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Bioremediation of Vegetable Oil Contaminated Soil with Two Microbial Isolates

Bukola Margaret Popoola*, Afolake Atinuke Olanbiwoninu, Rachael Oluyemisi Fashogbon

Department of Biological Sciences, Ajayi Crowther University, Oyo, Nigeria
Email: *bukolamargaret@yahoo.com

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Abstract

Vegetable oil spills are becoming frequent and are potentially more challenging than petroleum hydrocarbon spills. Microbial lipases occupy a place of prominence among biocatalysts and are often used for the remediation of vegetable oil spills. There is a need for extensive characterisation of lipase for the treatment of vegetable oil-polluted sites. This work was carried out to monitor the degradation pattern of vegetable oil. Two microbial isolates previously isolated from an oil mill in Ibadan, Nigeria were used for the bioremediation experiment. Soil samples (with some purposely contaminated with 2 different vegetable oils) collected from the Nursery section of the Microbiology department as well as soil samples from the oil mill were all subjected to varied treatment processes. Field bioremediation using the isolates was carried out for 12 weeks. The isolates were identified, and microbial load and residual oil weight were determined during the degradation period using standard methods. The two isolates were identified as *Pseudomonas fluoresecens* and *Candida parapsilosis.* The result of sterile soil samples with and without mixing option, from palm oil and palm kernel purposely contaminated soils for all the various treatments, showed a general increase in total viable counts from the 2nd week to the 12th week, however in the non-sterile counterpart there was a steady increase from the 2nd week to the 8th week and subsequently, a gradual decrease from the 10th to the 12th week. The residual oil weight in the sterile purposely palm oil-contaminated soil, treated with the consortium (POC) non-mixed gave a reduction of value from 0.335 g on day 0 to 0.13025 g by the 12th week. From the oil mill non-sterile, treated with (POC) non-mixed sample, the residual weight after 12 weeks of treatment was 0.0043 g from an initial weight of 0.01 g. The microorganisms *Pseudomonas fluoresecens* and *Candida parapsilosis* had the potential for the degradation of fatty waste. They could therefore be employed in the environmental cleanup of the vegetable oil spill sites.

Keywords

Vegetable Oil Spill, Bioremediation, *Pseudomonas fluoresecens*, *Candida parapsilosis*

1. Introduction

1.1. Environmental Impact of Edible Oil Pollution

Historically, vegetable oils have been considered relatively benign, non-toxic, and therefore of limited concern to the environment. However, this generalization has been demonstrated to be incorrect as previous experience has shown that both chronic and acute pollution incidents can lead to deleterious effects [1]. This awareness has led to the reclassification of many vegetable oils as category Y (hazardous) products under Annex II of the MARPOL Convention with associated limitations on their carriage.

1.2. Potential Environmental Impacts

Vegetable oils are harmful to the environment; like petroleum oils, they produce similar environmental effects. If spilled on the land, vegetable oils are almost undetectable. They cause devastating physical effects such as coating bird feathers, gills of aquatic animals, plants sediments, plant stomata, and other surfaces with oil and suffocating them by oxygen depletion (the oils cause depletion of oxygen in the water column to a level below what aquatic life needs) [1]. Experimentation has shown some effects such as reduced growth rates, poor food conversion, and liver impairment in fish and bivalves resulting from prolonged ingestion [1]. They destroy future and existing food supplies, breed animals, and habitats, and produce rancid odors. They create foul shorelines, clog water treatment plants, catch fire when ignition sources are present, and form products that linger in the waters and environment for many years.

1.3. Remediation of Polluted Environment in the Edible Oil Industry

Remediation is considered the management of the contaminant at a site so as to prevent, minimize or mitigate damage to human health, property, or the environment [2]. It is a broader term than Clean up in that remediation options can include physical actions such as removal, destruction, and containment.

Types of Remediation

1) Biological Treatment in the Edible Oil Industry

A wide variety of industries (e.g. the dairy industry and food processing) produce effluents rich in fats, oils, and greases (FOGs). High concentrations of FOGs in wastewater often initiate problems in wastewater treatment processes [3] [4]. FOGs often cause foul odours, as well as blockage of pipes and sewer

lines. These problems are solved by the preliminary refining equipment, so called grease traps. Grease traps may sometimes fail to retain dissolved and emulsified FOGs, allowing them to enter the water treatment system. An interesting strategy at the present time is the use of lipase-producing microorganisms in wastewater treatment systems [5].

2) Bioremediation and Its Advantages

Bioremediation is defined as the process whereby various microorganisms degrade environmental contaminants into less toxic forms. Compared with conventional treatment, the main advantages of bioremediation:

a) It uses relatively low-cost, high efficiency, no additional nutrient requirement, site disruption is minimal it can often be done on site and has greater public acceptance.

b) It uses primarily microorganisms or microbial processes to degrade and transform environmental contaminants into harmless and less toxic forms.

c) It uses bacteria, fungi, and plants obtained from natural habitats.

3) Chemical Remediation

This involves the treatment of any contaminated soil, water, or air with a chemical substance that locks up changes, or breaks down the toxic compound into a safe form. Remediation by oxidation involves the injection of oxidants such as hydrogen peroxide, ozone, potassium permanganate, persulfates, oxygen gas, or plain air. Another example is the use of absorbent clays which are mixed, into contaminated soil to absorb pollutants, or incorporated into filters to cleanse water or air passing through.

4) Physical Remediation

A number of the technologies have been adapted from general commercial uses in other industrial sectors. These are considered "conventional" technologies. The term "innovative" refers to technologies that have been developed, especially for the site remediation industry.

1.4. Factors Impacting Perfect Remediation

Many factors affect the selection of potential remediation technologies [6]. These include:

- Contaminant type and characteristics (Properties, volume, location, exposure risk);
- Site characteristics (soil types, permeability, surface and groundwater properties, climate, site infrastructures, topography, location);
- Costs (capital, operating, maintenance);
- Regulatory and public acceptance, and;
- Remediation schedule—Approaches, consideration, and prioritization.

1.5. Biodegradation Characteristics of Vegetable Oils

Lipids (fats, oils, and greases) form a major part of domestic and industrial waste; hence they contribute their fair share towards environmental pollution.

Sources include wastewater from the edible oil refinery, slaughter houses, and dairy industry products. These waste products are responsible for clogging sewer networks and unsettling the balance of wastewater treatment plants [7].

The first step in the degradation of vegetable-based oils is the enzyme-catalysed cleavage of the ester bond to a fatty acid. The enzymes which catalyse this biodegradation reaction include esterases and lipases which are synthesized by a wide range of microorganisms [8]. Lipases are hydrophobic proteins that catalyze the cleavage of carboxyl esterbonds in tri-, di-, and monoacylglycerols (the major constituents of animal, plant, and microbial fats and oils) [7]. Due to their degradability effects, lipases are applied in remediation efforts to degrade lipid-rich water. Drawbacks, however, include the thermal instability of the enzyme and the high cost of the single use of the enzyme [7]. Following the first step of degradation both saturated and unsaturated fatty acids biodegrade via a process of β-oxidation.

Indeed, the biodegradation of materials also is dependent on the nature of the environment, as documented by [9] where pH adjustments of soils for instance were found to have a not incurable effect on the biodegradation of certain compounds [9]. Microbial communities are also prone to adapt to a substrate when it is a regular contaminant which has led to the identification and documentation of increases in rates of transformation of hydrocarbons associated with oil-contaminated environments [10]. Modified strains of bacteria emerge which are characterized by the ability to degrade the substances which induce the modification [11] [12]. However, biodegradability primarily is a function of the chemical nature of the substrate in question.

That natural and vegetable oils are biodegradable is not in doubt as documents show that "vegetable oils and synthetic esters have a much better biodegradation capacity than mineral oil under aerobic as well as anaerobic conditions" [8]. Tests carried out severally indicate that vegetable oils undergo about 70% - 100% biodegradation in 28 days [13].

In a specific comparative study carried out by [14], vegetable-derived lubricants were established to be more biodegradable than comparable mineral-derived lubricants in the presence of tropical mangrove or coral reef microbial communities [14]; while some others have examined the biodegradation of vegetable oils under spill conditions [15] [16]. Several authors have examined the biodegradability and oxidative stability of industrial fluids obtained from vegetable oils. These include; methyl esters [17]; hydraulic fluids [18] [19] and lubricant [20].

However, though they are biodegradable, ongoing research suggests that vegetable oil spills are becoming more common and are potentially more dangerous than hydrocarbon spills because of their toxicity. Records show that the toxicity of products such as canola oil and soybean oil actually increases significantly during aerobic biodegradation [1]. The effects of such a process in a confined, shallow environment could be significant [1]. This study, therefore, aims at monitoring the degradation patterns of the vegetable oil contaminated soils using *Pseudomonas fluoresecens* and *Candida parapsilosis*.

2. Materials and Methods

2.1. Physico-Chemical Analysis

The physico-chemical properties of soil samples collected were determined for the following parameters using the procedure in the method of soil analysis [21].

2.1.1. pH in Water

The soil samples were air-dried and 10 g of the samples were placed in beakers. The suspension was stirred several times over a 30 min interval and the pH of the soil in each beaker was thereafter measured with the glass electrode of a pH meter.

2.1.2. Water Activity a_w

The soil sample was air-dried for three days, thereafter 50 grammes of the soil were placed in a pre-weighed aluminum tray. The aluminum tray containing the soil sample was carefully placed in an oven and dried at 110˚C for 48 hrs. The tray and the oven-dried soil were kept thereafter to a dessicator for 1 hr to cool. The weight of the tray and the oven-dried soil were then taken. The following formula was used to calculate the water activity in the soil.

$$a_w = \frac{\text{Weight of soil before oven drying}}{\text{Weight of oven dried soil}} \times 100\%$$

2.1.3. Percentage of Organic Carbon

The soil sample was grounded to pass through a 60 mm mesh sieve and 1 g of the sieved samples was measured in duplicates into two 250 ml conical flasks. Ten millilitres of 1 N $K_2Cr_2O_7$ solution was pipetted into each flask and swirled gently to disperse the soil. 20 ml of concentrated H_2SO_4 was promptly added directly to the suspension and gently swirled immediately until the soil and the reagents have mixed. It was thereafter shaken vigorously for one minute. The flask was rotated again and allowed to stand on the bench for 30 minutes. 100 ml of distilled water was subsequently added and 3 to 4 drops of ferroin indicator were added and titrated with 0.5 N $FeSO_4$ solution in the burette. Greenish cast coloration in the solution which changed to dark green, marked the end point of the titration.

The $FeSO_4$ solution was at this point added drop by drop until the colour changed rapidly from blue to red. The blank titration (without soil) was carried out in the same manner to standardize the dichromate. The percentage of organic carbon in the soil sample was calculated thus.

$$\text{Percentage of Organic Carbon} = \frac{\text{me. } K_2Cr_2O_7 = \text{me. } FeSO_4 \times 0.003}{\text{Weight of air-dried soils}} \times 100(F)$$

Correction factor, $F = 1.33$.

[Where me. = Normality of solution × ml of solution used]

2.1.4. Total Nitrogen

Five grammes of air-dried soil sample were grounded and transferred to an 11

cm^3 Whatman No. 2 filter paper, wrapped, and dropped into a 500 ml Kjedahl flask. One heap-full of a teaspoon (about 11 g) of the digestion mixture was added into the flask through a long wide stern funnel; 25 ml of concentrated H_2SO_4 was added and swirled gently until the sample and acid are thoroughly mixed. The flask was then placed in the Kjeldahl digestion apparatus and the fume aspirator turned on. The mixture was heated at low heat until the organic matter was destroyed, evidenced by the light gray or straw colour of the mixture. The heat was turned off, and the flask was removed and capped immediately. The whole unit was cooled until distilled water can be added without explosive result. Two hundred and fifty millitres of distilled water was carefully added and mixed thoroughly. While holding the flask at an angle of 45˚, 75 ml of 40% NaOH was carefully and slowly added down the side of the flask to the bottom without mixing. Three pieces of mossy Zn were added and the flask was attached immediately to the distillation unit at the receiving end, with a 500 ml flask containing 25 ml of 4% boric acid (plus indicator) and 250 ml of water. The Kjeldahl flask was gently swirled to mix the content at low heat.

About 200 ml of the mixture was distilled into the receiving flask. The distillate was then titrated with a standard 1 M sulphuric acid until the blue colour disappeared. The end point was indicated by the solution turning pink. The volume of the acid required to titrate the blank (set up as a parallel) was subtracted from the amount required to titrate the sample. This gave the amount of nitrogen in the sample.

2.1.5. Available Phosphorus

Five grammes of the soil samples and a scoop of refined charcoal were placed in a 250 ml conical flask. One hundred millitres of the extracted solution containing 0.5 M $NaHCO_3$ solution adjusted to pH of 8.5 with 0.1 N sodium hydroxide was added and shaken for 30 minutes. The soil suspension was then filtered through a No. 40 filter paper and 5 ml of the filtrate was pipetted into a 25 ml conical flask. 5 ml of 0.5 M ammonium molybdate solution was slowly added. The flask was shaken gently to mix the contents and the neck of the flask was subsequently washed down, diluting the content simultaneously with 22 ml of distilled water. 0.1 ml of dilute $SnCl_2$ solution was added and mixed. The transmittance of the solution was measured in the calorimeter at 660 nm, 10 minutes after adding $SnCl_2$ solution giving the value of the available phosphorus in the soil.

2.1.6. Exchangeable Cations (Na and Mg)

Ten grammes of the soil sample were weighed (10 g) into a 250 ml conical flask. 1 ml of 1 N ammonium acetate was added and the flask stoppered. The suspension was manually shaken intermittently for 30 minutes. The suspension was then filtered through Whatman's No. 1 filter paper. The first 10 to 20 ml of the filtrate obtained was discarded and about 25 ml of it obtained subsequently was collected in a 50 ml conical flask. A flame photometer was used to determine the

concentrations of Na and Mg cations in "ppm" present in the soil sample. This was calculated as follows:

$$\text{ppm cation in soil} = \frac{\text{ppm cation in solution} \times \text{vol. of extractant}}{\text{Weight of soil}} (\times \text{any further dil})$$

$$\text{mg}/100\text{ g of soil} = \frac{\text{ppm cation in soil}}{10 \times \text{mg wt. of cation}}$$

2.2. Bioremediation of Vegetable Oil Polluted Soil

The bioremediation experiment for the treatment of vegetable oil-polluted soil was carried out for 12 weeks from January to April during the dry/raining season in a nursery shed under natural environmental conditions.

2.3. Experimental Design for the Bioremediation Process

The bulk soil from the polluted site was measured out in 500 g each into 12 plastic containers of one litre capacity with perforated bottoms in duplicates. These cups were then divided into two sets. The first set of six cups (in duplicates) represented the sterile soil while the other represented the non-sterile soil.

Bulk soil from unpolluted sites in the nursery area was also collected into 24 plastic containers of one litre capacity with perforated bottoms in duplicates. These cups were further divided into four sets. The first set of six cups (in duplicates) represented sterile soil purposely polluted with 20 ml each of palm kernel oil, the second set represented its non-sterile counterpart, while the third set of 6 cups (in duplicates) represented sterile soil also purposely polluted with 20 ml each of palm oil, the fourth set represents its non-sterile counterpart.

All the sterile and non-sterile soils were further divided into four sets (each containing six cups in duplicate) representing the mixed and non-mixed in the sterile and non-sterile soils. Each of the three microbial isolates from an oil mill site (PO1, CO1, and POC) was then added to each set of cups containing the polluted soil.

The three microbial isolates were represented as follows:

PO1—*Pseudomonas fluorescens*

CO1—*Candida parapsilosis*

POC—*Pseudomonas fluorescens* plus *Candida parapsilosis*

The experimental set-up thus follows:

2 × 2 × 3 design for all the combinations of treatments used.

The treatment options used on sterile and non-sterile for all polluted and controlled soils in this experiment were:

- Addition of microbial inoculum

  PO1, CO1, and POC
- Tilling

  Mixing and non-mixing

The unpolluted control soil samples were combined in the same way for all

the treatments highlighted above.

2.4. Inoculum Preparation and Addition

The modified method of [22] was used. The two isolates (*Pseudomonas fluorescens* and *Candida parapsilosis*) from the polluted soil were used for this bioremediation experiment. They were re-introduced singly (PO1, CO1) and in combination (POC) to inoculate the polluted and the control soils for the biodegradation process. Each of the isolates was first cultured in 100 ml Erlenmeyer flasks containing nutrient and potato dextrose broths at room temperature and 100 rpm using orbital shaker Stuart SSLI for 48 hours to increase the inoculum size.

The number of cells per ml was determined with a Marienfeld haemocytometer and standardised to obtain an inoculum size of about 10^7 per ml. The culture in each flask was then dispersed in normal saline solution to make 1 litre of cell suspension, following which 20 mls of the cell suspension was inoculated into the soil in each cup. The inoculated soils were then mixed thoroughly to ensure the uniform distribution of the added microbial cells.

2.5. Tilling

Soils with the "MIXING" treatment option were tilled twice a week throughout the 12 weeks experimental study period to improve the aeration of the soil and enhance biodegradation. The soil without MIXING was left undisturbed in the cups throughout the study period.

The sterile and non-sterile polluted as well as control soils with and without tilling were represented as follows:

- Sterile with mixing (MS)
- Sterile without mixing (NMS)
- Non-sterile with mixing (MNS)
- Non-sterile without mixing (NMNS)

2.6. Soil Sterilisation

Polluted and unpolluted (control) soil samples were sterilised at 121˚C for 15 minutes for three consecutive days in order to exclude all viable microorganisms present. This was done to ensure that only the inoculated bacterial isolate will degrade the edible oil contaminant present in the soil in order to determine their biodegradability ability.

2.7. Addition of Water

Twenty millilitres of water was added twice a week to all the soils in the cups to prevent their drying throughout the 12 weeks experimental period.

2.8. Bioremediation

Soil samples were taken bi-weekly for 12 weeks from each bowl of each treat-

ment option by scooping the soil at the top, middle, and bottom with a sterile metal spoon so that the sample taken will be a true representation of the treated soil samples. The biodegradation process was determined by the following methods.

2.9. Total Viable Count Enumeration

The total viable microbial count of the soil sample was determined for 12 weeks in order to enumerate the ability of the microbial isolates present in the polluted soil to utilise the edible component for their growth and metabolic activity.

The pour plate technique was used, and incubated at room temperature for 24 and 48 hours. Colonies that developed were counted and recorded. This was repeatedly done for 2nd, 4th, 6th, 8th, 10th, and 12th week.

2.10. Gravimetric Analysis

The modified method of [23] was used to determine the residual oil present in the soils during the bioremediation experiment. The residual oil present in the soil after 4, 8, and 12 weeks of bio-treatment was extracted as follows: 5 g of each soil sample was extracted with 10 ml n-Hexane and allowed to stand for a few minutes. The n-hexane was used as the extracting solvent at 50˚C. The residue obtained was then quantified in grams.

3. Result

3.1. Physico-Chemical Parameters

The physico-chemical parameters of the polluted and unpolluted soil samples are shown in Table 1. The oil mill polluted soil sample was blackish, while the unpolluted garden soil was dark-brown. As can be seen, all the parameters measured showed values higher in oil mill polluted soil than in unpolluted soil.

Table 1. Physico-chemical analysis of soil samples.

Parameter	Unpolluted Garden Soil (Control)	Oil Mill Polluted Soil (Sample)
Nitrogen (g/kg)	1.34	2.21
Total organic carbon	11.4	23.4
Sodium (Cmol/kg)	0.49	1.35
Magnesium (Cmol/kg)	1.72	11.04
Available phosphorus (mg/kg)	16.20	24.15
pH in water	6.5	7.7
Water holding capacity (%)	12.2	42.4
Colour	Dark-brown	Blackish

3.2. Bioremediation Experiment

Total Viable Count Enumeration

The growth of microbial species in all the soil samples increased from 2 to 12 weeks in almost all the experiments. However, by the 12th week, there was recorded a decrease in growth in the non-sterile soil of the oil mill sample with and without mixing.

The total viable microbial count in sterile soil samples from oil mills with and without mixing shows there to be an averagely significant increase from the second week to the twelfth week (Figure 1). For that the total viable microbial count in non-sterile soil samples from oil mill with and without mixing, there was also an increase in microbial count from the second to the tenth, but on the twelfth week, there was a decrease in microbial counts (Figure 2).

Comparatively, the microbial count in sterile control soil samples with and without mixing presented in Figure 3 was almost constant between the 2nd and 4th week and then gradually decreased from the 4th to the 8th week. Thereafter, a gradual increase was observed from the 10th to the 12th week, although there were some exceptional cases as in soil treated with *Pseudomonas fluorescens*, mixed on the 6th week. The total viable microbial count in non-sterile soil samples (control) with and without mixing also shows a similar growth pattern to the sterile counterpart (Figure 4).

The result of sterile soil sample with and without mixing option, from palm oil, is shown in Figure 5. There was a general increase in total viable counts from the 2nd week to the 12th week, in the non-sterile counterpart (Figure 6). There was a steady increase from the 2nd week to the 8th week and subsequently, a gradual decrease from the 10th to the 12th week.

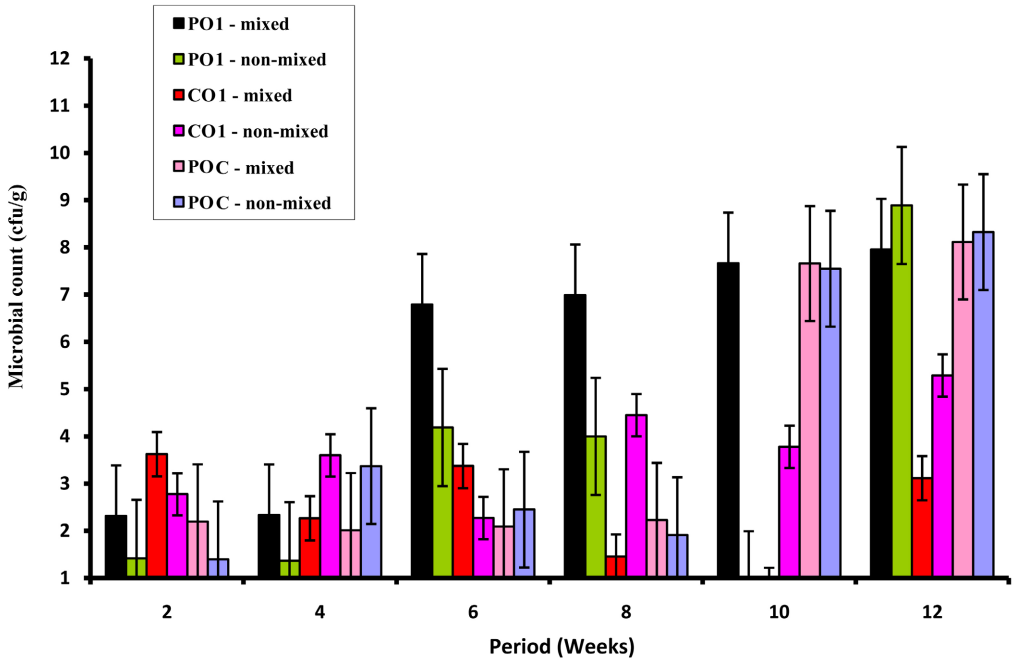

Figure 1. Total viable microbial count in sterile soil samples from oil mill with and without mixing.

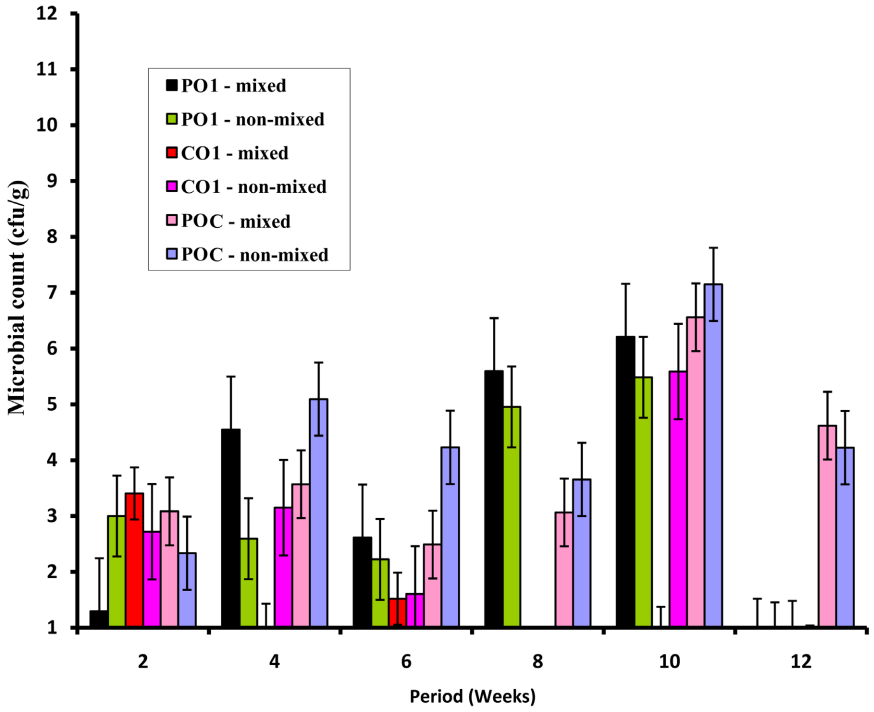

Figure 2. Total viable microbial count in non-sterile soil samples from oil mill with and without mixing.

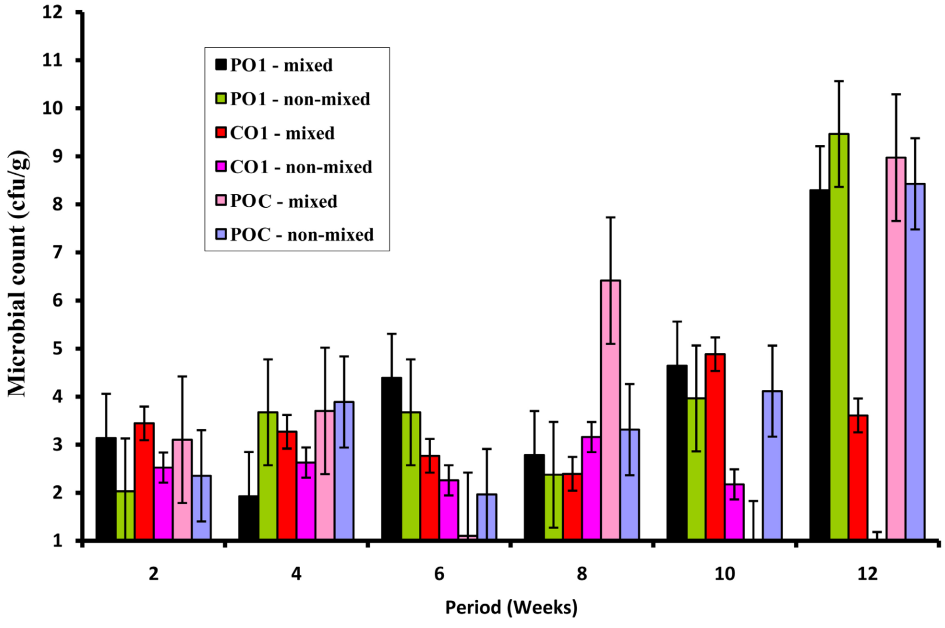

Figure 3. Total viable microbial count in sterile soil samples (control) with and without mixing.

The counts recorded in the sterile soil samples from palm kernel oil-contaminated samples with and without mixing relatively increased over the 12 weeks of bioremediation (Figure 7). In the non-sterile soil sample counterpart, there was also a steady increase from the 2nd week to the 8th week. However, from the 10th week, there was a gradual decrease in counts (Figure 8).

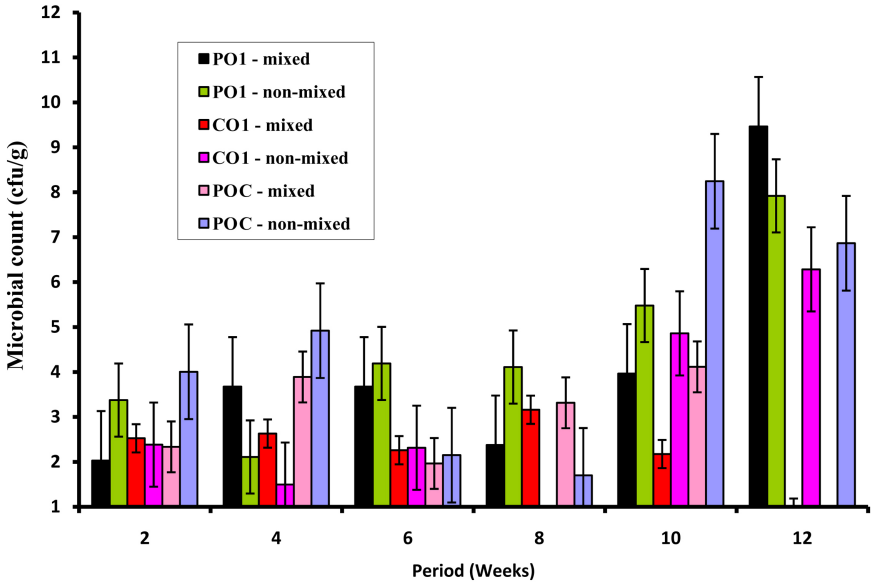

Figure 4. Total viable microbial count in non-sterile soil samples (control) with and without mixing.

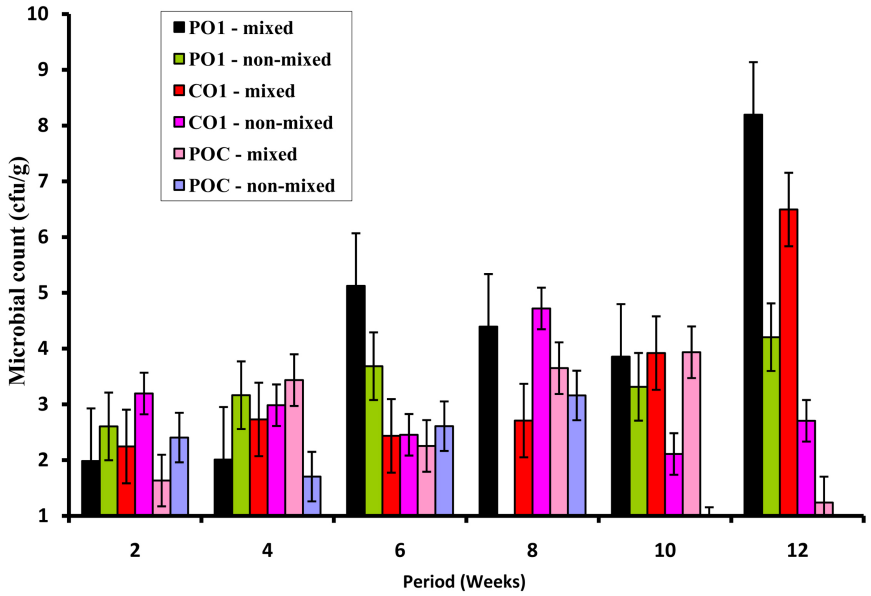

Figure 5. Total viable microbial count in sterile soil samples contaminated with palm oil with and without mixing.

Gravimetric analysis of residual oil from bio-remediated soil samples

The total recoverable residual oil weight in sterile oil mill sample with and without mixing at the beginning of the experiment, shows the weight to be 0.025 g. They all reduced by the 4th week but decreased slightly by the 8th week before increasing on the 12th week, although the weight difference is negligible (**Figure 9**). However, there were some exceptions as shown in the figure. The non-sterile counterpart shows an initial increase by the 4th week followed by a decrease in their residual weight in the 8th week, thereafter an increase again by the 12th week,

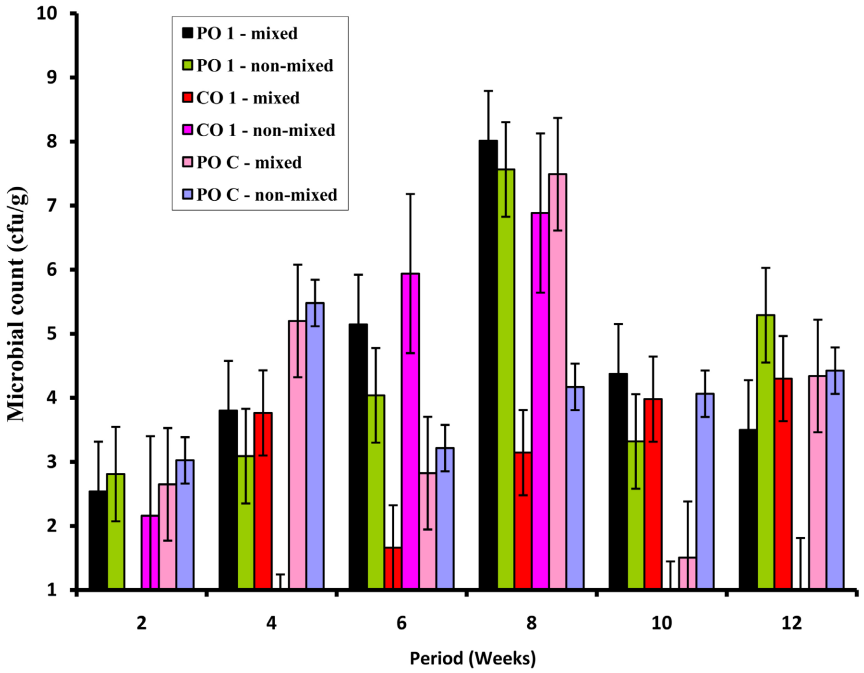

Figure 6. Total viable microbial count in non-sterile soil samples contaminated with palm oil with and without mixing.

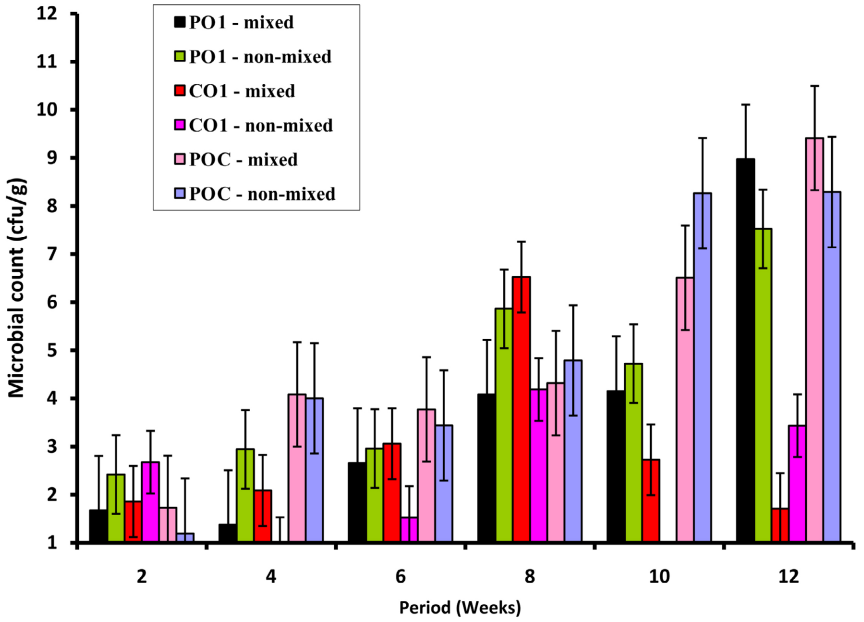

Figure 7. Total viable microbial count in sterile soil samples contaminated with palm kernel oil with and without mixing.

whereas others showed a decrease in residual weight by the 12th week (e.g. POC non-mixed) (**Figure 10**).

The total recoverable residual oil in the sterile unpolluted sample with and without mixing shows that at the beginning of the experiment, the weight of extract from the unpolluted soil sample to be 0.025 g. All were reduced by the 4th week and

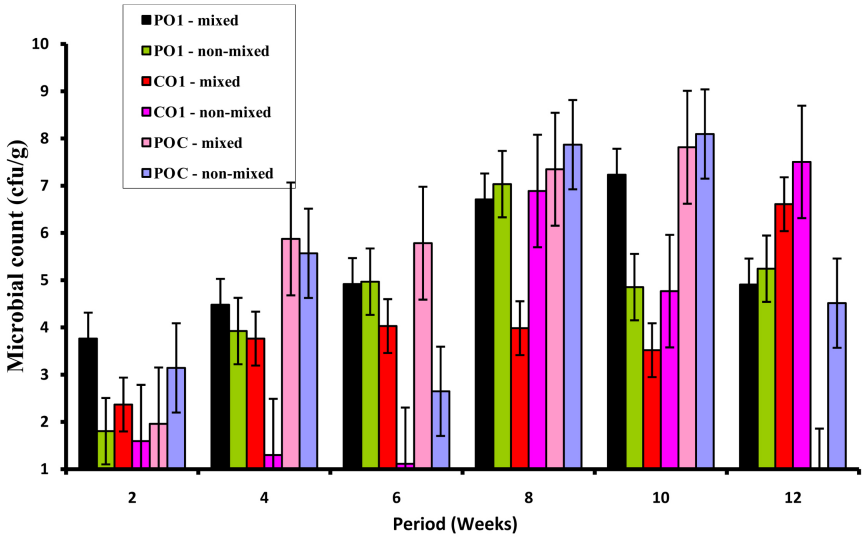

Figure 8. Total viable microbial count in non-sterile soil samples contaminated with palm kernel oil with and without mixing.

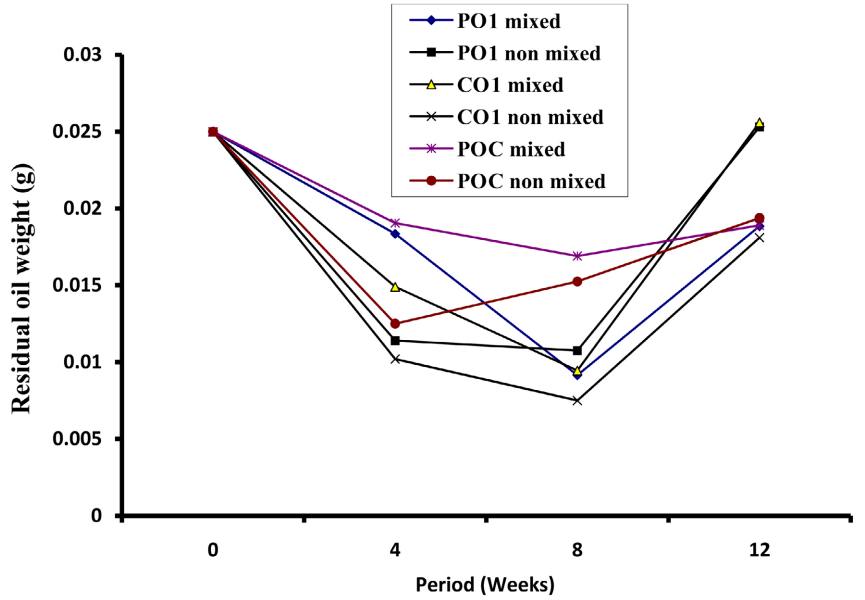

Figure 9. Total recoverable residual oil in sterile oil mill sample with and without mixing.

then increased slightly over the 12 weeks period, although the weight difference is negligible, however, PO1 non-mixed reduced over the period of the experiment (**Figure 11**). The non-sterile counterpart shown in **Figure 12**, there was a gradual decrease in residual weight over the 12 weeks period of remediation with an exceptional case seen in PO1 mixed which showed an increase by the 8th week before a final drastic reduction by the 12th week, also the weight differences are almost negligible.

In the case of sterile soil samples purposely polluted with palm kernel oil with and without mixing, the total residual oil weight as shown in **Figure 13**, progressive decreases in weight from start through eight weeks before it is significantly

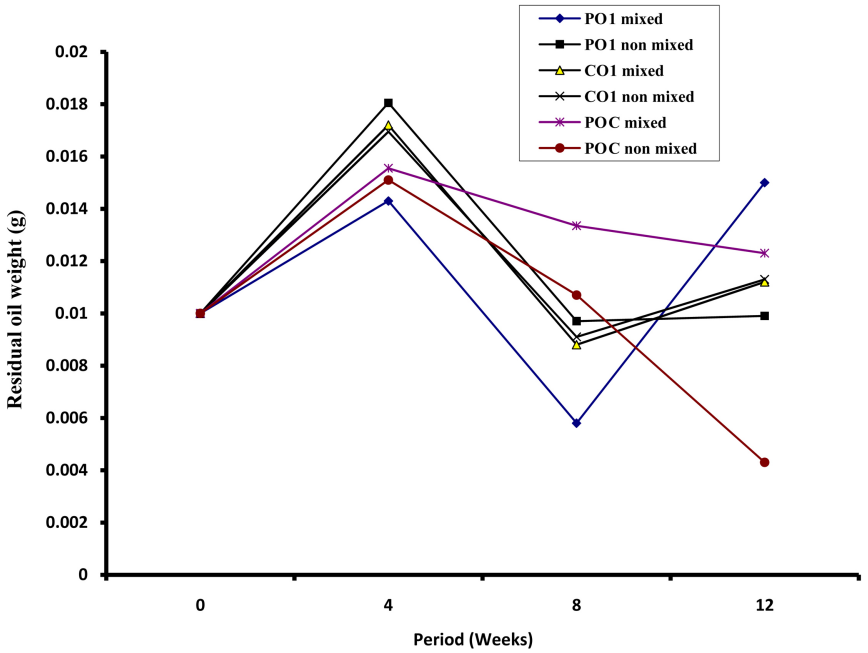

Figure 10. Total recoverable residual oil in non-sterile oil mill sample with and without mixing.

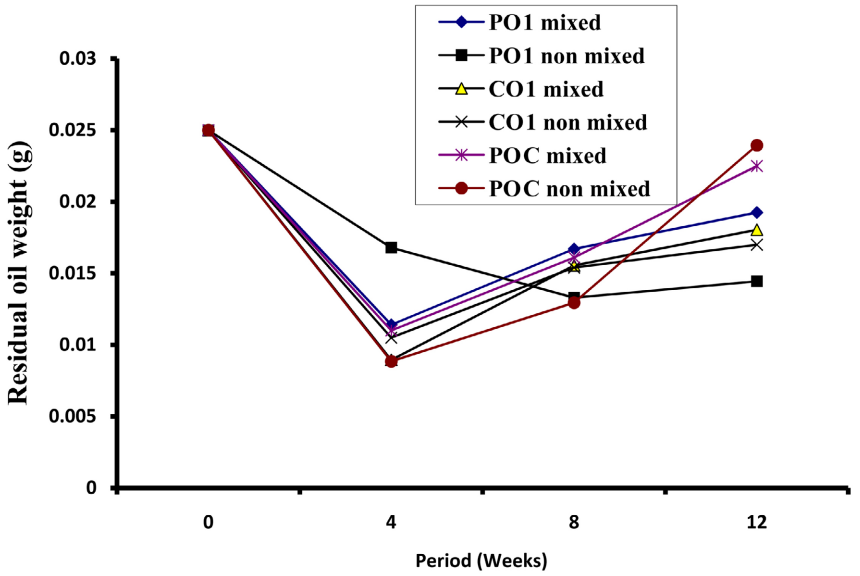

Figure 11. Total recoverable residual oil in sterile unpolluted sample with and without mixing.

reduced by the 12th week, however, CO1 mixed is an exception. In the non-sterile counterpart (**Figure 14**) the same pattern of reduction was seen.

The total recoverable residual oil in sterile palm oil-contaminated soil sample with and without mixing, a progressive reduction over the 12 weeks period of remediation was noted, although some had a slight increase from 4th to 8th week before the reduction in weight on the 12th week (**Figure 15**). The same progressive reduction over the 12 weeks period of remediation was observed in the non-sterile

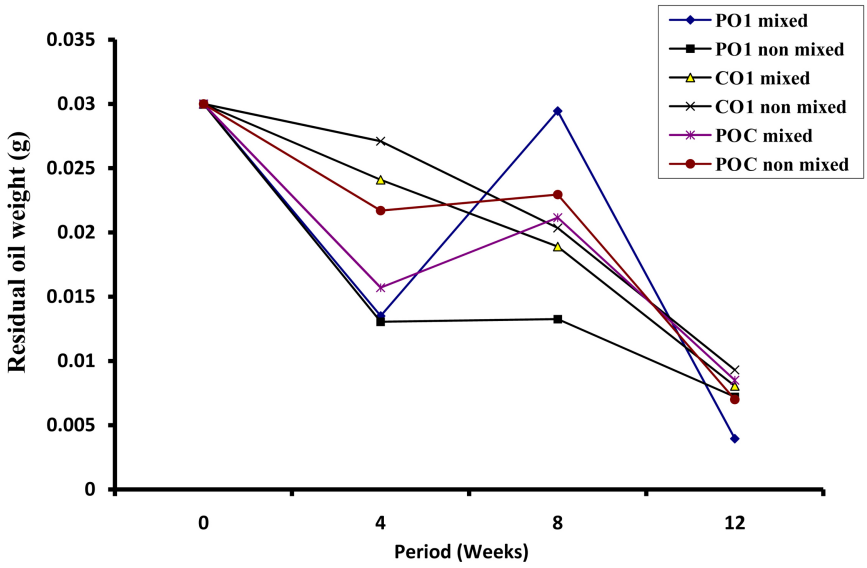

Figure 12. Total recoverable residual oil in non sterile unpolluted sample with and with out mixing.

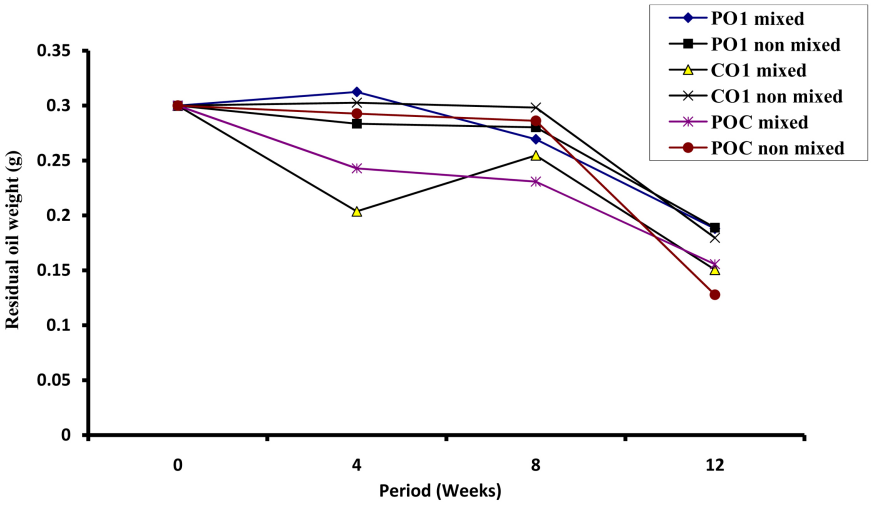

Figure 13. Total recoverable residual oil in sterile palm kernel oil contaminated sample with and without mixing.

counterpart however, PO1 non-mixed is an exception, its weight increased from the 4th week to the 8th week, before reducing drastically on the 12th week (**Figure 16**).

The observed black colour of the oil mill polluted soil samples may be due to the chronic pollution of this site, as it has been known as the site of vegetable oil production for over 12 years. This is evidenced by all the physicochemical parameters higher in the sample than all the unpolluted counterparts. [24] also report the black with humus colouration of palm oil mill effluent (POME) in the soil. However, the observed dark brown colour of the unpolluted botanical garden soil may be due to the high organic matter content of the soil, which has been reported by [25] to be a major component in the top soil of virgin soil.

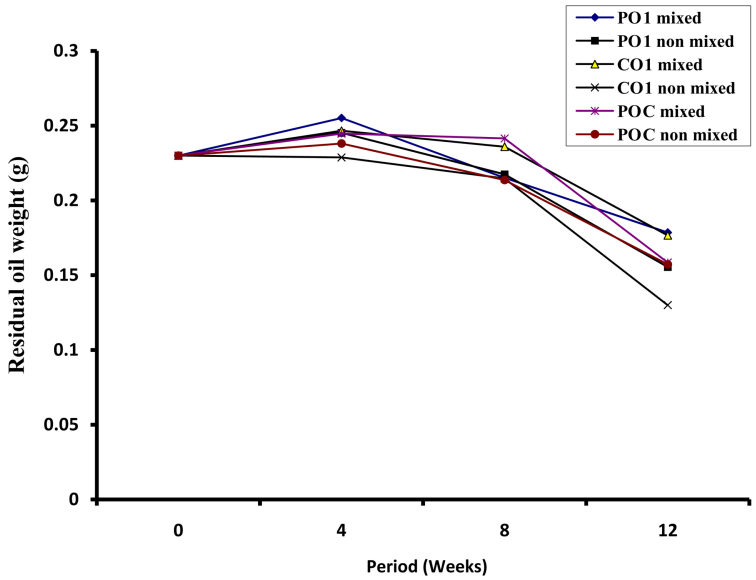

Figure 14. Total recoverable residual oil in non-sterile palm kernel oil contaminated sample with and without mixing.

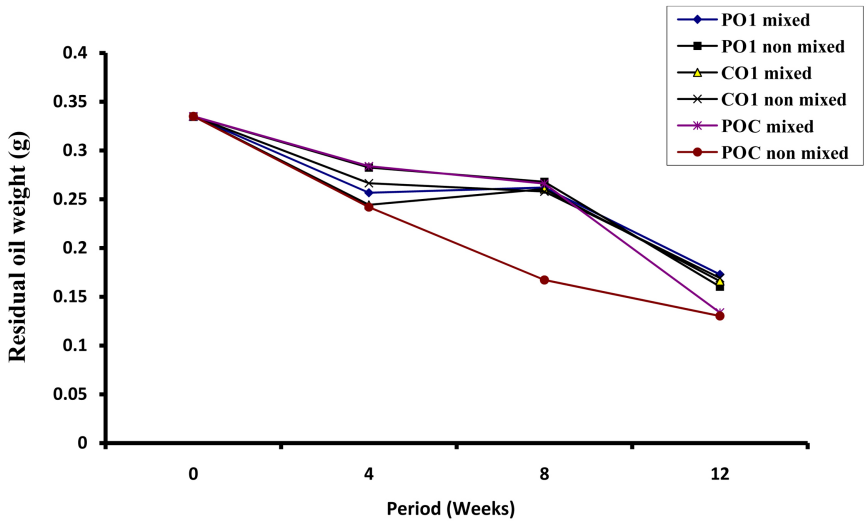

Figure 15. Total recoverable residual oil in sterile palm oil contaminated sample with and without mixing.

The total nitrogen contents and organic carbon of the oil mill polluted soil was higher than that of the unpolluted soil an observation which correlates with the findings of [24]. The higher organic carbon value for the oil mill polluted soil can be related to the constituents of raw and inadequate treatment of the palm kernel effluent released into the soil. It is possible that a slow decomposition of organic matter in the palm kernel oil mill polluted soil, under water-saturated conditions, particularly when mean soil temperatures are low contributed significantly to the higher organic carbon of the polluted soil.

The cation exchange capacity (CEC) (which is the total capacity of a soil to hold exchangeable cations) values observed in this study (sodium and magnesium)

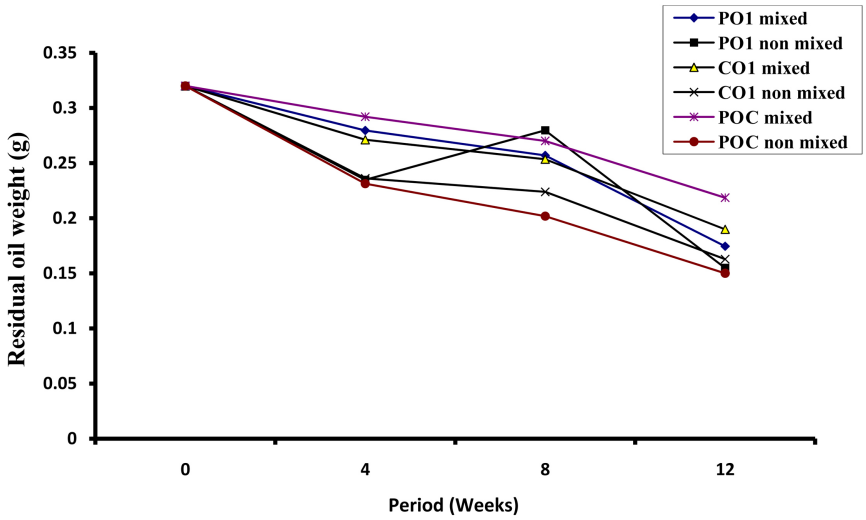

Figure 16. Total recoverable residual oil in non-sterile palm oil contaminated sample with and without mixing.

when considered along with the exchangeable cations were higher in the polluted soil than in the non-polluted soil. In a similar study by [26], as well as [24], the results showed an overall increase in the CEC of POME soils, especially in the area close to its source which this study supports.

The polluted oil mill soil was observed to be richer in phosphorus than the non-polluted soil. There is ample evidence to suggest that phosphorus is the dominant element controlling carbon and nitrogen immobilisation [27]. The increase in the available phosphorus in the polluted soil suggests a possible high absorption in the soil or possible precipitation of phosphate [28]. This may be due to the gradual biodegradation of the oil mill polluted soil, which leads to a delayed effect on the soil.

The water holding capacity of the polluted soil was also higher than that of the unpolluted soil. [29] stated that organic matter content strongly affects soil fertility by increasing the availability of plant nutrients, improving the soil structure and the water holding capacity, and also acting as an accumulation phase for toxic, heavy metals in the soil environment. [24] also reported an increase in the water holding capacity of the POME sample.

The pH in water showed that non-POME soil had its pH near neutral (6.5) however the POME soil was 7.7. This corroborates the work of [24] who observed the non-POME soil to be more acidic. Thus, no pH adjustment was required for the biodegradation experiment.

In the bioremediation experiment, the total viable microbial count generally increased from the 2nd to the 12th week of bioremediation. This was probably due to the fact that untreated vegetable oil in the soil contained high concentrations of free fatty acids, starches, proteins, and plant tissues [30] and are also non-toxic [31] [32]. Organic matter plays an important role in soil productivity and polluted soil samples are good sources of organic matter as observed in this

work. Numerous organisms invade and grow in palm oil-contaminated soil [33], as well as palm oil mill effluent, breaking down complicated molecules into simple ones. The high organic matter in the polluted soil sample which has been shown to be higher than in control soil samples may have a major role in the proliferation of aerobic microorganisms.

In the sterile soil samples from the oil mill (whether mixed or unmixed), where basically there was an increase throughout the 12 weeks, in addition to the effect of organic matters on proliferation, sterilisation of the soil, removing the probable competition of other indigenous micro-flora naturally present in the soil. The competitive relationship of *Pseudomonas fluorescens* and *Candida parapsilosis* may account for the recorded growth basically not higher than when treated only with *Pseudomonas fluorescens*. From this treatment, it was observed that in the 2nd week microbial growth was better in the 'tilling' (mixed option) for all the treatment options but by the 12th week, it was observed that the "no-tilling" option gave better growth. Normally and like [34] observed, adequate oxygen supply was a necessary condition to enhance growth and metabolism in aerobic organisms and oil degraders. However, as revealed in the 12th week, a simile anaerobic condition was favoured. A similar study by [35] on jatropha oil degradation by *Enterococcus faecalis* and *Burkholderia cepacia* W-1 under anaerobic conditions recorded close results. Hence, the experimental result suggests that it may be possible to use anaerobic rather than aerobic conditions as a treatment strategy.

In the non-sterile counterpart, there was an increase in the microbial count up till the 10th week beyond which there was a decrease up till the 12th week. This may be due to unfavourable environmental conditions, generated by the production of certain metabolites on the microbial cells.

The viable microbial count in the control soil samples shows a gradual decrease from the 4th to the 8th week and then increased again from the 10th to the 12th week. This could be a result of nutrient exhaustion by the 6th and 8th weeks. It could also be a result of unfavoured environmental conditions generated by the production of certain metabolites (Biosurfactants) which are amphipathic compounds excreted by microorganisms that exhibit surface activity [36] [37]. However, soil microbiological and biochemical properties respond rapidly to small changes that occur in soil, thereby providing immediate information on changes in soil quality. This could be a result of soil microbial activity having a direct influence on soil fertility. Hence, in this study the complex molecules like glycolipids, peptides, phospholipids, and fatty acids in the biosurfactants (excreted by the microorganisms) could be converted as substrates for the microbial cells again, thereby causing a further increase in biomass again by the 10th to 12th week.

The residual edible oil present in the soil during bioremediation by gravimetric analysis showed that the weight recorded at the beginning of all the experiments indicated a high level of the different edible oil contamination in the soil.

The high value recorded by the 4th week in most of the different treatments in the polluted soil sample was due to microbial degradation of the edible oil present in the soil. This is evident in the reduced quantity of residual oil obtained in the gravimetric analysis (especially in the sterile samples). The degradation of the vegetable oil into fatty acids and other simple inorganic chemical compounds may also be responsible for the values of some of the recoverable oil and grease by the 4th week.

By the 8th week, high residual oil values were recorded in most of the soil samples. This could be a result of biosurfactants excreted by the microorganisms which contain many complex molecules such as glycolipids, polysaccharide-protein complexes, peptides, phospholipids, lipopeptides, and fatty acids. The release could be due to the acclimatisation of the inoculated organism and indigenous organisms (in the non-sterile soils) degrading most of the complex products into alcohols and acids. These products have been reported to be more soluble thus, increasing the number of extractable materials in the n-hexane. Thus, the increased weight in some of the residual oil recorded and the total viable counts were recorded for most of the soils in the 8th week.

[38], in considering the coconut oil biodegradation per day observed that *Pseudomonas aeruginosa* had the highest biodegradation rate from 6 to 9 days. Further observation revealed that the rate of degradation by *Pseudomonas aeruginosa* decreased sharply with an increase in the incubation period from the 4th day to the 12th day and then started to slow from the 24th day. When relating this trend to the increase in microbial cell count, it was reported that there was a rapid increase in the cell biomass of *Pseudomonas aeruginosa* within the first 12 days of incubation. [22] also observed a similar result. The low residual oil values recorded again in the 12th week was due to a reduced biodegradation activity of the isolates, which also resulted in the high total viable microbial count recorded. Some exceptional cases with high residual oil values were observed as in the case of edible oil of sterile oil mill sample without mixing in *Candila parapsilosis* where an initial increase was noticed in the 4th week as opposed to the general reduction in the 4th week. This could mean that normal hydrolysis was not favoured. [39] observed that the lipases from *Candida parapsilosis* have been shown to catalyse transesterification in the presence of a large molar excess of water in the biphasic aqueous/lipid reactant medium with the hydrolysis of the ester being inhibited by a proposed competition of the acyl acceptor with water. Hence, it means these organisms actually acclimatised, and degraded the oil to alcohol and other more soluble compounds, thus increasing the number of extractable materials in the n-hexane. However, transesterification most probably caused a reduction in the weight of residential oil recorded. This generally could be the reason for the low record of count, especially in soil treated with *Candida parapasilosis* only.

Some of the samples in the treatment involving a mixture of *Pseudomonas fluorescens* and *Candida parapsilosis* also showed a similar trend; for example,

residual edible oil of sterile palm kernel oil sample without mixing. Possibly, an antagonistic reaction of *Candida parapsilosis* with *Pseudomonas fluorescens* could also cause similar reactions. However, this was not observed in the oil mill sample, most likely due to the fact that natural degradation has been on in the sampling environment before this experiment.

4. Conclusions

Microbial species such as *Pseudomonas fluorescens* and *Candida parapsilosis*, among other microbiota, were found present in the oil mill-polluted site. This implies that with time, given favourable conditions, these microorganisms could naturally aid the degradation process in vegetable oil-polluted soil. It was established that some of these microorganisms could metabolise complex triglycerides present in oil into fatty acids and glycerol which could be further mineralised into simple inorganic chemical compounds that might not be toxic to the environment. Effective bioremediation could be achieved within 2 to 8 weeks of bio-treatment after which an additional measure like additional inoculum application would be required for a prolonged biodegradation process. This could be applicable in areas of need such as Malaysia. Hence, these microorganisms (*Pseudomonas fluorescens* and *Candida parapsilosis*) could become potential candidates in the development of microbial bioaugmentation products for the treatment of vegetable oil mill effluents and soils, particularly in polluted areas and sites. Finally, a balanced process of microbial seeding and nutritional supplementation, and tilling/non-tilling would be required for an effective bioremediation programme. In the near future, this would definitely help to organise a cleaner environment with waste utilisation by these microbial species.

There is a need to produce lipases of *Pseudomonas fluorescens* and *Candida parapsilosis* with improved properties by protein engineering to further enhance the usefulness of these organisms.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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