Original Research Article

Variance in Protease, Dehydrogenase, Phosphatase and Respiratory Activities during Phytoremediation of Crude Oil Polluted Agricultural Soil Using *Schwenkia americana* and *Spermacoce ocymoides*

ABSTRACT

Aims: To investigate the variation in the activities of some soil enzymes and microbial respiration during phytoremediation of crude oil polluted agricultural soil.
Study design: Indigenous plants of a crude oil polluted agricultural farmland were harvested and identified. Two species (*Schwenkia americana* and *Spermacoce ocymoides*) were selected for the study. Nursery was set up using sterile soil and mature and viable seeds of the selected species, and germinated seedlings were transplanted into an 8 kg potted homogenized polluted soil for remediation.
Place and Duration of Study: Polluted agricultural soil from Ogoniland Nigeria, University of Port Harcourt ecological garden, between May 2017 and February 2018.
Methodology: Protease activity was determined based on the amino acids released after incubation of the soil with sodium caseinate. Phosphatase activities determination was based on determining the extent of degradation of p-nitrophenol phosphate (PNPP) by the samples. Dehydrogenase activity was determined based on the estimation of the rate of reduction of 2,3,5- triphenyltetrazolium chloride (TTC) to triphenyl formazan (TPF) in soils after incubation. The substrate induced method was adapted to estimate the respiratory activity. Organic matter was determined by loss of weight on ignition method.
Results: Dehydrogenase activities of the remediated groups increased after 4 weeks but decreased at the end of the remediation period. Protease and phosphatase activities, and soil organic matter of the remediated groups reduced over time while the soil microbial respiratory activity reduced at the end of the 12 weeks remediation. A trend between organic matter and enzyme and respiratory activities was revealed.
Conclusion: Soil microbial activities can reflect soil quality, and soil enzyme activities can directly reflect the metabolic need and nutrient availability of soil microorganisms. The extracellular enzymes (protease, dehydrogenase, acid and alkaline phosphatase) were shown to vary with crude oil pollution relative to time thus indicating ameliorative effects.

Keywords: Protease, Dehydrogenase, Phosphatase, Respiratory Activity, Phytoremediation, *Schwenkia americana, Spermacoce ocymoides*

1. INTRODUCTION

Soil is an abode for life [1] and its contamination by petroleum hydrocarbons emanating from commercial exploration and spillage [2] from the activities of the petroleum industry (including oil exploration, drilling, production, transportation, processing and storage), well blow-outs, pipeline rupture, tanker accidents, and pipeline vandalization by saboteurs and hoodlums poses a risk challenge in many oil producing areas ascribable to their environmental consequences to man [3].

Oil pollution dreadfully affects the soil ecosystem through adsorption and surface assimilation of soil particles purveying of an excess carbon which might be unattainable for microbial use and the

investiture of a constraint in soil nutrients [4]. During oil spillages, non-organic compounds, carcinogens, and growth inhibiting chemicals obtainable in crude oil are introduced to the environment [3], and protracted exposure to acute oil contamination could result to the instigation of kidney and liver diseases, mutilation of bone marrow and intensified risk of cancer [5]. There is a proportional reduction in contaminant extraction and biodegradation as the interaction between particles of soil and pollutants increase [6]. Biodegradation makes use of bacteria, fungi or various biological means to disintegrate materials. Microorganisms possess a great ability to metabolize degradable contaminants by employing them as energy source and/or converting them to non-toxic product such as carbon dioxide, biomass and water. This relies on the nature and amount of hydrocarbons present [7].

Microbial and enzymatic activities of the soil can reveal succinctly the quality of soil [8]. The activities of soil enzymes can be used to reveal the metabolic need and nutrient availability of soil microorganisms which are essential in the processing and recovery of key nutrients from detrital inputs and accumulated soil organic matter [9]. Extracellular enzymes such as proteases, dehydrogenases and phosphatases are involved in the process of organic matter decomposition and cycling of key elements such as carbon, nitrogen and phosphorus [10]. Studies have revealed that enzyme activities in the soil are related to heavy metal contamination. Almost all enzyme activities in soils are significantly reduced by 10 to 50 times with the increase of the concentration of heavy metals in the soil [11]. Heavy metal toxicity affects microbial population size, diversity, and activity and also affects their genetic structure. It also alters the nucleic acid structure, disrupts cell membrane, and causes functional disturbance thereby inhibiting the enzyme activity and oxidative phosphorylation, causing lipid peroxidation and altering osmotic balance and protein denaturation [12]. This study thus assays for the presence of some soil enzymes in crude oil polluted agricultural soil and their activities with respect to remediation of the soil using *Schwenkia americana* and *Spermacoce ocymoides*.

2. METHODOLOGY

A polluted agricultural farmland located in Ogoniland, Nigeria was identified at Bodo community, Gokana L.G.A. of Rivers state and assessed to ascertain the types of contaminants involved and to determine the most appropriate technologies for its restoration. In the assessment, the site was mapped to determine its physical characteristics, size and location of contaminants as well as the plant ecological community. Thereafter, indigenous plants of the polluted site were harvested and taken to the Department of Plant Science and Biotechnology, University of Port Harcourt, Nigeria for identification. Two species (*Schwenkia americana* and *Spermacoce ocymoides*) were selected for the study owing to existing reports on their survival ability in polluted environments. Soil samples from crude oil polluted site and agricultural soil from natural matrix within the University of Port Harcourt were collected following the described method [13]. Nursery was set up using sterile soil and mature and viable seeds of the selected species. Three to four weeks after germination, 4 seedlings each of the plants were transplanted into an 8 kg potted homogenized polluted soil set up in triplicate alongside unvegetated polluted and unpolluted control soils. Soil sampling was carried out prior to the transplant and subsequently at 4th, 8th and 12th week. Fresh soil samples were collected and taken immediately to the laboratory. The activities of acid and alkaline phosphatase, dehydrogenase and protease were assayed and the organic matter content of the soil was determined.

The method [14] with modification was employed for all enzyme assays and respiratory activity performed in triplicate and compared to controls while soil organic matter was determined by loss of weight on ignition method [13].

The assay for protease activity is based on determining the amino acids released after incubation of the soil with sodium caseinate for 2 hours at 50^{o}C using Folin-Ciocalteu reagent. Two grams of moist, sieved (2 mm) soil was weighed into a 15-mL centrifuge tubes designated as test and control. Aliquot (5 mL) of 1% substrate, prepared a night before and kept in a refrigerator, was added to the test tubes. For the controls, only 5 mL of TRIS HCl buffer at pH 8.1 was added. The tubes were shaken for 2 hours at 50^{o}C and cooled immediately in cold water. An aliquot of 2 mL 17.5% trichloroacetic acid was added into test and control tubes and centrifuged at 3000 rpm for 2 minutes. The supernatant (2 mL) was dispensed into test tubes, and 3 mL 1.4M $NaSO_4$ was added in both the test and control tubes. The tubes were shaken thoroughly and 1 mL of dilute Folin-Ciocalteu reagent, prepared by diluting three times, was added and the content of the tubes centrifuged at 200 rpm for 2 minutes. An

aliquot from each tube was taken and read on a spectrophotometer at a wavelength of 578 nm. TRIS HCl buffer at pH 8.1 was used as blank and calibration curves were prepared.

Enzyme activity (mg tyrosine kg^{-1} dry matter h^{-1}) = (C x 10.5) / dw

where C = measured tyrosine concentration; dw = dry weight of 2g moist soil.

Acid and alkaline phosphatase activities were assayed based on the determination of the extent of degradation of ρ-nitrophenol phosphate (PNPP) by the samples. One gram each of air-dried and homogenized soil samples was placed in centrifuge tubes and 0.25 mL toluene was added, and placed in a fume chamber for 10 minutes. Thereafter, 4 mL of ACP (or ALP) buffer solution was added followed by the addition of 1 mL of ACP (or ALP) PNP substrate. The contents of the tubes were incubated for 1 hour at 37°C. Aliquots of 1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.5 M NaOH were added to the tubes, whose contents were shaken with a rotator mixer for 3 minutes and centrifuged at 4000 rpm for 10 minutes. The supernatant was read spectrophotometrically at 485 nm using ACP (or ALP) buffer solution as blank. The controls were prepared similarly but without the substrate while the calibration curve was prepared using standard p-nitrophenol solution. The ρ-nitrophenol per mL of filtrate was calculated by reference to the calibration curve. The *ρ*-nitrophenol released after incubation was used to calculate the enzyme activity as follows:

Enzyme activity (mmol PNP kg^{-1} dw h^{-1}) = (C x V) / (dw x SW x t)

where C = measured concentration of *ρ*-nitrophenol in *μ*g ml^{-1} filtrate; V = total volume of soil suspension in ml; dw = dry weight of 1 g moist soil; SW = weight of the soil sample used; and t = incubation time in hours.

The assay of dehydrogenase (DH) activity is based on the estimation of the rate of reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to triphenyl formazan (TPF) in soils after incubation at 30 °C for 24 hours. Five grams of field-moist soil was prepared in centrifuge tubes designated as test and control tubes. Using Eppendorf pipette, 5 mL of the 1% TTC was added to the tes es while 5 mL of TRIS HCl buffer at pH 7.4 was added to the control tubes. The content of the tubes was mixed thoroughly and incubated at 30 °C for 3 d. After incubation, 20 mL of methanol was added in all the tubes and shaken for 5 minutes on a turnover shaker at room temperature and then centrifuged at 300 rpm for 3 minutes. An aliquot from each of the tubes was taken and analyzed using a spectrophotometer at a wavelength of 485 nm, using TRIS HCl at pH 7.4 as blank. The *ρ*-nitrophenol contents of the filtrates were extrapolated from the calibration curve. The amount of TPF formed was used to evaluate the enzyme activity as follows:

Enzyme activity (mg TPF kg^{-1} d^{-1}) = (C x v) / (dw x SW x t)

where C = measured concentration of TPF in mg ml^{-1} filtrate; v = total volume of soil suspension in ml; dw = dry weight of 1 g moist soil; SW = weight of the soil sample used; and t = incubation time in hours.

The substrate (glucose) induced method was adapted to estimate the respiratory activity. A 10 g screened (2 mm sieve) soil sample was added into the outer jar of respiratory flask. An aliquot of 2.5 mL 0.2 M NaOH was added into the inner jar. Glucose solution (1 mL) was added to the soil. For the control flasks, screened (2 mm) 10 g of soil sterilized in an autoclave at 121 °C for 20 minutes was used. An aliquot of 2.5 mL 0.2 M NaOH was added with 1 mL 40% glucose as describe above. The flasks were allowed to stand for 24 hours at 25 °C. Afterwards, all the NaOH was transferred to a 25 mL beaker containing 1 mL 1 M $BaCl_2$ and the solution was titrated using 0.1 M HCl and phenolphthalein indicator. The titre values were noted for calculation.

To calculate the mass of CO2 generated:

$CO_2.C$ (mg) = [(HCl_b – HCl_s) / 1000 mL/L] × HCl molarity (mol/l) × 12g C/mol × 1000 mg/g

Where HCl_b = ml HCl used in titration of blank; HCl_s = ml HCl used in titration of sample; CO_2•C = mass of CO_2-carbon generated (mg).

This simplifies to:

$CO_2.C$ (mg) = (HCl_b – HCl_s) × 12

To determine the organic matter content of the soil samples, five grams of sieved (2 mm) soil samples were weighed into crucibles. The crucibles with the soil samples were placed in a drying oven, set at 105 °C and allowed to dry. After 4 hours, the crucibles were removed from the drying oven and placed in a dry atmosphere. When cooled, the crucibles with the soil samples were weighed to the nearest

0.01 g. Again, the crucibles with the dried soil samples were placed in a muffle furnace, set at 400 °C. After 4 hours of ashing, they were removed from the muffle furnace, cooled in a dry atmosphere, and reweighed to the nearest 0.01 g.

The percentage organic matter is given by:

$$\% \text{ OM} = [(W_1 - W_2) / W_1] \times 100$$

where W_1 = the weight of soil at 105°C; W_2 = the weight of soil at 400°C.

3. RESULTS AND DISCUSSION

The protease activities of the various soil samples are presented in Table 1. Compared to baseline values, the protease activities of the remediated groups reduced over time. This may be due to the inhibitory influence of the remediating plants on the soil microorganisms. It may however be due to the limiting effect of nutrients in the pots, since they have been depleted over time, with the resultant reduction in microbial activity. The later argument may account for the reduction observed for the unpolluted group. The former contention can be substantiated by the findings of [15] that plant extracts of *M. alternifolius* and other plants inhibited the growth of certain fungi and bacteria, with *M. alternifolius* strongly inhibiting the fungi *P. chrysogenum* and bacteria *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhi*. This study also revealed a trend between organic matter and enzyme and respiratory activities. Pearson's correlation coefficient (PCC, a measure of the linear correlation or dependence between two variables) of -1.00, -0.98 and -0.80 (Table 7) for protease activity in unpolluted control, polluted control and *S. americana* treated groups, respectively, showed substantial negative correlations with organic matter (OM) where as the soil treated with *S. ocymoides* showed fair positive correlation (+0.47 PCC) as shown in Table 7.

Table 1. Protease activity (in mg tyrosine kg^{-1} dry matter h^{-1}) of unpolluted control, polluted control, *Schwenkia americana* and *Spermacoce ocymoides*.

Group	Before	Week 4	Week 8	Week 12
Unpolluted Control	32.9021±3.86^{a}	38.6255±0.77^{a*}	20.3461±1.59^{a*}	11.9031±1.00^{a*}
Polluted Control	44.4372±0.77^{b}	21.9986±8.06^{b*}	13.6596±43.47^{a,b*}	2.6944±2.88^{b*}
Schwenkia americana	44.4372±0.77^{b}	24.1303±3.32^{b*}	10.5389±1.18^{b*}	1.3813±0.47^{b*}
Spermacoce ocymoides	44.4372±0.77^{b}	23.6187±16.81^{a,b*}	6.2756±5.56^{b*}	2.4046±1.19^{b*}

Values are mean ± standard deviations of triplicate determinations.
Values in the same column with different letters (a,b) are significantly different at P = .05.
**P = .05 compared to the corresponding values before treatment.*

The dehydrogenase activities of the various soil samples are presented in Table 2. Compared to baseline values, the dehydrogenase activities of the remediated groups showed a significant (p<0.05) rise in activity after 4 weeks but reduced at the end of remediation. The increase may have been a result of an initial increase in microbial population within the first 4 weeks which afterwards reduced with depletion of carbon source or available nutrients, since they have been depleted over time, with the resultant reduction in microbial activity. Though there might be available nutrients in the unpolluted group, the absence of carbon source may account for the insignificant activities observed. [16] Reported an undesirable reduction in the dehydrogenase activity and associated that with the low activities of microorganisms in polluted soil. [17] Made a clearer and more acceptable report that both the microbial population, activity of the microbial population and the kind of microbe present in the soil determine the enzyme activity. This trend as observed in Table 2 follows similar trends [18], [19], [20]. Substantial positive correlation (+0.96 PCC) for dehydrogenase activity and OM was observed only in the soil treated with *S. americana.* While the unpolluted control and soil treated with *S. ocymoides* showed a fair positive correlation of +0.36 and +0.55 respectively, the polluted control soil showed a substantial negatively correlation (-0.90 PCC).

Table 2. Dehydrogenase activity (in mg TPF kg^{-1} d^{-1}) of unpolluted control, polluted control, *Schwenkia americana* and *Spermacoce ocymoides*.

Group	Before	Week 4	Week 12
Unpolluted Control	1.1837±0.95^{a}	7.6737±6.72^{a}	0.6091±0.08^{a}
Polluted Control	0.1528±0.21^{b}	11.4075±6.66^{a*}	0.6318±0.11^{a}
Schwenkia americana	0.1528±0.21^{b}	5.2486±2.72^{a*}	0.9236±0.40^{b}
Spermacoce ocymoides	0.1528±0.21^{b}	7.4165±0.61^{a*}	0.1450±0.06^{c}

Values are mean ± standard deviations of triplicate determinations.
Values in the same column with different letters (a,b) are significantly different at P = .05.
**P = .05 compared to the corresponding values before treatment.*

The acid and alkaline phosphatase activities of the various soil samples are presented in Tables 3 and 4. Compared to baseline values, the acid phosphatase activities (Table 3) of the remediated groups reduced over time. This may be due to the inhibitory influence of the remediating plants on the soil microorganisms. It may however be due to the limiting effect of nutrients in the pots, since they have been depleted over time, with the resultant reduction in microbial activity. The later argument may account for the reduction observed for the polluted and unpolluted groups, since the pots were unvegetated. However, the increase observed at week 8 for unpolluted group may indicate a rise in peak in microbial activity which may have reduced owing to the depletion in available nutrients. Likewise, compared to the baseline values, alkaline phosphatase activities of the remediated groups, as shown in Table 4, reduced over time albeit a recorded increase in *S. ocymoites* treated group at week 8. The reduction may be due to the hampering influence of the remediating plants on the soil microorganisms. Nonetheless, it may be due to the limiting effects of nutrients in the pots as depletion may have taken place over the period of time, thus resulting to reduction in microbial activity. If the later argument is true, it may therefore account for the reduction observed for unpolluted and polluted groups. However, the population and/or the presence of certain microorganisms specific for alkaline phosphatase secretion may have influenced the increase in activity recorded in *S. ocymoides* treated group at week 8. This finding is supported by the report [17], that microbial population, activity and the kind of microbe present in the soil determine the enzyme activity. Acid phosphatase activity showed a substantial positive correlation with OM for the unpolluted control and *S. americana* treated soil (+0.84 and +0.80 PCC, respectively). However, whilst the soil treated with *S. ocymoides* showed a fair positive correlation (+0.55PCC); the polluted control soil indicated almost no correlation (+0.05 PCC). On the other hand, alkaline phosphatase activity revealed a fair positive correlation with OM for unpolluted control, polluted control and soil treated with *S. americana* (+0.61, +0.52, and +0.36 PCC, respectively), and its correlation with OM for *S. ocymoides* treated soil revealed a fair negative correlation of -0.57.

Table 3: Acid phosphatase activity (mmol PNP kg^{-1} dw h^{-1}) of unpolluted control, polluted control, *Schwenkia americana* and *Spermacoce ocymoides*.

Group	Before	Week 4	Week 8	Week 12
Unpolluted Control	1.5190±1.06^{a}	1.6357±0.15^{a}	7.5831±1.22^{a}*	0.0485±0.06^{a}
Polluted Control	5.4736±1.74^{b}	1.5739±1.00^{a}	2.8370±4.61^{a,b,c}	0.1502±0.04^{a*}
Schwenkia americana	5.4736±1.74^{b}	4.5386±1.21^{b}	6.1315±1.72^{c}	0.2076±0.39^{b,c*}
Spermacoce ocymoides	5.4736±1.74^{b}	6.5304±1.51^{c}	0.1773±0.50^{b}*	0.2127±0.25^{a,c*}

Values are mean ± standard deviations of triplicate determinations.
Values in the same column with different letters (a,b) are significantly different at P = .05.
**P = .05 compared to the corresponding values before treatment.*

Table 4: Alkaline phosphatase activity (mmol PNP kg⁻1 dw h⁻1) of unpolluted control, polluted control, *Schwenkia americana* and *Spermacoce ocymoides*.

Group	Before	Week 4	Week 8	Week 12
Unpolluted Control	3.2944±1.11[a]	0.1846±0.16[a,b*]	3.2186±1.20[a]	0.6330±0.43[a*]
Polluted Control	4.3601±1.06[a]	0.5912±0.26[a,c*]	3.3636±1.05[a]	1.2072±0.96[a*]
Schwenkia americana	4.3601±1.06[a]	0.4942±0.07[c*]	0.1835±0.04[b*]	0.6294±0.31[a*]
Spermacoce ocymoides	4.3601±1.06[a]	0.2753±0.64[b*]	11.4072±2.44[c*]	0.8385±0.69[a*]

Values are mean ± standard deviations of triplicate determinations.
Values in the same column with different letters (a,b) are significantly different at P = .05.
**P = .05 compared to the corresponding values before treatment.*

Assessment of oxidation of organic matter by aerobic microorganisms, known as respiration, confirmed microbial activity in all the soils. According to [21], soil respiratory activities and microbial abundance are sensitive to contamination with petroleum derivatives. [22] Associated decline of respiratory activity similar to what is represented in Table 5 to depleted available carbon substrates. Additionally, respiration declines in soils that lack nutrients and other supporting factors for microbial and other biological activities [23]. While respiratory activity showed a substantial negative correlation with OM for the unpolluted control (-0.96 PCC), its correlation with OM for polluted control showed arguably no correlation (-0.07 PCC). Nonetheless, a fair positive correlation was recorded in *S. americana* treated and *S. ocymoides* treated soils (+0.51 and +0.54 PCC, respectively).

Table 5: Respiratory activity (CO_2.C) (in mg) of unpolluted control, polluted control, *Schwenkia americana* and *Spermacoce ocymoides*.

Group	Before	Week 12
Unpolluted Control	1.4400±0.01[a]	0.800±0.37[a*]
Polluted Control	1.9200±0.01[b]	0.24±0.12[a*]
Schwenkia americana	1.9200±0.01[b]	0.40±0.37[b*]
Spermacoce ocymoides	1.9200±0.01[b]	0.28±0.18[c*]

Values are mean ± standard deviations of triplicate determinations.
Values in the same column with different letters (a,b) are significantly different at P = .05.
**P = .05 compared to the corresponding values before treatment.*

As shown in Table 6, the soil organic matter in the remediated and polluted control groups, when compared with the baseline values, reduced over time. Organic matter is the major source of plant nutrients [24] and its mineralization depends on the interaction between the chemicals present in the soil [4]. [25] Reported that the decomposition of organic matter is largely a biological process that occurs naturally and determined by soil organisms, the physical environment and the quality of the organic matter. The reduction in organic matter in the groups may therefore be associated with its utilization by the microorganisms to release nutrients for use by plants and microorganisms.

Table 6: Organic matter (in %) of unpolluted control, polluted control, *Schwenkia americana* and *Spermacoce ocymoides*.

Group	Before	Week 8	Week 12
Unpolluted Control	2.4900±0.01[a]	2.4700±0.22[a]	2.2267±0.19[a]
Polluted	4.800±0.10[b]	4.0200±0.09[b*]	3.7767±0.14[b*]

Control			
Schwenkia americana	4.800±0.10[b]	3.8467±0.24[b*]	3.7267±0.11[b*]
Spermacoce ocymoides	4.800±0.10[b]	3.8067±0.25[b*]	3.5767±031[b*]

Values are mean ± standard deviations of triplicate determinations.
Values in the same column with different letters (a,b) are significantly different at P = .05.
**P = .05 compared to the corresponding values before treatment.*

Table 7: Pearson's correlation coefficient (PCC) of observed enzyme activities versus organic matter (OM)

Enzyme Activity	Unpolluted control	Polluted control	*S. americana*	*S. ocymoides*
Protease	-1.00*	-0.98	-0.80	+0.47
Dehydrogenase	+0.36	-0.90	+0.96	+0.55
Acid phosphatase	+0.84	+0.05	+0.80	+0.55
Alkaline phosphatase	+0.61	+0.52	+0.36	-0.57
Respiratory	-0.96	-0.07	+0.51	+0.54

4. CONCLUSION

Crude oil spillage presents deleterious effects on the environment. Both microbial activities of the soil can reflect sensitively the quality of soil, and soil enzyme activities can directly reflect the metabolic need and nutrient availability of soil microorganisms which are important key nutrients' processing and recovery from detrital inputs and accumulated soil organic matter. Microorganisms secrete degradative enzymes which can counter the effect poised by the spillage thus effecting amelioration of the pollutants' effects in the polluted soil. The extracellular enzymes; protease, dehydrogenase, acid and alkaline phosphatase activities are shown to vary with crude oil pollution relative to time thus indicating ameliorative effects.

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