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Enzyme-Based Assay for Toxicological Evaluation of Soil Ecosystem Polluted With Spent Engine Oil

Ebulue, M.M., Nwodo, O.F.C., Onwurah, I.N.E. Uwakwe, A.A. and Wegwu, M.O.

Ecotoxicology and Environmental Biotechnology Research Unit; Department of Biochemistry, University of Nigeria, Nsukka. Nigeria.

ABSTRACT: Soil enzymatic activities which have a central role in the soil environment are used as attractive bio-indicators for monitoring various impacts on the soil. They bio-transform toxic petroleum products, spent engine oil, hydrocarbons, heavy metals and pesticides into harmless compounds. This study investigated the impact of spent engine oil at various concentrations (0, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5% w/w) overtime based on the activities of soil dehydrogenases and catalase, pH and total microbial counts. The result indicated that while spent engine oil stimulated the activity of soil dehydrogenase significantly ($p<0.05$) in a concentration and time dependent manner: from 4.72 ± 0.015 mol/min to 9.78 ± 0.040 mol/min that cuts across days-zero to -28 at 1.0 – 3.5% contaminations; the activity of soil catalase was in the same wise inhibited significantly ($p<0.05$) from 0.195 ± 0.005 mol/min to 0.042 ± 0.002 mol/min. These two enzymes have demonstrated that they could be used in concert as biomarkers of hydrocarbon-contaminated soil ecosystem. The oiled soil was acidic as the pH was significantly reduced from 7.1 ± 0.0 at 1.0% contamination to 6.6 ± 0.0 at 3.5% contamination on day-zero; and from 6.8 ± 0.0 at 1.0% contamination to 6.2 ± 0.0 at 3.5% contamination on day-28. At increased concentrations, 3.5% w/w of contamination, hydrocarbons increased the abundance of hydrocarbon-degrading microorganisms, (the hydrocarbonclastics), from 3.26 $x10^7$ ± 0.02cfu/g on day-zero to 6.55 x 10^8 ± 0.04cfu/g on day-28; but on the other hand, induced a limitation on microbial diversity. The concentration of the hydrocarbonclastic bacteria in the spent engine oil-contaminated soil correlated with the enzyme induction and activity. These effects which altered the entire soil biochemistry could disrupt ecosystem dynamics by slowing soil organic matter mineralization and associated nutrient re-mineralization.

KEY WORDS: Soil, Spent engine oil, pH, Catalase, Dehydrogenase and Microorganisms

INTRODUCTION

Environmental pollution arising from the spillage of petroleum derivative (spent engine oil) contributes to soil toxicity and degradation. The soil is of prime important to human existence for various reasons, especially agriculture. However, the soil represents the primary recipient of oil pollution, as it has been subjected to several abuses from spillage of petroleum derived products (Osam, 2011; Nwaugo *et al.*, 2006, 2009).

Spent engine oil is a-brownish-black mineral-based crankcase oil produced when new mineral-based crankcase oil is subjected to high temperature and high mechanical strain (ATSDR, 1997). It is constituted of several different chemicals (Wang *et al*., 2000), low and high molecular weight C_{15} – C_{20} aliphatics and aromatics hydrocarbons, polychlorinated biphenyls, chlorodibenzofurans, lubricative additives, decomposition products and heavy metals such as aluminium, chromium, tin, lead, manganese, nickel, barium and silicon which some of them come from engine parts as they tear and wear down (ATSDR, 1997).

Spent engine oil is a common environmental toxicant not found in the natural environment (Dominguez-Rosado and Pichtel, 2004). It is an anthropogenic toxicant which its contamination of the existing and potential agricultural lands arises from the exhaust system during engine use and engine leakage (Anoliefo and Edegbai, 2000; Osuborand Anoliefo, 2003); and when the motor engine oil is changed and disposed into gutters, water drains, and farmlands, as practiced by motor and generator mechanics (Odjegba and Sadiq, 2002).

Spent oil, though it is biodegradable, when present in the soil creates an unsatisfactory condition for life in the soil. It alters the soil energy balance and disturbs the ecological equilibrium, hypoxia and oxygen tension, disruption of the ecosystem dynamics by slowing soil organic matter mineralization and associated nutrient re-mineralization. This culminates into a reducing environment as the pH is reduced with alteration in microbial succession and enzymatic activities. Thus, spent oil alters the entire soil biochemistry (Atuanya, 1987; Achuba and Peretiemo-Clarke (2008).

This contaminant which is one of the limiting factors to soil fertility and hence crop productivity due to its toxicity on soil organisms and to plants, can bio-accumulate in food chains where they disrupt biochemical or physiological activities (Onwurah *et al*., 2007). The presence of spent oil in the natural environment can display potential carcinogenic and mutagenic activities in the soil (Krahl *et al*., 2002; Onwurah *et al*., 2007); alter the succession of microorganisms (Kaplan and Kitts, 2004), which is directly associated with the induction and activities of soil enzymes (Wyszkowska *et al*., 2002; Wyszkowska and Kucharski, 2004).

Soil enzymes which are produced by microorganisms are responsible for catalyzing the transformation of the contaminants (substrates) to harmless products. The degradation of the internalized spent oil (substrate) will depend on the binding of the substrate to the enzyme and conformational changes at the active site of the enzyme. The activities of soil enzymes provide an integrative measure of the biological status of the soil (Li *et al*., 2005).

Dehydrogenases (EC 1.1.1.1) which by their oxidative activities catalyze the removal of hydrogen atoms from substrates (Nelson and Cox, 2000) and this culminates in the degradation of soil organic matter (Margesin *et al*., 2000). The activity of soil dehydrogenases is an index of respiratory activity in response to organic matter input in the soil environment (Schinner *et al*., 1996).

Catalase (EC 1.11.1.6), an antioxidant enzyme generates oxygen from hydrogen peroxide (Nelson and Cox, 2000). The enzyme is widely present in nature, which accounts for its diverse

activities in the soil. The activities of catalase and dehydrogenase are used as biomarkers of hydrocarbon-polluted soil ecosystem as they give information on the microbial activities in the soil. Their values have been suggested to be used as simple toxicity test (Rogers and Li, 1985) in hydrocarbon pollution.

This study was aimed at substantiating the effect of time and various concentrations of the pollutant in the use of soil enzymatic activities as biomarker for establishing pollution.

MATERIALS AND METHODS

Materials

The soil sample used was dug about 15cm depth from Botanical garden of the Department of Botany, Faculty of Biological Sciences, University of Nigeria Nsukka, and spent engine oil was obtained from the Mechanic Village, Nsukka, Enugu State, Nigeria.

Methods

Experimental design

This study was designed for a-thirty-five-day investigation:
Day-zero
Day-14
Day-28 and
Day-35;
within which the effect of time and various concentrations of spent engine oil on the aforementioned objectives were determined.

Experimental soil

The virgin sandy soil from the Botanical Garden of University of Nigeria Nsukka was sieved with a cheese cloth to remove tiny stones and other particulate matters. Soil analysis revealed: sand - 80 %, clay - 4 %, silt - 2 %, tiny stones and other particulates - 14 %. The pH was 7.1

Determination of pH

Into small beakers, 1 – 7 were introduced 10g of the sieved soil sample; and into the first beaker, 0.1g of spent engine oil corresponding to 1.0% concentration (w/w) was added and mixed thoroughly. This procedure was carried out at increasing concentrations of 1.5, 2.0, 2.5, 3.0 and 3.5 % into all the beakers except the 7^{th} beaker, the control. The mixture was allowed to stand for 10min before determining the pH with a pH meter.

Bacterial culture and isolation of organisms

The hydrocarbon-degrading organisms were isolated from the spent engine oil-contaminated soil. A mineral salt medium of Zajic and Supplison (1972) made up of the following (g/l): KH_2PO4 (1.8), K_2HPO4 (1.3), NH_4CI (4.00) $MgSO_4.7H_20$ (0.2) and agar-agar (15.0) was supplemented with 1.0% spent engine oil. The medium was sterilized and a pour plate method was used with 1ml aliquot from the diluted soil sample obtained from the spent engine oil (soil-oil mixture). After 48h of incubation, three most abundant species from the plates incubated were sub-cultured and identified. The total viable counts were determined by counting the colony forming units (cfu) and distinct colonies were isolated using the method of Bergey's Manual of

Systematic Bacteriology (Baumann and Schubert, 1994).This procedure was carried out at various percent concentrations 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 % of spent engine oil.

Preparation of extract for enzyme determination

The extract was prepared using the method of Achuba and Peretiemo-Clarke, (2008) where 100ml of phosphate buffer, pH 7.4, was added to 10 g of soil, homogenized gently, filtered with cheesecloth, and the filtrate was centrifuged at maximum speed of 7000 g for 10 min to obtain the supernatant.

Determination of the activity of catalase

The method of Cohen *et al*. (1970) was adopted where the decomposed substrate (hydrogen peroxide) was measured by reacting it with excess of potassium tetraoxomanganate (VII), $KMnO_4$ and residual $KMnO_4$ was measured spectrophotometrically at 480 nm.

One tenth ml of the supernatant was introduced into differently labeled small beakers containing 0.5 ml of 2mMol hydrogen peroxide and a blank containing 0.5 ml of distilled water. 1ml of 6N tetraoxosulphate (VI) acid (H_2SO_4) was added to each of the labeled beakers containing different percent concentrations of spent engine oil:1.0, 1.5, 2.0, 2.5, 3.0, 3.5% w/w (oil-soil mixture) to initiate enzymatic reactions ; and also to the blank. And 7 ml of 0.1N $KMnO_4$ was added within 30s and mixed thoroughly before taking absorbance.

The concentration of catalase was determined using the Beer-Lambert's law, A = ECL with the molar extinction coefficient of catalase of $4.02Mol^{-1}$ cm^{-1}; and the activity was determined thereafter.

Determination of the activity of soil dehydrogenase

The activity of dehydrogenase was determined using the method described by Tabatabai (1982); where dehydrogenases convert 2,3,5-triphenyl tetrazolium chloride (TTC) to formazan. The asbsorbance of formazan was read spectrophotometrically at 485nm.

10g of the sieved soil sample was placed into each of the labeled beakers containing different percent concentrations of spent engine oil: 1.0, 1.5, 2.0, 2.5, 3.0, 3.5% w/w (oil-soil mixture), and into the blank, distilled water. Then, 5ml of 3% (w/v) aqueous 2,3,5-triphenyl tetrazolium chloride was added into all the beakers, mixed, stirred with a glass rod and incubated for 96h at 27^oC. Thereafter, 10ml of ethanol was added and the suspension was vortexed for 30s, incubated for 1h to allow suspended soil to settle. The absorbance of the resulting supernatant was read spectrophotometrically at 485nm and the concentration of formazan and the activity of dehydrogenase was determined using the molar extinction coefficient of $15433Mol^{-1}$ cm^{-1} (Dushoff, 1965).

Statistical analysis

The results were expressed as mean ± standard error mean (SEM). 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 Analysis of Variance (ANOVA). Differences at $P < 0.05$ were considered significant.

Results

Effect of spent engine oil on soil pH

The pH of the oiled soil is shown in Figure 1. Relative to the control, there was a progressive reduction in pH which was significant as the concentration of the spent engine oil and its duration of contact increased. Thus, the soil was acidic. The acidity increased up to day-28and began to decline.

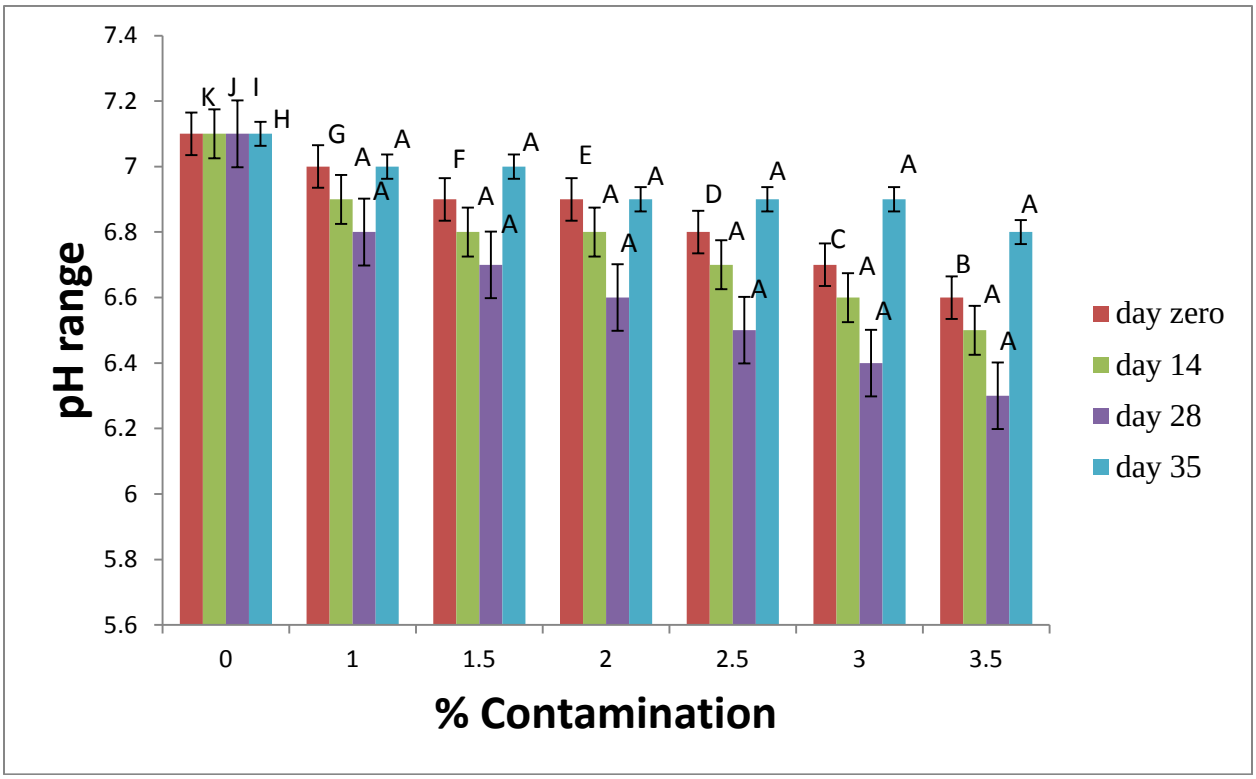

Fig. 1: pH of the soil polluted with spent engine oil
Comparison between groups: bars with different letters are not significantly different ($p<0.05$).

Microbial population of the oil-impacted soil

The mean total aerobic and anaerobic viable bacterial count present in the soil sample from the Botany garden of University of Nigeria, Nsukka was 1.22 x 10^9cfu/g. The total aerobic and anaerobic bacterial count observed in spent engine oil-contaminated soil sample on days; -zero, -14, -28, and -35 are presented on Table 2. There was a decline in total microbial biomass following oil impaction on day-zero. However, on days-14 and -28 there was an appreciable increase in biomass which thereafter began to decline on day-35.

Table 1: Microbial population of the oil-impacted soil (× 100)

% Contamination	Day-Zero (cfu/g)	Day- 14 (cfu/g)	Day- 28 (cfu/g)	Day- 35 (cfu/g)
0.0	1.22×10^9	1.22×10^9	1.22×10^9	1.22×10^9
1.0	3.55×10^8	3.68×10^8	3.80×10^8	2.00×10^8
1.5	3.05×10^8	3.80×10^8	3.99×10^8	2.22×10^8
2.0	4.11×10^7	3.92×10^8	4.76×10^8	2.38×10^8
2.5	3.98×10^7	4.01×10^8	5.01×10^8	2.60×10^8
3.0	3.80×10^7	4.25×10^8	5.44×10^8	2.89×10^8
3.5	3.26×10^7	4.55×10^8	6.55×10^8	3.04×10^8

Effect of spent engine oil on the activity of soil catalase

Relative to the control, spent engine oil inhibited significantly the activity of soil catalase in a concentration and time dependent manner up to day-28 as shown in Figure 2.

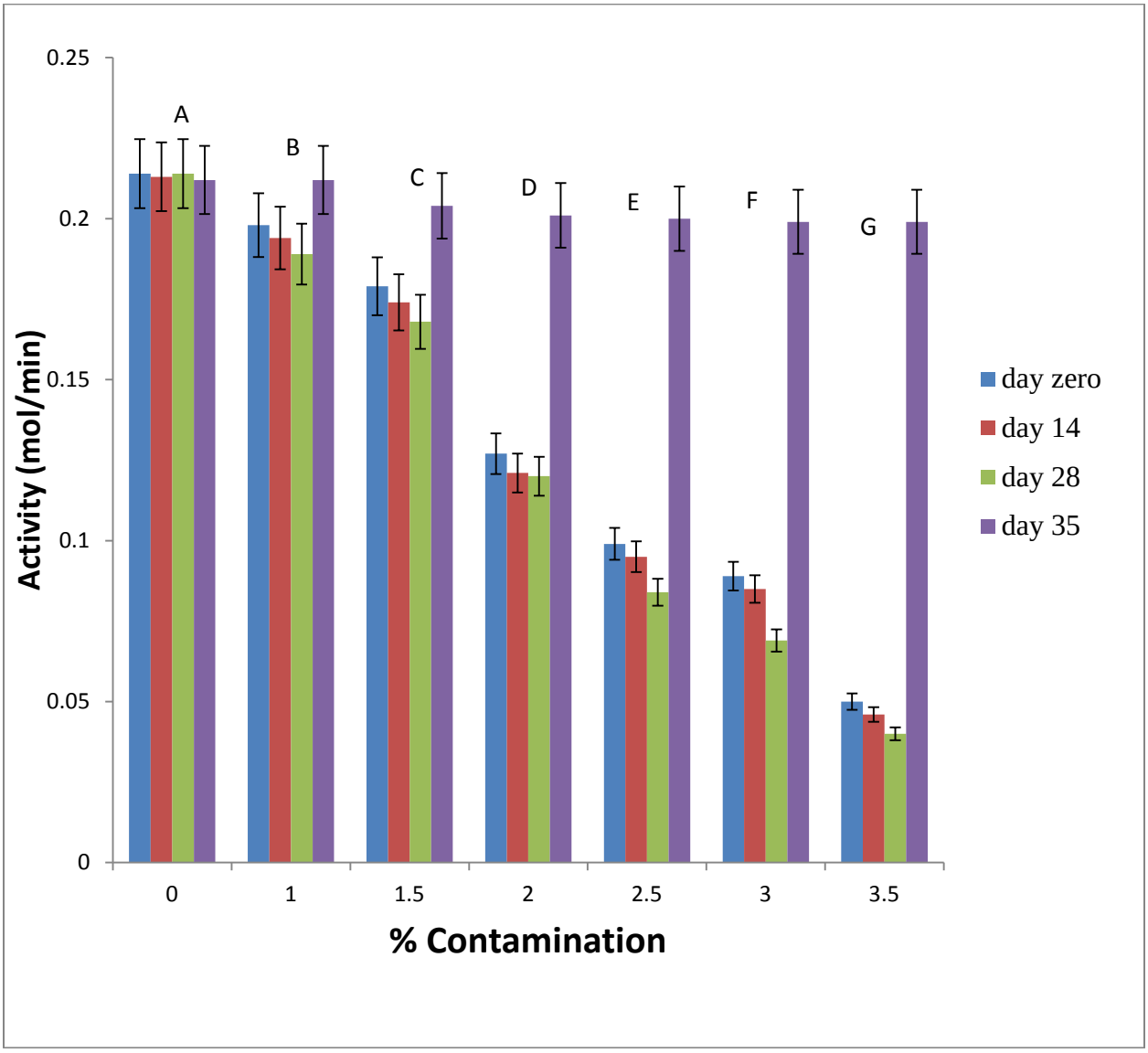

Fig. 2: The activity of soil catalase in the spent oil-impacted soil
Comparison between groups: bars grouped in different letters differ significantly ($p<0.05$).

Effect of spent engine oil on the activity of soil dehydrogenase

Figure 3 presents the effect of spent engine oil on soil dehydrogenase as it contrasted sharply with that obtained in soil catalase. Relative to the control, the oil stimulated the activity of soil dehydrogenase significantly ($p<0.05$) overtime up to day-28 as the concentration increased.

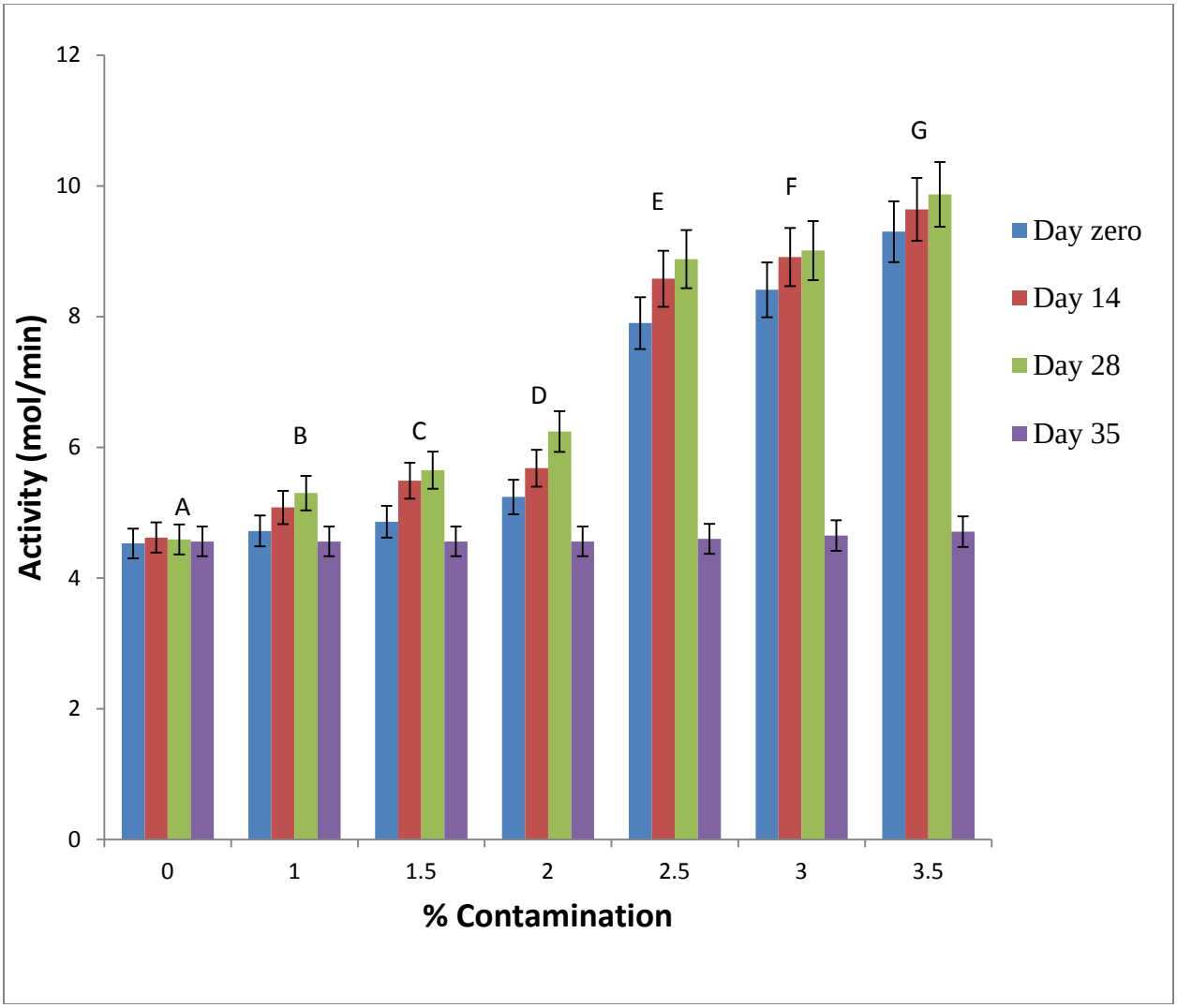

Fig. 3: The activity of soil dehydrogenase in the spent oil-polluted soil
Comparison between groups: bars grouped in different letters differ significantly ($p<0.05$).

DICUSSION

Spent engine oil caused a reduction in soil pH in a concentration and time dependent manner. This may be attributable to microbial metabolism of the hydrocarbon present in the oil, which consequently gave rise to the production of organic acids. 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. Soil pH governs the rate and extent of microbial succession and degradation of the added petroleum hydrocarbons. The influence of soil pH on oil decomposition was also evaluated by McGill and Nyborg (1975) who found that oil decomposition and microbial activity was slow under acidic soil conditions. Bacteria are

probably the most important group at least in the early stages of oil decomposition; but as their actions from this investigation lowered the pH, the degradation of the hydrocarbon input was delayed.

Following an oil spill, the microbial population in the soil passed through a short period of adaptation or lag phase on day-zero. The lag phase encountered in this study upon the application of spent oil was caused by the toxicity of low boiling aromatic hydrocarbons. Walker *et al.* (1975) and Rowell (1975) reported similarly the microbial lag phase following the introduction of hydrocarbon from oil and attributed it to the toxicity of the later where they concluded that the time lag was equivalent to the time required for the active oil degrading microbial populations to grow and synthesize the enzymes required for oil decomposition. Thus, a decrease in the bacterial population in the soil sample contaminated with spent engine oil supports the report that spent engine oil is prejudicial to soil environments. The oil elicited its acute toxicity effects on some bacterial strains and those which could not withstand this toxicity were eliminated, some became extinct; while the hydrocarbonclastic strains survived it. However, at increased concentrations overtime, there was increase in bacterial population. The implication in this upsurge was attributable to the ability of some of the extinct ones to adapt to the unfavourable conditions and multiply along with the hydrocarbonclastic strains and thereby increased the biomass.

Overall, the result implicated that at increased contamination, hydrocarbons increased the abundance of hydrocarbon-degrading microorganisms (the hydrocarbonclastics), which comprised *Pseudomonas aeruginosa, Micrococcus varians and Bacillus subtilis*, but on the other hand, induced a limitation on microbial diversity.

In this study, spent engine oil did not only reduce the total aerobic bacterial count of the fresh soil sample in a concentration and time dependent manner; soil catalase suffered a similar fate. This may not be surprising because the activity of catalase in the oil-polluted soil was gradually inhibited. Thus, there was a progressive decline in the enzyme activity which was significant as the concentration and contact time of the contaminant increased. This could be because catalase being an enzyme, its activity was altered by unfavourable conditions such as hypoxia, unavailability of nutrients and changes in pH. This portrays that soil catalase is irritable to hypoxic changes, and any condition that creates oxygen tension adversely affects its activity. It then follows that the aerobic bacterial status / population has a correlation with the activity of soil catalase. This finding is in consonance with the report of Waarde *et al*. (1995), Margesin and Schinner (1997), Achuba and Peretiemo-Clarke (2008) on the inhibition of the activity of soil catalase following an insult of soil ecosystem with spent oil.

On the other hand, our investigation revealed that spent engine oil increased significantly the activity of soil dehydrogenases in a concentration and time dependent manner. The stimulatory effect of the oil on the aforesaid enzyme and its induction was stronger as the contamination rates and duration of contact increased; which in turn correlated with the upsurge of hydrocarbonclastic organisms. This finding is in harmony with the work of Schinner *et al*. (1996), Wyszkowska *et al*. (2002), Wyszkowska and Kucharski (2005), Achuba and Peretiemo-Clarke (2008) who reported that dehydrogenase activity in the soil was a function of microbial activity and respiration rate.

CONCLUSION

Spent engine oil which permeates the soil has adverse influence in the soil environment; which was concluded from the observations of the upset of the biochemical equilibrium of the soil measured as the activities of dehydrogenase and catalase, pH and total microbial counts. The present study demonstrated that the actual effect of spent engine oil on the biochemical activities of the soil depended largely on the degree of contamination and duration of contact with this petroleum derivative. The concentration of the hydrocarbonclastic bacteria in the spent oil-contaminated soil correlated with the degree of hydrocarbon input, contact time, enzyme induction and its activity. The degradative activity of these hydrocarbonclastic organisms is the hallmark of detoxification and hydrocarbon decontamination of the ecosystem insulted with spent oil. The stimulatory, and or inhibitory effect produced by spent oil pollution on the activities of the aforementioned soil enzymes, and other parameters was particularly evident in the soil polluted with the highest degree of this petroleum product. The result indicated that soil enzyme activities are biomarkers and attractive indicators of soil ecosystem polluted with spent engine oil.

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