

Investigating the Potential of Sunflower Species, Fermented Palm wine and *Pleurotus ostreatus* for Treatment of Petroleum-contaminated Soil

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ABSTRACT

Phyto- and myco-remediation have been identified as sustainable options for treatment of petroleum-contaminated soils. To appraise the benefits thereof, the potentials of 3 sunflower species, 2 palm wine types and *P. ostreatus* to treat petroleum-contaminated soils was investigated. The study involved sampling of petroleum-contaminated soils and treatment with the phyto- and myco-remediation agents for a period of 90-days. Agents used for the remediation were 3 species of sunflowers (*Helianthus annus*-pacino gold, *Helianthus sunsation* & *Helianthus annus*-sunny dwarf), fermented palm wine (from 2 species of palm trees -*Elaeis guineensis* & *Raffia africana*), and oyster mushroom (*Pleurotus ostreatus*). The study further investigated variation in remediation efficiency among the sunflower and palm wine species, as well as different substrates and conditions for optimal application of *P. ostreatus*. The results obtained revealed up to 340 g/kg dry weight of Total petroleum hydrocarbons (TPHs) in the soils, with remediation outcomes of up to 69% by the sunflower-*Helianthus annus* (Pacino gold), 70% by fermented palm wine, and 85% by *P. ostreatus*. While the remediation efficiency of sunflower species was proportional to biomass, there was no significant difference in remediation efficiency of the palm wines. It was also found that substrates type and method of application has a significant impact on the remediation efficiency of *P. ostreatus*. The study further revealed available nitrate and electrical conductivity as possible useful indicators of TPHs concentration and remediation progress in soils.

Keywords: Total Petroleum Hydrocarbons, Sunflower species, Palm wine, *Pleurotus ostreatus*, Petroleum-contaminated soil, Soil remediation.

1. Introduction

Environmental issues arising from the petroleum sector are well known (Al-Nasrawi, 2012; Adenipekun *et al.*, 2015). These can be particularly serious in remote communities and developing countries where resources for effective management are not readily available (Albert *et al.*, 2018). Even with the current drive for greener energy sources, it will be difficult to completely obviate the need for petroleum (Gatfaoui, 2016). The scale of environmental pollution by hydrocarbons requires concerted efforts to develop techniques for its effective

management. This is necessary to create a balance between resource utilization and environmental sustainability (Rhodes, 2014).

Phyto- and myco-remediation are promising techniques for management of petroleum-contaminated soil (Kogbara *et al.*, 2016; Liduino *et al.*, 2018). Yet these techniques have not been utilised commercially in many tropical regions. Dickson *et al.* (2019) reported that for optimal utilisation of these techniques, ideal conditions for their applications should be investigated. There are several reports on remediation potential of sunflowers (Dominguez-Rosado *et al.*, 2004; Diab, 2008). Sunflowers species with different biomass are also known (Rigi, 2018). However, there are no reports relating phytoremediation of sunflowers to species or biomass. To maximise the potentials of sunflower plants for clean-up of petroleum-contaminated soils, it is necessary to investigate the variation of the remediation efficiency of different species. This will help in the choice of the plant type for use in remediation projects.

Palm trees are abundant in many tropical regions such as the Niger Delta, Nigeria (Izah & Seiyaboh, 2018). The juice of palm trees (palm wine) is used as food and ceremonial drink. The drink becomes sour and unfit for drinking if left overnight due to fermentation and is often discarded (Santiago-Urbin & Ruiz-Teran, 2014). Palm wine consists of a consortium of microorganisms principally the yeast- *Saccharomyces* species (Nwaiwu *et al.*, 2016). Consortia of microorganisms have been used for the effective treatment of petroleum-contaminated soils (Robichaud *et al.*, 2019). Consequently, it is needful to investigate the potential of fermented palm wine in remediation of petroleum-contaminated soil.

P. ostreatus are ubiquitous and are known agents for treatment of petroleum-contaminated soils (Stamet, 2005; Kogbara *et al.*, 2016; Kogbara *et al.*, 2017). Adapting these mushrooms for remediation of petroleum-contaminated soils in the tropics, will require substrates, which are abundant in such region (Mamiro and Royse, 2008). It will also require appropriate techniques for successful in situ application.

The aim of this research was to investigate the potential of some locally available sunflower species, fermented palm wine and *P. ostreatus* for treatment of petroleum-contaminated soils that can be adapted to the Niger Delta, Nigeria. The research specifically evaluates the variation of remediation efficiency of the agents with respect to their species, and application methods. This information is important in the choice of the agents among their numerous

species. Adapting *P. ostreatus* for use in the clean-up of petroleum-contaminated soils has been problematic (Stamet, 2005; Dickson *et al.*, 2019). The present study also seeks to develop a realistic approach and investigate a novel substrate (Palm tree which is abundant in tropical regions) for application of *P. ostreatus* in the clean-up of petroleum-contaminated soils.

The phytoremediation agents used for the study were 3 species of sunflower plants namely, *Helianthus annuus* (Pacino gold), *Helianthus sunsation*, and *Helianthus annuus* (Sunny dwarf). The mycoremediation agents were (1) fermented palm wine from 2 species of palm trees (*Elaeis guineensis* and *Raffia africana*); and (2) white rot mushroom- *Pleurotus ostreatus* grown on palm tree substrates. These agents are either ubiquitous or abundant in the Niger Delta region of Nigeria. Soils from a petroleum-contaminated site at Tibshelf, Derbyshire, United Kingdom (temperate soils) were used for the initial pilot study. The outcomes would be applied to petroleum-contaminated soils from a tropical region (the Niger Delta, Nigeria), to evaluate the adaptability of the methods for both temperate and tropical soils.

2. Materials and Method

2.1 Sample collection

Soil samples were collected from an oil spill site near a decommissioned British oil well at Tibshelf, Derbyshire, UK (359414N, 444927E). The soils samples include petroleum-contaminated soils collected at the immediate vicinity of the oil well, and uncontaminated (control) soil samples collected at 200 m uphill from the oil well. These samples were packaged, transported to the glasshouse and stored under airtight conditions (BSI ISO/DIS 18400-203; 2016). Sunflower seeds of *Helianthus annuus* (Pacino gold), *Helianthus sunsation*, and *Helianthus annuus* (Sunny dwarf) were purchased from Nicky's Nursery Ltd, Broadstairs, Kent, UK and pre-grown on Ericaceous compost for a period of 2 weeks. The Ericaceous compost was purchased from Amenity Land Solutions, Allscott Park, Shropshire, UK. Palm wine was purchased from African grocery shops in Nottingham, United Kingdom, while grain spawns of *Pleurotus ostreatus* were purchased from Ann Muller's Mushrooms Ltd, Aberdeenshire, UK. Palms stump of *Trachycarpus fortunei* was purchased from Brookfields garden centre, Mapperley, Nottingham, UK.

2.2 Glasshouse experiments with the petroleum-contaminated soils

Glasshouse experiments were carried out using the methods of Ciurli *et al.* (2014) in the glasshouse facility at Nottingham Trent University, Brackenhurst, UK. 1.5-litre terracotta plant pots were used for the study. The pots were placed in plant trays (Grow bag standard 100 X 40 X 5 cm) to avoid seepage. The soil samples were air dried, ground, extraneous materials removed, sieved through a 2 mm sieve and homogenised. 300 g of the homogenised soil was weighed out and placed in each 1.5-litre plant pots. Three set of glasshouse pots were prepared to consist of uncontaminated soils set, contaminated soils without amendment and contaminated soils with amendment. Cow manure was used as soil amendment and was added to the contaminated soils to provide nutrients and as a diluent. Each Pot with the amendment was prepared by the addition of cow manure to the soils in a ratio of 1:6 (50 g of cow manure compost added to 300 g of soils). The amendment was properly mixed with soils for homogeneity. The amended petroleum-contaminated soils was used for growing sunflowers, treatment with fermented palm wine as well as the white rot mushrooms *P. ostreatus* (Table 1). All glasshouse setups were carried out in triplicates for a period of 3 months (90 days).

Table 1: Glasshouse set ups for remediation of petroleum contaminated soils from Tibshelf, UK using sunflower species, palm wine and *P. ostreatus*

Samples	No of samples	Soil Quantity	Cow manure	Sunflower	Palm wine	Mushroom Substrate	Mushroom Spawn
Set1: (Control 1) uncontaminated soil	9	300.00 g	-	-	-	-	-
Set 2: (control 2) contaminated soil without amendment	9	300.00 g	-	-	-	-	-
Set3: (control 3) contaminated soil with amendment	9	300.00 g	50.00 g	-	-	-	-
Set 4: contaminated soil + Sunflower	9	300.00 g	50.00 g	1 seedling	-	-	-
Set 5: contaminated soil + Palm wine	9	300.00 g	50.00 g	-	0.20l	-	-
Set 6: contaminated soil + <i>P. ostreatus</i>	9	300.00g	50.00 g	-	-	20.00 g	10.00 g

2.3 Physicochemical properties of the soils

Physicochemical properties of the soils such as particle size analysis, temperature, pH, electrical conductivity and available nitrate were monitored at the start of the glasshouse experiments and the end of the 90 days treatment period. Soil particle compositions was determined using a laser diffraction system - Beckman LS 13 320 laser diffraction particle size analyser. Temperature, pH and electrical conductivity were determined in situ with soil

conductivity meter model HI-98129 (Liebig *et al.*, 1996; Scoggins & van Iersel, 2006). Available nitrate was measured with a Horiba - LAQUAtwin NO3-11 - NO3-11C - NO3-11S Compact portable nitrate meter (Hampton *et al.*, 2019).

2.4 Glasshouse experiments with sunflowers

Sunflower seedlings of *Helianthus annuus* (Pacino gold), *Helianthus sunsation* and *Helianthus annuus* (sunny dwarf) were first pre-grown on the Ericaceous compost for a period of 2 weeks. The 2 weeks old seedlings were then transplanted to the experimental pots containing the amended petroleum-contaminated soils in the glasshouse (section 2.2). A control set of sunflower plants were also grown on compost. Soil moisture content was maintained by watering of the containing trays and uptake by capillary rise, and also by vertical spraying every 4 days.

2.5 Glasshouse experiments with fermented palm wines

Two of the dominant species of palm trees in the Niger Delta, Nigeria from which palm wine is tapped are *E. guineensis* and *R. africana*. The remediation efficiency of palm wine from these two species of palm trees on petroleum-contaminated soils was therefore investigated. The palm wines were left in the open overnight (12-18 hours) to ferment (Santiago-Urbina & Ruíz-Terán, 2014). Specified volumes of the fermented palm wines (200 ml) were measured out and added to glasshouse pots containing the amended petroleum-contaminated soils (Table 1). A further 200 ml of each freshly prepared fermented palm wines were added to the pots each week. The use of the 2 species of palm wine was to evaluate any variability in their remediation efficiency.

2.6 Glasshouse set with *P. ostreatus*

Two set of experiments were carried out with *P. ostreatus*. First, was an investigation for substrates for growing white rot mushroom under unsterilized conditions. This was followed by assessing the effect of different application procedures on the remediation efficiency of *P. ostreatus* on the petroleum-contaminated soils.

For the first part, 6 substrates (Table 2) were investigated for growing *P. ostreatus* under sterilised and unsterilized conditions. Based on the outcomes, Palm substrates of *Trachycarpus fortunei* was selected for the study. The palm substrates were prepared by maceration of plant parts (stems, roots, branches) and air drying for one week. These parts

were further pulverized to sawdust form after air drying and used as substrate for growing *P. ostreatus*.

Table 2: Growth of *P. ostreatus* under sterilised and unsterilized conditions using different substrates. Estimated lignin content of substrates are also given.

S/N	Substrate Type	Time Taken for mushrooms to germinate and produce fruity body (weeks)		Temperature of growth = 10 - 15°C	
		Without sterilisation	With sterilisation	Extent of germination of mushroom mycelia	Estimated lignin content of substrates
1	Cassava peels	6	3-4	Very extensive	8 % (Daud <i>et al.</i> , 2014)
2	Pine barks	3-4	2	Very extensive	53% (OLÁR <i>et al.</i> , 1998)
3	Palm tree	3-4	2	Very extensive	33% (Abdul Khalil <i>et al.</i> , 2006)
4	Maize cob	8	3-4	Very extensive	16–18% (Wang <i>et al.</i> , 2011)
5	Saw dust	8	4	Extensive	Varies according to wood type (Joshua, 2016).
6	Hay	8	4	Extensive	7 and 8% (Whitehead & Quicke, 1964).

For assessing the effects of application procedures, 3 subsets were created. Firstly, mushroom spawns (10 g) were applied directly on petroleum-contaminated soils by mixing without the substrates. Secondly, mushrooms spawns (10 g) were applied on the substrates (20 g) layered on top of the contaminated soils (Adenikpekun and Fasisdi, 2005). Lastly, part of the substrates and spawns was mixed with the contaminated soils, with some parts also layered on top of the soils. This last set were prepared as follows: The substrates (10 g) were mixed with the amended soils. The mushroom spawn (5 g) were then added by uniformly spreading into the soils. This was then followed by layering of another 10 g of substrates on top of these soils. The layered substrates were also inoculated with 5 g of mushroom spawns.

2.7 Sample collection, preparation and analysis

After glasshouse set up, composite soil samples were collected at the start of the study (Time= 0 days) and after a 3-month treatment period (Time = 90 days). Samples were air-dried, sieved through a 2 mm mesh and extraneous materials removed, ground and homogenised prior to laboratory treatment and analysis according to methods of BS ISO 11464 (2014). Extraction of TPHs in samples was carried out using a microwave assisted extraction with a Milestone MA182-001 ETHOS UP Microwave system, using a 1:1 acetone – heptane (USEPA METHOD 3546; Punt *et al.*, 1999). Commercial TPHs diesel range standard was used for quantification of TPHs. Sample extracts, and the TPHs standards were all

analysed in a GC-MS (model Agilent Technologies 7000 GC/MS Triple Quad with 7890 GC and 7693 Autosampler) (USEPA 8270E).

Quantification of TPHs in the soils was carried out using the methods of BS EN ISO 16703 (2011). Initial calibration of the instruments, followed by evaluation of the concentration of TPHs, alongside calibration verification were carried out.

2.8 Data treatment and validity

Values for Total petroleum hydrocarbons (TPHs) are expressed in g/Kg dry weight of soils. Each sample was extracted and analysed in triplicate and the values were reported as mean \pm standard deviation (2δ). Consistencies in triplicate analysis were evaluated by precision. Accuracy in analysis was evaluated by calibration verification run in each set of sample analysis.

T-test was used to evaluate if differences in TPHs at different treatment times (T=0 days and T= 90 day) with the same sample treatment method was significant (Zheng *et al.*, 2019). Analysis of variance (ANOVA) were carried to evaluate variability in TPHs content among soils samples from different sampling points, glasshouse pot and the different remediation treatment sets.

3 Results

3.1. Physicochemical properties of the soils

Particle size analysis of the soil samples revealed higher clay contents (50%), followed by silt (30%) and sand particles (20%) in control soils from Tibshelf, Derbyshire. For the petroleum-contaminated soils, the percentage composition was 65, 25 and 10 for clay, silt and sand particles, respectively. Levels of clay particles were higher in petroleum-contaminated soils than those of controls.

Soil pH was significantly affected ($p < 0.05$) by petroleum contamination (Table 3). The pH of control soil samples was near neutral (7.35-7.55) and were not significantly different during the study. The pH of untreated petroleum-contaminated soil samples was acidic (pH 6.53 – 6.58) and were significantly different ($p = 0.000$ at T=0, 0.002 at T=3) from those of uncontaminated soils. The pH of cow manure was in the range of 9.42- 9.49. The addition of cow manure to the petroleum-contaminated soils increased soil pH from acidity to alkalinity (Table 3). The pH values were maintained at slightly alkaline levels (8.50 – 8.90) throughout

the duration of the remediation. pH in soils treated with cow manure were not statistically different ($p>0.05$) during the treatment period.

A significant decrease in available nitrate was observed in untreated petroleum-contaminated soils compared to controls (Table 3). The addition of cow manure significantly increased ($p<0.05$) levels of available nitrate in the soils (Table 3). Levels of available nitrate also increased as remediation progressed and correlated positively with % decrease in the soils' TPHs (Figure 1). A similar trend was also observed for electrical conductivity. Electrical conductivity values increased as remediation progressed and correlated positively with % decrease in TPHs (Figure 2). Variation in temperature values (19- 23°C) was also observed during the treatment but no trend could be deduced for temperature.

3.2 TPHs levels in the soils

Initial concentrations of TPHs in glasshouse pots varied from 130 to 340 g of TPHs/Kg dry weight of soil with the highest (340 g/Kg) observed in pots of untreated soils. Variations were also observed among the treatment sets such as those of sunflower species, palm wine and those treated with *P. ostreatus* (Figure 3). For the set involving the three-sunflower species, initial TPHs concentrations were 200 g/Kg for *H. annus-sunny dwarf*, 250 g/Kg for *H. sunsation* and 150 g/kg for *H. annus-pacino gold*. Glasshouse pots treated with fermented palm wine from *E. guineensis* had initial TPHs levels of 340 g/Kg while that of *R. africana* was 280 g/Kg dry soil. Levels of TPHs in the soils treated with *P. ostreatus* at time = 0 days were 290, 130, and 210 g/Kg dry soils for *P. ostreatus* applied without substrates, applied by layering substrates on topsoil and that applied by a combination of mixing substrate with soil and layering.

After the 90 days remediation treatments, TPHs levels decreased to 290, 90, 120 and 50 g/Kg dry soil for untreated soils and those treated with *H. annus-sunny dwarf*, *H. sunsation* and *H. annus-pacino gold*. For the set treated with palm wine TPHs levels were 100 for *E. guineensis* and 90 g/Kg for *R. africana*. Treatments with *P. ostreatus* resulted in 210, 60 and 30 g of TPHs per Kg dry soil for the applications without substrates, layering substrates on topsoil and those by a combination of mixing substrate with soil and layering, respectively.

Table 3: Variation in Physicochemical properties of petroleum contaminated soils and controls from Tibshelf, UK during Glasshouse remediation treatment with sunflower species, fermented palm wine and *P. ostreatus*. Values are given as average of triplicates with standard error (S.E)

Samples/Treatment	Temperature °C				pH				Electrical conductivity				Available Nitrates (ppm)			
	T=0	S.E	T=3	SE	T=0	S.E	T=3	SE	T=0	S.E	T=3	SE	T=0	S.E	T=3	SE
Untamminated soil samples	20.43	0.17	22.80	0.14	7.35	0.04	7.55	0.05	7.35	0.03	1.03	0.03	621.3	5.21	610.0	4.96
Cow manure	18.83	0.18	21.20	0.47	9.42	0.04	9.49	0.08	9.42	0.02	3.24	0.02	700.3	4.95	700.0	3.77
Untreated petroleum-contaminated soil without amendment (Control 1)	21.75	0.13	21.74	0.30	6.40	0.02	6.44	0.11	6.40	0.01	0.23	0.01	33.33	0.27	36.00	0.47
Untreated petroleum-contaminated soil + amendment (Control 2)	21.17	0.01	22.43	0.44	8.54	0.06	8.70	0.05	8.54	0.05	2.85	0.08	436.7	2.72	469.0	4.78
Petroleum-contaminated soil + <i>Helianthus annuus</i>	20.60	0.25	22.67	0.28	8.61	0.11	8.53	0.03	8.61	0.04	2.68	0.01	486.7	2.72	793.3	2.72
Petroleum-contaminated soil + <i>Helianthus sunsation</i>	20.85	0.12	21.96	0.04	8.63	0.11	8.70	0.07	8.63	0.05	2.81	0.03	486.0	2.49	756.7	15.15
Petroleum-contaminated soil + <i>Helianthus annus</i> (sunny dwarf)	19.50	0.25	20.70	0.74	8.65	0.19	8.90	0.00	8.65	0.04	3.07	0.05	465.0	4.08	910.0	4.71
Petroleum-contaminated soil + fermented palm wine from <i>Elaeis guineensis</i>	21.00	0.00	20.80	0.66	8.82	0.02	8.90	0.02	8.82	0.08	3.47	0.05	416.7	17.84	903.3	2.72
Petroleum-contaminated soil + fermented palm wine from <i>Raffia Africana</i>	20.57	0.46	20.84	0.36	8.55	0.15	8.89	0.01	8.55	0.05	3.80	0.08	393.3	5.44	873.3	5.44
Petroleum-contaminated soil + <i>P. ostreatus</i> without substrates	20.60	0.17	21.00	0.47	8.57	0.01	8.60	0.00	8.57	0.07	2.77	0.03	440.0	4.71	483.3	2.72
Petroleum-contaminated soil + <i>P. ostreatus</i> + substrates layered on soil	21.03	0.03	20.30	0.09	8.55	0.13	8.90	0.00	8.55	0.00	3.00	0.00	430.0	0.00	720.0	0.00
Petroleum-contaminated soil + <i>P. ostreatus</i> + substrates mixed with soils and layered	21.01	0.08	20.60	0.33	8.79	0.04	8.73	0.12	8.79	0.02	3.83	0.03	483.3	11.86	973.3	5.44

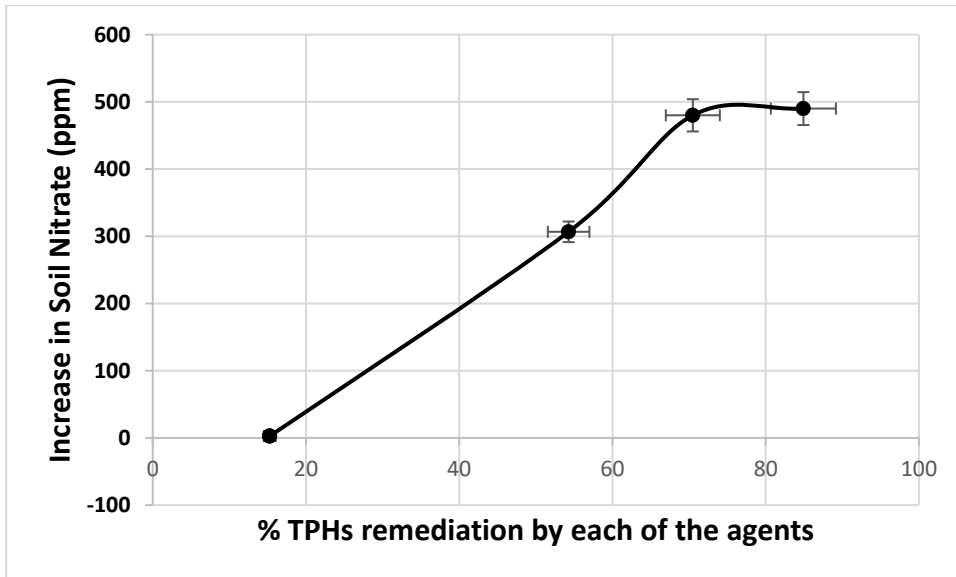


Figure 1: Variation in soil nitrates with % reduction in TPHs during remediation of petroleum-contaminated soils from Tibshelf, UK (Error bars represents standard deviation δ). The figure is a plot of the % reduction in TPHs by each of the agents against the corresponding increase in soil nitrates associated with each agent during such remediation. For instance, % remediation was obtained by subtracting the levels of TPHs at time=0, and time=90 days for a particular agent. The increase in nitrate level as also similarly evaluated (at time=0, time=90 days for each of the agents). The curve shows a steady rise of available nitrate with increase remediation and peaks at remediation efficiency of 70%. These points correspond to TPHs concentrations in uncontaminated soils.

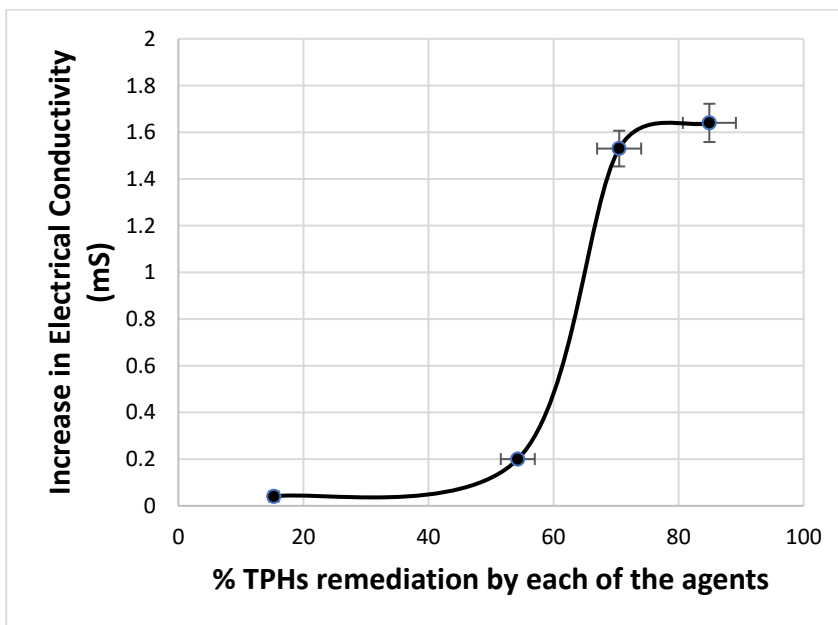


Figure 2: Variation of soil's EC with % reduction in TPHs during remediation of petroleum contaminated soils from Tibshelf, UK (Error bars represents standard deviation δ). The figure is a plot of the % reduction in TPHs by each of the agents against the corresponding increase in soil electrical conductivity associated with each agent during such remediation. For instance, % remediation was obtained by subtracting the levels of TPHs at time=0, and time=90 days for a particular agent. The increase in electrical conductivity level as also similarly evaluated (at time=0, time=90 days for each of the agents). The curve shows a steady rise of electrical conductivity with increase remediation and peaks at remediation efficiency of 70%. These points correspond to TPHs concentrations in uncontaminated soils.

3.3 Remediation efficiencies of sunflower species, fermented palm wine and *P. ostreatus* on the petroleum-contaminated soils

All the agents used in the treatment of the petroleum-contaminated soils demonstrated noticeable remediation efficiency after 90 days (Figure 3). A comparison of remediation efficiencies of the agents revealed the following order: *P. ostreatus* > palm wine > sunflower species (Table 4). This was based on the enhanced application method of mixing substrates and mushrooms with soils followed by layering. With respect to the typical method of layering mushrooms and substrates on soils, remediation efficiency of the agents was observed as Palm wine > *P. ostreatus* > sunflower species.

Table 4: Percentage reductions in TPHs levels in petroleum contaminated soils treated with sunflower species, fermented palm wine and *P. ostreatus*

Samples/Treatment	% reduction In TPHs contents of soils between T=0 days and T=90 days	p-values (@ 95% CI) of T=90 days values against T=0 days
Untreated Petroleum-contaminated soil (Control)	15	0.294
Petroleum-contaminated soil + <i>H. annus</i> (<i>Sunny dwarf</i>)	54	0.001
Petroleum-contaminated soil + <i>H. sunsation</i>	53	0.003
Petroleum-contaminated soil + <i>H. annus</i> (<i>Pacino gold</i>)	69	0.011
Petroleum-contaminated soil + fermented palm wine from <i>E. guineensis</i>	70	0.001
Petroleum-contaminated soil + fermented palm wine from <i>R. africana</i>	69	0.000
Petroleum-contaminated soil + <i>P. ostreatus</i> without substrates	29	0.164
Petroleum-contaminated soil + <i>P. ostreatus</i> + substrates layered on soil	60	0.000
Petroleum-contaminated soil + <i>P. ostreatus</i> + substrates mixed with soils and layered	85	0.000

p-values are for T-test on TPHs concentrations at T=0 days against Time = 90 days, to see if TPHs concentrations at Time = 90 days (after remediation) are significantly different from T=0 days (before remediation). Raw data are in Appendix III-2. P-values highlighted in bold signify results where there is no significant differences in TPHs values at Time =0 days and 90 days($p>0.05$). Those not highlighted in bold signify results where there is significant differences between in TPHs values at time= 0 days and 90days.

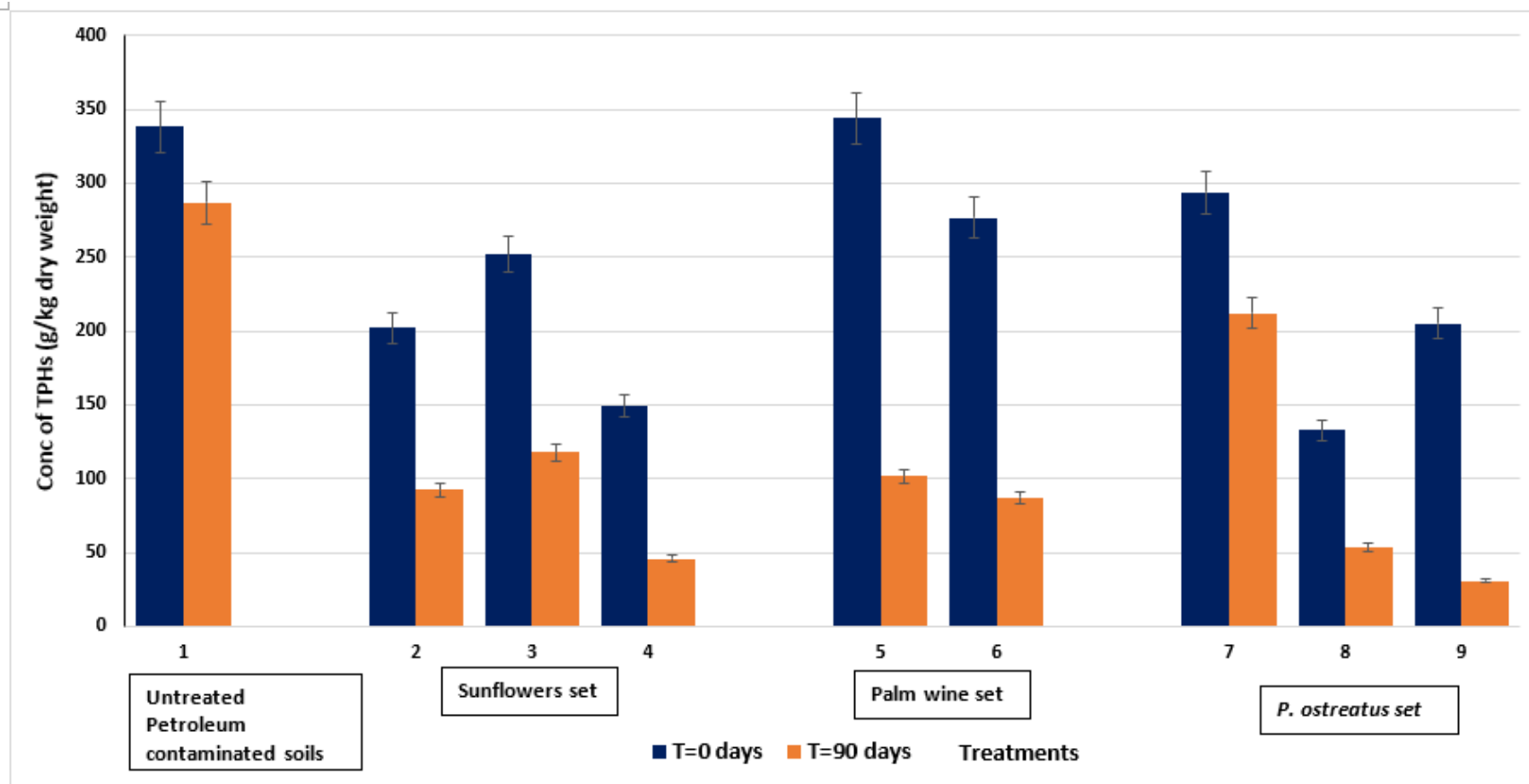


Figure 3: Levels of TPHs in soils at start of remediation (T= 0 days) and after T= 90 days of treatments with the different agents. Errors bars represents standard deviation from the mean of triplicate analysis. Raw values are available in Appendix III-2: Treatment numbers are as follows:

(1). Untreated Petroleum-contaminated soil (Control); **(2).** Contaminated soil + *Helianthus annus* (Sunny dwarf); **(3).** Contaminated soil + *Helianthus sunsation* **(4).** Contaminated soil + *Helianthus annus* (Pacino gold); **(5).** Contaminated soil + fermented palm wine from *Elaeis guineensis* **(6).** Contaminated soil + fermented palm wine from *Raffia Africana* **(7).** Contaminated soil + *P. ostreatus* without substrates **(8).** Contaminated soil + *P. ostreatus* + substrates layered on soil **(9).** contaminated soil + *P. ostreatus* + substrates mixed with soils and also layered

For the sunflower species, highest percentage reduction in TPHs was obtained with *H. annus* (Pacino gold) and was 69% (Table 4). This was followed by *H. annus* (Sunny dwarf) (54%) and lastly by *H. sunsation* (53%). Phytoremediation efficiencies of the sunflower species were significant ($p < 0.05$) compared to those of control soils. Similar remediation efficiency was observed in the remediation potential of *H. sunsation* and *H. annus* (Sunny dwarf) ($p > 0.05$). Remediation efficiency of *H. annus* (pacino gold) was observed to be significantly higher ($p < 0.05$) than those of both *H. sunsation* and *H. annus* (Sunny dwarf) (Table 4).

Physiological parameters such as height and biomass of sunflower plants were negatively affected by presence of petroleum contaminants in soils. Plants growing in contaminated soils had lower height and biomass. Positive correlation was also observed between phytoremediation efficiency of sunflower species and their biomass (Figure 4). *H. annus* (Pacino gold) had both the highest biomass and remediation efficiency with respect to TPHs on the petroleum-contaminated soil.

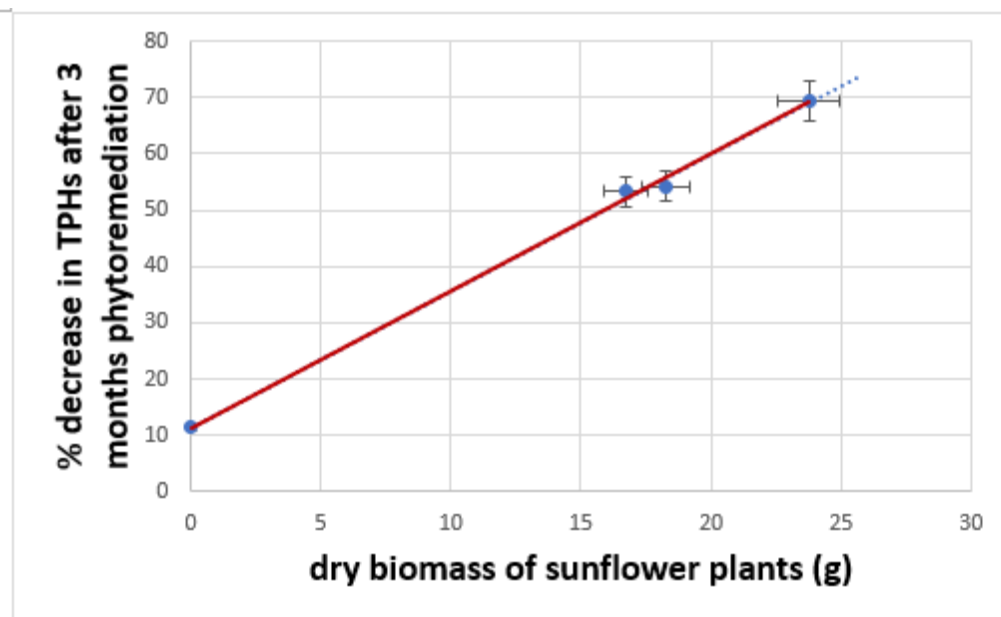


Figure 4: Correlations between remediation efficiencies (% decrease in TPH) and dry biomass of sunflower plants. Errors bars represents standard deviation of the mean.

Fermented palm wine from the two species of palm trees (*E. guineensis* and *R. africana*) each demonstrated significant remediation potential ($p < 0.05$) on the petroleum-contaminated soils compared to controls (Figure 3). Palm wine from *E. guineensis* effected up to 70% reduction of TPHs while that from *R. africana* was 69% (Table 4). There was however, no significant difference ($p = 0.000$) between remediation efficiencies of fermented palm wine obtained from *E. guineensis* or *R. africana*.

Investigation of alternative substrates for growing *P. ostreatus*, revealed that the mushroom germinated and grew faster under sterilised conditions (Table 2). Growth of *P. ostreatus* was still achieved under unsterilised conditions at temperatures of 10-15°C. Shorter timeframe was observed for germination and fruiting of *P. ostreatus* with substrates such as palm tree and pine bark compared to others. The effectiveness of these substrates to support the growth of the mushroom can also be related to their lignin contents (Table 2). *P. ostreatus* was able to grow faster on substrates with higher lignin contents. Pine bark and palm tree substrates exhibited better growth even in unsterilised conditions.

The highest remediation efficiency of 85% for TPHs in soils treated with *P. ostreatus* was obtained by the method of mixing both mushrooms and substrates with soils combined with layering. This was followed by layering substrates and mushrooms on soil (60%), while the method of mixing mushrooms spawn with soils without substrates resulted in 28% TPHs reduction (Table 4). Application of *P. ostreatus* using the combined method of mixing mushrooms and substrates with soils, and layering resulted in 25% increase in remediation efficiency compared to the usual method of layering. There was also a significant difference in remediation efficiency between contaminated soils treated by layering *P. ostreatus* with substrates and the control (Table 4). Although there were reductions in TPHs in untreated soils and those treated with *P. ostreatus* without substrates (Table 4), the difference was not significant ($p = 0.294$) when compared to the TPHs values in soils at Time = 0 and Time = 90 days.

4. Discussion

4.1 Physicochemical properties of the soils

From particle size analysis, the soil from Tibshelf, Derbyshire, can be categorised as clayey loam. Higher levels of clay particles in the petroleum-contaminated soils suggest soil particle degradability by crude oil on soil (Okoro *et al.*, 2011). The soil acidification by petroleum contaminants observed in this study corroborated those of Gogoi *et al.* (2003) and Osuji &

Nwoye (2007) and is related to the constituents (acids and acids anhydrides) of the contaminating petroleum (Sarkar *et al.*, 2005). As observed in this study, Whalen *et al.* (2000) reported that cow manure can be used to amend acidic soil to near neutrality. Therefore, the pH condition (8.50-8.9) induced by the addition of cow manure in the present study provided suitable conditions for the remediation to proceed (Gogoi *et al.*, 2003). The increased pH favoured the solubility and bioavailability of the hydrophobic petroleum contaminants which in turn increased their degradation (Jardine *et al.*, 1989).

From the present investigations, it was observed that high levels of petroleum contamination resulted in low levels of nitrate in soils and vice versa which is in line with the findings of John *et al.* (2016). When these contaminants were taken out of the soils during remediation, more nitrate that were immobilised, were released (Table 2). The observed reduction in available nitrate with high petroleum contamination is related to the great affinity for organic compounds such as organic matter by soil nitrate (Taylor & Townsend, 2010). Therefore, some of the nitrate is sequestered by the high organic content of the petroleum making them unavailable in soil solutions. In addition, the petroleum contaminants also take up much of the soil pores thereby reducing nitrate availability. The addition of cow manure increased organic matter (with nitrate-attracting functional groups such as carboxylic acids and hydroxyl groups); and moisture contents (Raviv *et al.*, 2004) and consequently diluted the concentration of the petroleum contaminants. Thus, more nitrates were available in soil solutions.

Khamehchiyan *et al.* (2007) described increase in physicochemical properties of petroleum-contaminated soils on addition of organic manure. In this study, the addition of cow manure to the petroleum-contaminated soils increased soil pH, electrical conductivity and available nitrate. As remediation progressed, levels of soils nitrate and electrical conductivity increased and was proportional to the remediation efficiency of the agents (Figure 1). Thus, available nitrate and electrical conductivity can serve as possible useful indicators of TPHs remediation progress in petroleum-contaminated soils.

4.2 Phytoremediation efficiency of the agents

Variation in TPHs levels in the soil samples of the glasshouse is due to the non-homogeneity of the petroleum contaminants in the soil matrix. Variable concentration of TPHs is expected

in soils due to factors such as proximity to contaminants source, duration of impact, constituent soil particles, soil structure and chemistry (Yang *et al.*, 2015). Furthermore, the soil samples from Tibshelf had predominant clay particles (Table 2) which are crumbly and difficult to break. These crumbs each accumulate peculiar levels of the petroleum contaminants. In this study, the soil samples were mixed by hand, there is also the chances of poor homogeneity. These factors all resulted in the observed variability in TPHs levels at T= 0 days in the soil samples (Xing *et al.*, 2011).

The general observation in this study was that the levels of TPHs at Time = 90 days was proportional to their respective levels at Time = 0 days. For instance, for the sunflower species, TPHs concentrations at time = 0 was 200 and 90 g/Kg at time = 90 days for *H. annus-sunny dwarf* and 250 and 120 g/Kg for *H. sunsation*. This trend also holds for the palm wine set (340 and 100 g/Kg for *E. guineensis*; 280 and 90 g/Kg dry soil for *R. africana* at time = 0 and 90 days, respectively). Winqvist *et al.* (2014) demonstrated a 96% degradation of PAHs with an initial concentration of 3500 mg/kg in a glasshouse, and 94% during a field study with an initial concentration of 1400 mg/kg of soil, after three months. Thus, it is reasonably assumed that the initial levels of TPHs at the start of the remediation does not affect the remediation potentials of the agents. Hence, the variability in initial concentrations of TPHs among sample treatment sets can be accommodated.

Although the samples labelled 1 (figure 3) were not treated, natural attenuation plays a part in reduction of TPHs in soils, however, the progress is very slowed, and most times insignificant (O'Brien *et al.*, 2019).

Sunflower species

Plants can phyto-extract, phyto-degrade or phytostabilise organic contaminants in soils (Pilon-Smits, 2005). Thus, the sunflowers could utilise any of these mechanisms. Hassan *et al.*, (2018) demonstrated a phytoremediation efficiency of up to 56% with sunflower (*H. annus*) in the remediation of crude oil contaminated soils supplanted with inorganic fertilisers. Liduino *et al.* (2018) demonstrated up to 58% and 48% reduction of TPHs by sunflowers (*H. annus L.*) supplanted by biosurfactants on petroleum-contaminated soils after three months. A similar range of phytoremediation efficiencies (53%, 54%) was observed for 2 of the

sunflower species used in the present study, substantiating the potential of sunflowers for remediation of petroleum-contaminated soils. The sunflower plants could not grow in the high concentrations of crude oil in the soils of the present study without amendment, but only on the amended soils. The use of cow manure as a source of amendment in this study demonstrated that organic manures can be used to successfully initiate phytoremediation of petroleum-contaminated soils.

The reduction in height and biomass of the sunflower plants observed in the present investigation are comparable to that of Brandt *et al.* (2006). These reductions can be attributed to difficulties in adapting to the stress environment, insufficient nutrients or toxicity of the petroleum contaminants (Merkl *et al.*, 2005). John (2016) reported that soil contamination by crude oil leads to a reduction in nutrient parameters such as available nitrate with a marked reduction in water uptake. These are essential factors for plants growth. Thus, any constrain which negatively affects nutrients and water availability would invariably result in poor growth and biomass yield.

Robinson *et al.* (1998) stated that phytoremediation efficiency is influenced by biomass of plants. Plant species with higher biomass are known to exhibit better phytoremediation potential (Chekol *et al.*, 2004). Both *H. annus* (*sunny dwarf*) and *H. sunsation* exhibited similar heights and biomass. The measured height and biomass of *H. annus* (*Pacino gold*) was observed to be significantly higher than those of *H. annus* (*sunny dwarf*) and *H. sunsation*. Therefore, the remediation efficiency of the sunflower species with respect to TPHs is related to their biomass (Figure 4). This finding is important for real life in-situ application. It implies that using sunflower species with high biomass can produce better results in the treatment of petroleum-contaminated soils. In addition to the effectiveness at remediating TPHs, the use of sunflowers will provide the additional benefits of removing trace metals and other pollutants, with aesthetic appeal (Hull *et al.*, 2000; Chauhan & Mathur, 2018).

Fermented palm wine

Chandrasekhar *et al.* (2012) stated that fermented palm wine consists principally of yeast of the *Saccharomyces* species. A consortium of microbial species such as yeast, *candida*, *pichia*, *lactobacillus* and *acetobacter* are also found in palm wine (Santiago-Urbina & Ruíz-Terán,

2014; Nwaiwu *et al.*, 2016). Therefore, the remediation potential of palm wine may be a synergy among the different species of microorganisms present in the fermentation product (Santiago-Urbina & Ruíz-Terán, 2014). Enhanced remediation of petroleum-contaminated soils by synergistic microbial relationships is well known (Fan & Qin, 2014). Chandrasekhar *et al.* (2012) and Nwaiwu *et al.* (2016) also reported that fermented palm wine chemically consists of mixtures of alcohol such as ethanol, propanol and methanol; esters like ethyl propanoate; and organic acids such as ethanoic, methanolic and propanoic acids. These compounds are organic solvents and can act as surface-active agents (Stjerndahl *et al.*, 2019). Therefore, the observed remediation potentials of fermented palm wine could be the result of the combined actions of a consortium of microorganisms and that of surfactants organic compounds.

The observed similarity in the remediation potentials of fermented palm wine on petroleum-contaminated soils from the two species of palms trees indicated that palm wine obtained from these sources may be of similar microbial or chemical constituents. This implies that palm wine from other palm trees can be used for the treatment of petroleum-contaminated soils. The use of fermented palm wine for remediation of petroleum-contaminated soils is very promising because there are varieties of palm trees for the supply of palm wine in tropical climates like the Niger Delta, Nigeria (Asuk *et al.*, 2018). Application of fermented palm wine for remediation of petroleum-contaminated soils as observed in this study did not required much expertise and preconditioning, substrates or nursery activities. The fermented Palm wine can easily be applied directly to the soil. This makes it a method of choice compared to the other agents used in the study.

P. ostreatus

Investigation of alternative substrate for growing *P. ostreatus*

Adenipekun and Lawal (2012) stated that substrates sterilisation helped in the decontamination of other microflora, which can compete and slow down the growth of white rot mushrooms. Thus, the mushrooms could grow faster in sterilised substrates due to absence of competition from other microflorae. However, the destruction of these natural microflorae by sterilisation can eliminate their activities which may be useful in the remediation processes. It was, therefore, necessary to investigate the feasibility of growing the mushrooms without sterilisation. This investigation revealed that *P. ostreatus* can still

grow in substrates without sterilisation (Table 2). The outcome is useful for potential in situ application since it saves energy and removes the need for sterilisation equipment. It would also allow for the contribution of natural microflora towards the remediation process.

White rot mushrooms such as *P. ostreatus* naturally feed on lignin (Rabinovich *et al.*, 2004); therefore, substrates with higher lignin contents should favour its growth. *P. ostreatus* germinated and fruited faster in substrates with higher lignin contents (Table 2). A further study to also investigate the types of lignin present in these substrates and possible application of biotechnology for extraction is required. This would aid their commercial preparations for prospective use in the treatment of petroleum-contaminated soil. The cultivation of white rot mushrooms on palm substrates is yet to be reported; therefore, this investigation has added palm trees as promising substrates for the cultivation of white rot mushrooms. Palm substrates will also serve as valuable substrates for the application of mycoremediation on petroleum-contaminated soil, particularly in tropical regions like the Niger Delta, Nigeria.

Effects of application procedures on mycoremediation efficiency of *P. ostreatus*

Typical method of application of *P. ostreatus* on petroleum-contaminated soils is by layering (Stamet, 2005; Adenipekun *et al.*, 2015). The results in this study demonstrated that mixing palm tree substrates and mushrooms with soil combined with layering on topsoil resulted in significant enhancement ($p=0.000$) of mycoremediation efficiency of *P. ostreatus* on the petroleum-contaminated soils even under unsterilized conditions (Table 5). The observations can be attributed to an increase in surface area of contact between the mushrooms and the hydrocarbon contaminants in the soil matrix. Since these substrates were not sterilised, activities of other microorganisms may also contribute to the observed results.

Adenipekun and Fasidi (2005) obtained a biodegradation of 20% after 3 months and 40% after 6 months on petroleum-contaminated soils. Mehrasbi *et al.* (2003) reported remediation efficiencies of 36%, 55% and 60% after 6 months. The results obtained by layering in this study under unsterilised conditions are comparable to those of Adenipekun and Fasidi (2005), Mehrasbi *et al.*, (2003), Chiu *et al.*, (2009) and Adenipekun *et al.*, (2015) using *P. ostreatus* under sterilised conditions. However, the remediation efficiency obtained by method of mixing the substrates and mushrooms with soils combined with layering, is much higher than those of these literatures. The soils used in this study had very high initial levels of TPHs and

were conventional petroleum-contaminated soils taken from a site with proximity to an exuding oil well at Tibshelf, UK. Thus, the remediation outcome of *P. ostreatus* (85%) under these conditions is unique.

Although mushrooms can grow straight from spawn without substrates, most times this is not ideal (Royse, 2002). Meysami and Baheri (2003) reported that substrates are required for action of white rot fungi on petroleum-contaminated soils. Mamiro & Royse (2008) stated that a small quantity of mushrooms spawn can inoculate a much greater amount of substrate resulting in better mushrooms growth and yield compared to using the spawn alone. The low remediation efficiency in TPHs contents obtained in untreated soils and those treated with *P. ostreatus* without substrates (Figure 3) are comparable to those of Meysami and Baheri (2003). The present investigation verified that a suitable substrate is required for the application of mycoremediation on petroleum-contaminated soils. The use of palm substrates resulted in up to 60% reduction in TPHs with layering and 85% when the substrates are both mixed with the soils and also layered under unsterilised conditions. A comparison of remediation efficiency for the methods of layering of substrates, with that of mixing the mushrooms and substrate with soils combined with layering, revealed an increase of about 25% for the later. Thus, substrates type, and method of application can influence mycoremediation efficiency of white rot fungi on petroleum-contaminated soils.

The application procedure of mixing the substrates and mushrooms with soils combined with layering is advantageous for in situ applications of mycoremediation on petroleum-contaminated soils. Palm substrates can be ploughed into contaminated soils followed by the inoculation of the mushroom spawns for remediation of petroleum-contaminated soils. The combination of the mixing procedures with layering would also allow for protection of the remediation process against precipitation agents such as heavy rains, which are common in tropical climates (Yabi & Afouda, 2012). Thus, the spawns underneath the layered samples could continue the remediation almost undisturbed even during rainy seasons.

Commercial mushroom spawns are usually available as grain or sawdust spawns (Chang & Hayes, 2013). There is therefore, the possibility for the development of sawdust spawn of *P. ostreatus* using palm tree substrates, which could be used for remediation of petroleum-contaminated soils. Overall, mixing the substrates and mushroom with soils followed by

layering, resulted in optimisation of the mycoremediation efficiency of *P. ostreatus* compared to the usual method of layering.

5. Conclusion

This study investigated the potentials of sunflower species, fermented palm wine and *P. ostreatus* for treatment of petroleum-contaminated soils. The main conclusions are as follows:

- Cow manure can be used to increase soil nutrients, decrease acidity in soils and provide buffer for soil during remediation of petroleum-contaminated soils.
- Soils available nitrate and electrical conductivity increases with remediation efficiency and can be used to monitor remediation progress of petroleum-contaminated soils.
- Phytoremediation efficiency of sunflower species could be related to their biomass, with those having higher biomass exhibiting better remediation potentials.
- Fermented palm wine can be used for the remediation of petroleum-contaminated soils.
- Palm tree substrates can be used for growing white rot mushrooms under unsterilized conditions. The substrate can also be used for the application of white rot mushrooms on petroleum-contaminated soils.
- Mycoremediation potential of *P. ostreatus* can be enhanced by an application method of mixing the substrates and mushrooms with the contaminated soil combined with layering.
- Mycoremediation efficiency of white rot mushrooms on petroleum-contaminated soil depends on the application method and type of substrates.

The concept of phyto-and myco-remediation has been developed overtime with many challenges and short-comings. The present study goes beyond the remediation of petroleum-contaminated soils by sunflowers to the assessment of the remediation efficiency with species; and has established that this varies with biomass. This information is important in the choice of sunflowers for remediation. The use of fermented palm wine for treatment of petroleum-contaminated soils is novel and is useful for tropical regions where palm trees are abundant. Adapting *P. ostreatus* for use in the clean-up of petroleum-contaminated soils has been problematic. The present study has developed a realistic approach and with a novel

substrate (Palm tree which is abundant in tropical regions) for application of *P. ostreatus* in the clean-up of petroleum-contaminated soils.

The applications of these phyto- and myco-remediation techniques can provide environmentally friendly options for treatment of petroleum-contaminated soils. These techniques would be beneficial to tropical regions like the Niger Delta, Nigeria because of the abundance of the phyto – and mycoremediation resources such as palm trees and palm wine. The methods would also provide readily available and cost-effective alternatives for the management of petroleum-contaminated soil.

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