical Package for Social Sciences (SPSS) version 20 and One-way Analysis of Variance (ANOVA). Differences at $p < 0.05$ were considered significant.

Results

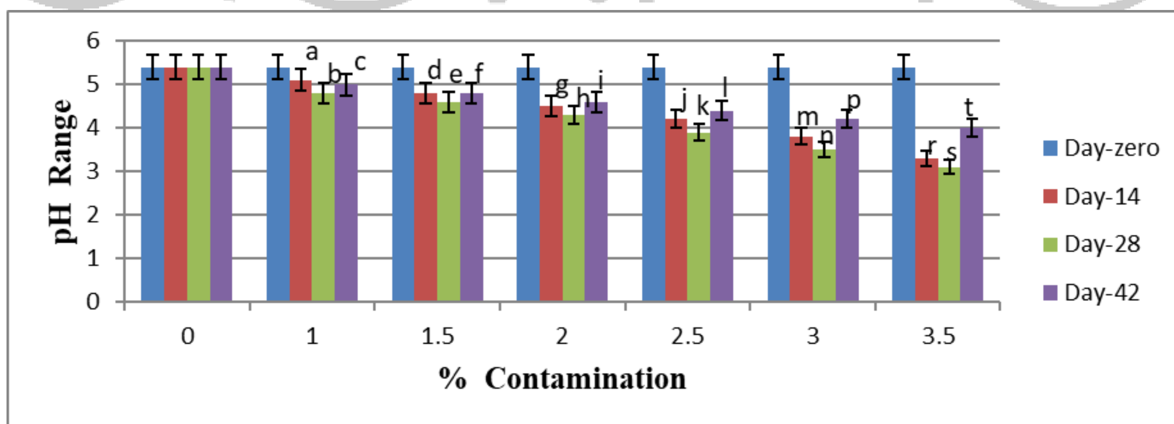

Fig. 1.0: pH of the soil polluted with crude oil

Comparison between groups: Bars with different letters differ significantly (p<0.05).

Effect of crude oil on soil pH

The pH of crude-oil contaminated soil is shown in Fig.1.0. Relative to the control, there was a progressive reduction in pH values which was statistically significant ($p<0.05$) between treatment groups.

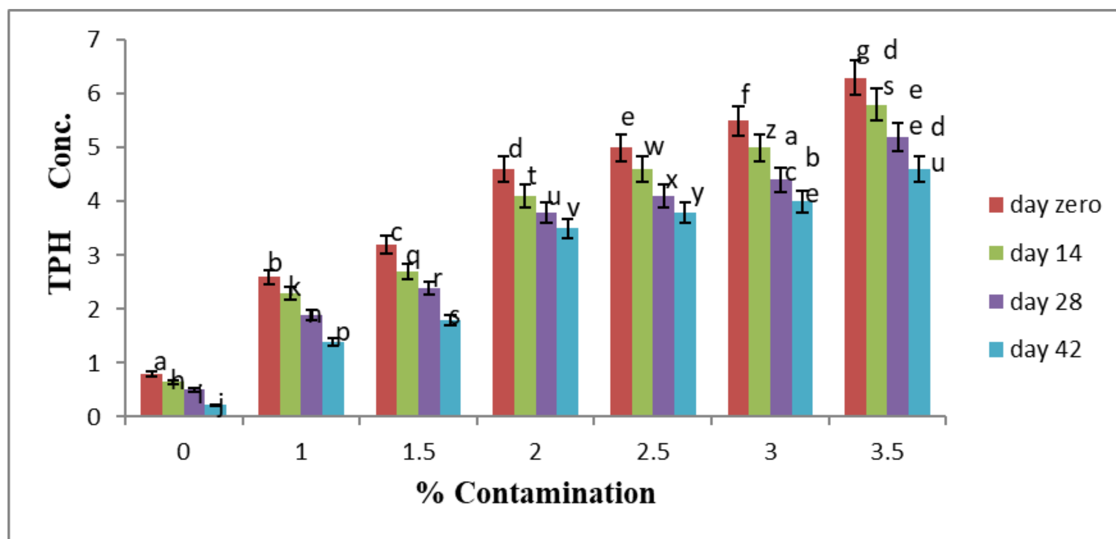

Fig. 2.0: Total petroleum hydrocarbon (TPH) of the crude oil-polluted soil Comparison between groups: Bars with different letters differ significantly ($p<0.05$).

Total petroleum hydrocarbon (TPH) of crude oil-polluted soil

Following crude oil contamination, there was a synergistic increase in total petroleum hydrocarbon (TPH) which was significantly ($p<0.05$) different between groups.

Table 1.0: Total microbial population in the crude oil-impacted soil (x 100)

% Contamination	Week One (cfu/g)	Week Two (cfu/g)	Week Four (cfu/g)	Week six (cfu/g)
Control 1.36×10^9				
1.0	4.06×10^8	4.18×10^8	4.31 x 10^8	3.80 x10^8
1.5	3.84 x10^8	4.28 x10^8	4.40 x10^8	4.01 x10^8
2.0	3.22 x10^8	4.40 x10^8	4.50 x10^8	4.20 x10^8
2.5	2.68 x10^8	4.48 x10^8	4.62 x10^8	4.55 x10^8
3.0	2.50 x10^8	4.44 x10^8	4.60 x10^8	4.82 x10^8
3.5	2.32 x10^8	4.10 x10^8	4.32 x10^8	5.26 x10^8

Microbial isolates in the crude oil-impacted soil

Bacterial strains: *Pseudomonas aeroginosa*, *Flavobacteria*, *Nocardia*, *Corynebacteria*, *Mycobacteria*, *Micrococcus sp*, *Rhodococcus*, *Streptomyces*, *Bacillus sp*, *Arthrobacter* and *Cyanobacteria*.

Fungal strains: *Fusarium sp*, *Aspergillus niger*, *Candida sp*, and *Penicillium*

Microbial population and isolates in the crude oil-impacted soil

The total viable microbial count present in the soil sample from Owerri Imo State Nigeria was 1.36 x 10^9cfu/g. The total microbial counts and isolates in crude oil-contaminated soil across the weeks are presented on Table 1.0. Relative to the control, there was a decline in total microbial biomass as the treatment increased in week one. However, across the weeks microbial population increased.

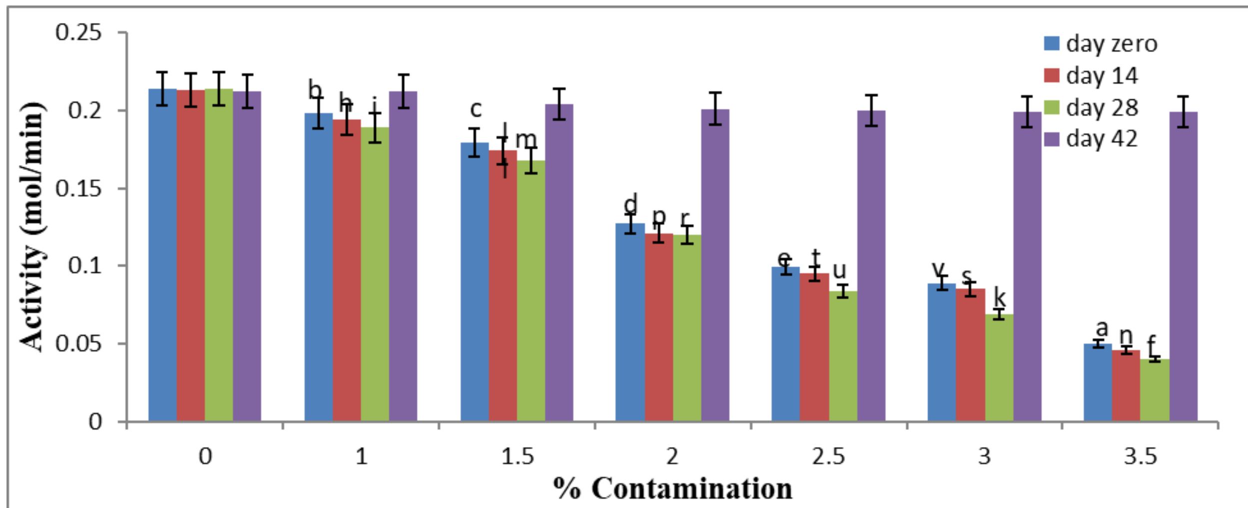

Fig. 3.0: Effect of various concentrations of crude oil on soil catalase activity Comparison between groups: Bars with different letters differ significantly (p<0.05).

Effect of various concentrations of crude oil on soil catalase activity

Relative to the control, the crude oil inhibited the activity of soil catalase as shown in Figure 3.0. The inhibition which was in a concentration and time dependent manner except on day-42 was significantly different ($p<0.05$) between groups.

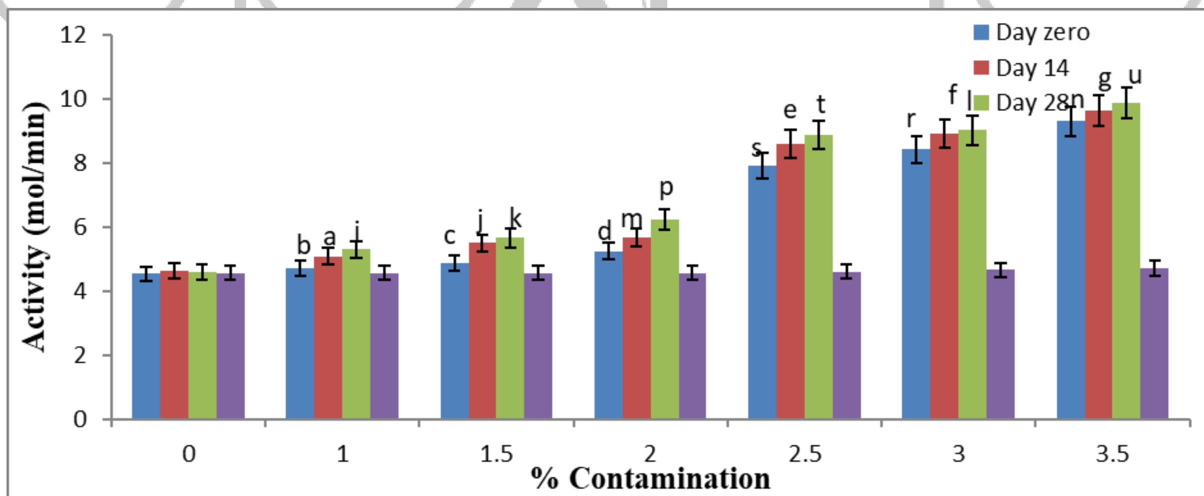

Fig. 4.0: Activity of soil dehydrogenase in the crude oil-polluted soil.

Comparison between groups: Bars with different letters differ significantly (p<0.05).

Effect of various concentrations of crude oil on soil dehydrogenase activity

The effect of crude oil on soil dehydrogenase activity contrasted sharply with that obtained on soil catalase. Relative to the control, the crude oil stimulated the activity of soil dehydrogenase in a concentration and time dependent manner up to day-28, but declined on day-42 as presented in Figure 4.0, which was significant ($p<0.05$) between groups.

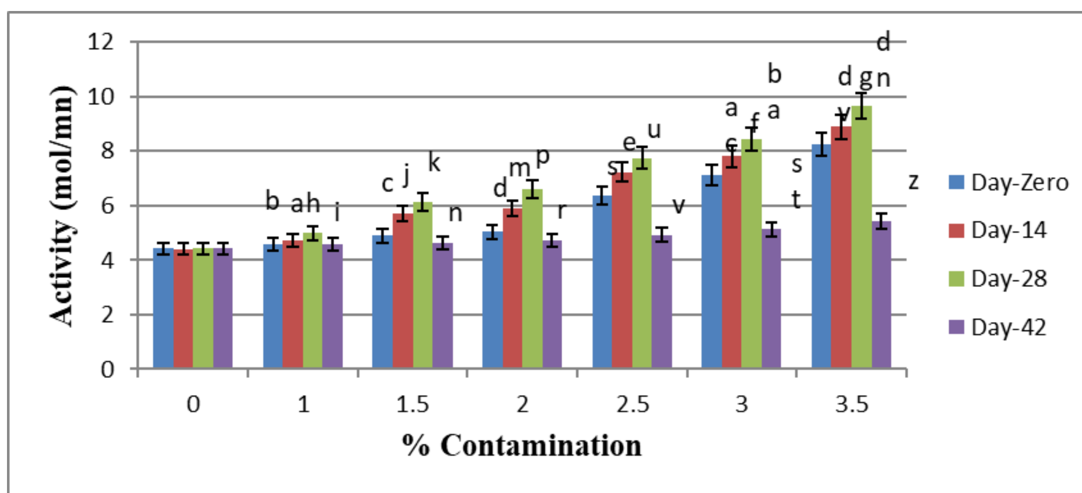

Fig. 5.0: Activity of alkaline phosphatase in the crude oil-polluted soil.

Comparison between groups: Bars with different letters differ significantly ($p<0.05$).

Effect of various concentrations of crude oil contamination on the activity of alkaline phosphatase

The crude oil stimulated significantly ($p<0.05$) the activity of alkaline phosphatase. This stimulation was in relation to concentration and duration of contact as presented in Figure 5.0.

Discussion

Maintenance of ecological equilibrium is a necessity of every natural ecosystem. Any biological disequilibrium as a result of impact of hydrocarbon from crude oil or any xenobiotics will provoke the insurgence of indigenous microbial communities to biodegrade the foreign compounds and bring the ecosystem to a balance and equilibrium. This is the hallmark of the entire ecosystem function.

Contamination of the natural environment with petroleum compounds poses an extremely serious problem. The stressor adversely affected the physicochemical status of the soil, pH and microbial communities which are the sole producer of soil enzymes that comprise the decomposer community. From this investigation, the positive correlation between the pH of the soil and the

amount of crude oil added may be an implication that crude oil pollution led to a reduction in soil pH in a concentration and time dependent manner from 5.4 ± 0.0 to 3.1± 0.0 which was statistically significant ($p< 0.05$) across days-zero to -28 at concentrations 1.0 – 3.5 %. The lowered pH reflected accelerated metabolism and accelerated demand for electron acceptors thus creating a reducing environment. This could be attributable to microbial metabolism of the hydrocarbon which consequently gave rise to the production of organic acids that resulted to the increase in the acidity of the affected ecosystem (Ebulue, 2020). This is replete with the report of Osuji and Nwoye (2007), Osam *et al*. (2013). This increase in acidity would likely affect plant growth, microbial succession and metabolism and leachability of metals (Ebulue, 2020).

The intense infusion of degradable petroleum hydrocarbon from 0.03 ± 0.0 to 0.86 ± 0.00mg/ml that cuts across days-zero to -28 at concentrations 1.0 – 3.5% observed in this study following an exposure of soil ecosystem to crude oil would have likely stimulated aerobic and anaerobic microbial metabolism. So, as oxygen became limiting, utilization of alternate electron acceptors produced an increased reducing environment. This report is in harmony with the findings of Osuji and Opiah (2007). High level of hydrocarbon causes oxygen deprivation and reduction in gaseous diffusion by the surface film of oil, and these usually have far reaching implications on the flora and fauna of the affected area, and hence, soil fertility (Osuji *et al*., 2004).

Following the oil spill, the microbial population in the soil passed through a short period of adaptation or lag phase and a limitation in microbial diversity from $1.36 \times 10^9 \pm 0.00$ to $2.32 \times 10^8 \pm 0.01$ cfu/g in week one at 3.5% contamination. The lag phase encountered in this study upon the application of the stressor may be attributed to the toxicity of hydrocarbons (Ebulue *et al*., 2017; Walker *et al*., 1975). The time lag was equivalent to the time required for the induction of hydrocarbonclastic organisms to synthesize the enzymes required for oil decomposition. Thus, this initial decrease in the microbial population in the crude-oiled soil is indicative of the prejudicial nature of the stressor on soil ecosystem. However, at increased concentrations overtime, there was increase in microbial population from $2.32 \times 10^8 \pm 0.01$to $5.26 \times 10^8 \pm 0.00$ cfu/g which cuts across weeks -one to -four at 1.0 – 3.5% contamination. The implication in this upsurge was attributable to the hydrocarbon-degrading organisms (the hydrocarbonclastics), which use hydrocarbon as source of carbon and energy thereby increasing the biomass. The significance of this insult has been shown to enhance microbial growth in the affected soil due to increase in the availability of degradable substrate, thus hydrocarbons increased the abundance of hydrocarbon-degrading microorganisms (the hydrocarbonclastics), but on the other hand, induced a limitation in microbial diversity; an effect that slows soil organic matter mineralization and associated nutrient re-mineralization (Ebulue *et al*., 2017).

The result revealed that the activities of the microbial exudates (dehydrogenases and alkaline phosphatase) in the crude oiled soil increased significantly ($p < 0.05$) and non-significantly ($p > 0.05$) in a concentration and time dependent manner, from 4.72 ± 0.015 to 9.78 ± 0.040 mol/min and from 3.82 ± 0.03 to 8.05 ± 0.01 mol/min respectively. This stimulatory effect of the crude oil on the activities of the aforesaid enzymes which was stronger as the rate of contamination and duration of contact increased was an indication of a stressed ecosystem. However, by day-42, the enzyme activities began to decline, a condition which is correlated with the depletion of the pollutants. The induction and repression in enzyme concentrations which reflected in the increase and decrease in the enzyme activities overtime is a function of the upsurge in hydrocarbonclastic organisms correlated with the concentration of the contaminant. This finding is in harmony with the work of Achuba and Peretiemo-Clarke (2008) on the activities of enzymes in hydrocarbon polluted soil ecosystem.

In this study, catalase suffered inhibition in hydrocarbon polluted soil (Ebulue *et al*. (2017), This alteration in the activities of the aforementioned enzymes could arise from unfavourable conditions such as hypoxia and a reduction in pH which occasioned in the oil-polluted environment indicating that oil biodegradation by microorganisms and metabolic enzymes could lead to production of organic acids. It could also imply that the amino acids at the active sites of soil catalase, are irritable to hypoxic and pH increases, and any condition that creates oxygen tension with a rise in acidic environment adversely affected the activity. It then follows that the aerobic bacterial status / population has a correlation with the activity of the enzyme. This finding is in consonance with the report of Waarde *et al*. (1995), Margesin and Schinner (1999), Achuba and Peretiemo-Clarke (2008) on the inhibition of the activity of soil catalase following an insult of soil ecosystem with crude oil.

Conclusion

Crude oil impacted soil lowered the pH. This increase in acidity emanated from microbial insurgence which their exudates (enzymes) in the course of hydrocarbon metabolism produced organic acids thereby making the ecosystem acidic. This acidic environment invariably caused a limitation in microbial degradation of the impacted hydrocarbon.

The activity of soil catalase was inhibited in hydrocarbon impacted soil. This could probably be as a result of the reducing environment, implying that soil catalase could be irritable to acidic environment which affected the transcription of its genes.

On the other hand, the activity of dehydrogenases and alkaline phosphatase were stimulated in the hydrocarbon impacted soil, an indication of gene transcription. This stimulated activity enhanced degradation of the crude oil. Dehydrogenase activity in soil is a measure of microbial activity and respiration rate (Schinner *et al*., 1996) which is attributable to microbial metabolism of polyaromatic hydrocarbons (Margesin *et al*., 2000).

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