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Effects of Soil Texture and Amendment Options on Bioremediation of Hydrocarbons in Soil

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Structured Abstract:

Purpose: To investigate the effects of particle size distribution of soils and various amendments on the bioremediation of diesel contamination.

Methods: Six treatments were carried out on soil manipulated into 2 distinct textural compositions. Each treatment was replicated twice and the process duration was 21 days. Bioremediation monitoring and analyses of both degradation and microbial activities were carried out after 7 days and at the end of the experiment.

Findings: Gas chromatographic results of the extracts indicated high degradation efficiency in all treatments ranging from 62% to complete decontamination. There was significant mortality of indigenous microbes in the seeded microcosms. Natural attenuation produced the best results throughout the entire process with complete decontamination in both sand and sandy-loam alongside high microbial growths and diversity. No significant difference was observed between the applications of different biostimulants. An abiotic amendment, zeolite amended sand produced the best results among the 3 seeded microcosms with complete degradation after 21 days.

Originality: The study found that soil physical and chemical properties are affected by the particle size of the soils with significant implications on limiting factors in bioremediation, especially if the textural difference is large.

Keywords: Degradation, texture, biostimulation, natural attenuation, bioaugmentation.

Paper Type: Research Paper.

Introduction

The industrialisation in the developed and emerging economies of the world has resulted in an increased demand for energy source. Consequently, the use of crude-oil as the source of

energy has increased. The extraction, transportation, storage, refining and the application of this source are accompanied by spills and waste generation (Zanaroli *et al*., 2010). Spills and wastes from these processes have caused organic substances such as monoaromatic, polycyclic aromatic hydrocarbons and many others to be deposited on soils (Lee *et al.,* 2008; Xu and Lu, 2010). More so, sustainable development on the other hand demands and will continue to require a friendlier technology to treat contaminations of soils due to petroleum products and allied substances (Juwarkar *et al.,* 2010).

The conventional remediation is very expensive and there is a problem of generating another form of waste (Xu and Lu, 2010). The development of more environmental friendly and cost-effective methods of dealing with oil contaminated sites is necessary and bioremediation is an alternative. It is simple in maintenance and relevance over large areas, cheap and depletes or degrades the contaminating substances to less harmful products (Lee *et al.,* 2008). Several researches in the past attempted to use a variety of methods in an effort to investigate how to further improve the application of bioremediation in a more efficient manner. However, these have been limited to laboratory and pilot studies because soil properties related to field applications of this technology have been superficially studied. The relationship between aeration, soil voids and texture under bioremediation was under reported in the literature, even though degradation is faster and affected by the above soil qualities (Levi *et al*., 2014).

Bacteria such as Achromobacter, Acinetobacter, Bacillus and fungus like Allescheria, Aspergillus, Candida and many others are well documented as degraders, widely distributed in the soil (Jooet al., 2008; Zanaroli et al., 2010). These are either found naturally in soils due to chronic contamination. It may require biostimulation to encourage the growth of degraders as the case with Exxon Valdez oil clean up (Lindstrom et al., 1991), orbioaugmentation to enrich the capabilities of degrading microorganisms as reported successful in several studies (Zhang et al., 2010; Jooet al., 2008; Xu and Lu, 2010).Diesel spills are of specific concern as they remained persistent in the soil and microbes are sensitive to it (Mooney et al., 2013; Redondo-Gomez et al., 2014). Due to their mobility, diesel are classified hazards for man and other living things (Zanaroli et al., 2010). Hydrocarbon compounds are popular as carcinogenic agents and their adverse effects on human nervous system are well documented (Sihag and Pathak, 2014).

This paper investigates the effects of particle size distribution on various bioremediation techniques: natural attenuation (NA), biostimulation (BS) and bioaugmentation (BA) in soil contaminated with diesel fuel.

Materials and Methods

Sampling

Samples were collected from Clive Farm Pattingham in South Staffordshire, UK and University of Wolverhampton Soil Research Site Hilton Village, Wolverhampton, UK. Both sites had no history of contamination due to crude oil or any of its products prior to sampling. Sampling was randomly done at the top soil going few inches below ground surface using a garden trowel.

Soil Preparation and Bioremediation Treatments

Precisely 6000 g of samples were shaken through 4-mm set of sieves and at the end of the particle separation, 8 particle sizes were obtained and only particles less than 2 mmsize were considered for manipulation. The bioremediation treatments used were the natural attenuation, biostimulationand sterilised samples as control. Another set of bioaugmented treatments were stimulated using poultry manure, zeolite and NPK. The bacteria selected for bioaugmentation were *Acinetobactercalcoaceticus*, *Burkholderiacapacia* and *Ralstoniaeutrophaand* were all present in the augmented treatments. These were selected because of their capabilities to degrade hydrocarbons specifically diesel (Liu et al., 2014). Sample pH, porosity and permeability were determined

Preparation of Microcosms

Sandy and sandy-loam soils were determined by textural triangle and prepared by manipulating the content of different soil particles for use in the microcosms. Twelve 100 ml flasks containing 50 g of the prepared samples were artificially contaminated with 1% (v/w) diesel which was followed by six treatments. A soil sterilised at 121^{o}C for 1 hour containing diesel without amendment and / or bioaugmentation was used as a control to the entire experiment. Soil that has not been sterilised and without amendment but spiked with diesel was prepared as natural attenuation to investigate the natural degradation process. Precisely 4% (w/w) of NPK added to un-sterilised soil to stimulate the natural degradation. Deionised

water was carefully added to aid nutrient transfer. These three treatments were not seeded with any microorganism. Three strains of bacteria were added to the three remaining treatments, each containing 4% (w/w) of different amendments (NPK, poultry manure and zeolite). The flask containing zeolite (clinoptilolite) was further treated with 4% (w/w) of NPK. The 12 treatments were prepared in 2 replicates and placed in a water bath and the temperature controlled at 30^{o}C in a fume cupboard. The flasks were covered with cotton wool to reduce evaporation and allow adequate aeration in the microcosms.

Inoculation

Three strains of hydrocarbon degrading bacteria namely *Acinetobacter*, *Burkholderiacapacia* and *Ralstoniaeutropha* were obtained from a stock in the University of Wolverhampton microbiology laboratory. These were grown overnight in 100 ml of Tryptic Soy Broth in shakers at 30^{o}C, and 0.5 ml of the turbid media was washed by a centrifuge at 4500 RPM for 15 minutes and suspended in ¼ Ringer's Solution. The microcosms were inoculated immediately.

Hydrocarbon Extractions and Analyses

At day 7 and 21, 12 flasks were removed and oven dried at 39^{o}C for extraction and subsequent analyses. Extraction was done using Soxhlet Extractor and dichloromethane as solvent (Lin *et al*., 2010), and the extracts were dried at 38^{o}C using a rotary evaporator. These were then mixed with known amount of solvent. All samples awaiting analyses were stored in refrigerator at 4 ±1^{o}C. Residual diesel was analysed by gas chromatography (GC) installed with Flame Ionisation Detector (FID) according to the method of Molina-Barahona*et al.* (2004) with slight modification. The rate of temperature change used was 9^{o}C.

Microbial Assays

Microbial assays were done in 3 dimensions and generally, viable counts using spread plate method was used to estimate the growth of bacteria in the microcosms. These were carried out at day 7 and day 21 after treatments, by taking out 2 g of samples and suspended it in ¼ Ringer's Solution. Exactly 0.5 ml of the solution was plated on TSA and CFUs were isolated using 8-fold serial dilution method. Plates were incubated at 30^{o}C and observed after 24 hours and counted 48 hours after plating.

Statistical Method

All data from viable counts were analysed using unpaired t test and f test in Graph Pad Prism statistical package. The t-test was carried out to test for significant difference based on the assumptions that the variances of these values were equal. Additionally, f-test was also run to test for significant difference were the variances of these groups appeared unequal for better estimate (Ruxton, 2006).

Results and Discussions

Effects of Particle Size Distribution on Soil Properties and Microbial Activities

Table 1.1 shows the particle size pattern of the soil sample used and it indicates that the soil is normally distributed and has an average pH value of 6.84 (Table 1.2). The manipulation of the samples resulted in two distinct soil types (Table 1.2) with slightly modified physical and chemical properties. The pH in sandy-loam has been more acidic (6.03) while the sand has shifted slightly alkaline (7.04). Although, this difference is small, it has significant implication if a wide range of soil types are considered. Textural triangle in Table 1.2 has grouped soils into 12 types based on their particle size compositions; sandy-loam close to sandy soils in the classification which means sandy and sandy-loam soils have slight difference in particle size distributions and have shown such pH variation. It could be assumed that if there is higher difference in the particle size, there will be high difference in the pH of soils. The difference in pH could be due to the fact that the finer particles contained more acidic substance of organic origin, otherwise the original sample would have been more alkaline than its present pH status. The pHs for optimal hydrocarbon bioremediation in soils for biological processes have been under reported in the literature. However, highest microbial population was reported more likely to be at pH of 7.0 – 7.5 and the same study showed reduced biodegradation at pH below 6.5 compared to a pH range of 7.0 – 8.0 (Khorasanizadeh, 2014).

Porosity of sandy-loam is measured to be 40% which is lower than sandy having a porosity of 54% (Table 1.2). This finding has important implications as the pore size is a function of soil structure, a 3-dimensional arrangement of solid particles and voids in which microbial communities reside (Juarez *et al*., 2013). Therefore, any slight difference in the texture could affect the volume of voids and the microbial activities taking place within the voids. Secondly, porosity might have resulted in the differences of permeability with both samples

having much different permeability to that of the initial sample prior to manipulation. The permeability of the sample prior to manipulation as shown in Table 1.2 is 0.1099 ml/s but upon manipulating the sand has exhibited high permeability of 0.3480 ml/s compared to sandy-loam with 0.0442 ml/s.

Soil pH, porosity and permeability affect movement of moisture, nutrients, oxygen and their availability in the microcosms during experiment. Sandy-loam having moderate porosity and permeability is likely to support microbial activities because of even distribution of oxygen, nutrients and moisture. Sandy samples in which case moisture and nutrients are more likely to be concentrated at the bottom with less or no oxygen leaving the top of the microcosm dry with excess oxygen. A study by Walworth *et al.* (2013) was very critical of the presence of excess or inadequate oxygen and suggested that oxygen above 10.4% tend to impede hydrocarbon degradation. Optimal O_2 requirements reported by Sihag and Pathak (2014) for microbial activities are 10% while optimal degradation oxygen requirement for hydrocarbons is between 10-40%. The implication of maintaining the balanced optimal oxygen level both for microbial function and hydrocarbon degradation is delicate as it has to be maintained either at 10% or slightly below or above it, and for ordinarily contaminated soils, this is difficult to understand and realised. However, for a larger remediation of contaminated soil, this could be achieved by soil analyses and tillage operation. Additionally, when nutrients and moisture are concentrated at the bottom of the flask, the contaminants and the degrading microorganisms would be in mutual contact and this increases bioavailability of the contaminants (Wolf *et al.,* 2013). However, if the adsorption between the contaminants and soil particles (a factor of soil type) is high, contaminant left at the top part of the microcosm would be left unavailable to degraders. Less or no degradation could take place at that point and thus reducing the efficiency. More so, moisture migrating from top to bottom may turn part of the process into anaerobic and is found to be a much slower process as observed by Levi *et al*. (2014) and Mori *et al.* (2013). Nevertheless, fungal activity is likely to increase at that matrix (Wolf *et al.,* 2013).

Table 1.3 shows the results of microbial viable count conducted 1 week after the commencement of the experiment. Generally, sandy-loam has shown higher microbial growth during the first seven days with about 10^{-3} to 10^{-7}colony forming unit (CFU) / 50g of soil probably due to the availability of nutrients and even distribution of moisture. The growth on sand is between 10^{-1} and 10^{-3} slightly lower than the values obtained for sandy-

loam except samples amended with zeolite. This could be due to lack of nutrients in sand and probably the amendments used lack some essential minerals to aid their growth in the first one week. Zeolite showed considerable bacterial growth on sand in the first one week estimated at 10^{-8} CFU / 50g but declined to 10^{-5} at 21 days which may be due to decline in nutrients or carbon and energy sources. This finding is contrary to a finding of a study by Andrejkovičová *et al*. (2012) that zeolite impedes microbial activities.

The decline in microbial population in zeolite at 21 days is unlikely to be associated with the antimicrobial properties of zeolite, but with the depletion of carbon source as there was complete degradation of the contaminant in sandy-loam. This is consistent with the finding of Kuran *et al*. (2014) with a new insight. In slight contrast to Kuran being sceptical about the rate of performance of microbes in zeolite amendment, this result indicated maximum growth was reached in 7 days. At 21 days, the bacterial growth increased in sand and declined in sandy-loam probably because the processes is generally faster in sandy-loam. The increase in biological process in zeolite amended sand and its decrease in loamy soil is therefore independent of its anticipated antimicrobial properties. These factors, pH, texture, permeability, oxygen, nutrient content and availability, and water holding capacity are the limiting factors in bioremediation which have been found to be affected by the particle size manipulation. This result further supports with the findings of research conducted by Sihag and Pathak (2014).

Effects of inoculation on Diesel Bioremediation

Part of the objectives of this work was to compare the performance of known degraders and indigenous microorganisms during the process. During the first one week, Ralstoniaeutropha became dominant species as revealed by viable count, followed by Pseudomonas capacia and Acinetobacter. No fungi were present which could have been suppressed by the bacteria, since they were present in the natural attenuation samples irrespective of its source which are certainly not from the soils. The results in Table 1.2 indicated lower bioremediation efficiency compared to those for natural attenuation and BS contrary to expectations. An exception where both sand and sandy-loam augmented with the 3 strains and stimulated with NPK and zeolite was found to remove up to 79% and 81% in the first week. These have been followed with insignificant degradation in sandy-loam and 100% removal in sand after 21 days which are comparable to those of natural attenuation and BS. On the other hand, the use of NPK and PM as amendments with the same microbial consortia have yielded 60-85%

degradation in sandy-loam within the first 1 week and about 72% degradation in PM amended soil with no significant increase in degradation after 21 days. These findings are contrary to findings of Nwaichi *et al*. (2011) in phytoremediation, where PM provided an average efficiency of 83% and NPK with average of 55%, and a study by Adams *et al*. (2014) where efficiency of more than 80% were reported for organic amendments of animal origin. Nevertheless, the results corroborate the findings of recent researches (Karamalidis *et al.,* 2010; Soleimani *et al*., 2013; Suja *et al*., 2014).

In NA, removal efficiencies were as high as 75% and 76% for sandy loam and sandy samples during the first week and after 21 days, there was complete degradation in both samples. High efficiency may have resulted due to the presence of several colonies of microorganisms revealed by the viable count. Thangarajan *et al*. (2011) demonstrated similar results from bioremediation of landfill disposal with high contaminant concentration. The NA samples in this study have diversity of microorganisms with growth of up to 10^{-6} alongside high degradation efficiency. These results have demonstrated the capabilities of indigenous microorganisms to effectively degrade hydrocarbons. However, the degraders may likely be present in the diesel used for the experiment. Fungi were present and might have contributed to the high efficiency in NA. Although, this results differed from some published studies (Thangarajan *et al*. 2011; Lebkowska *et al*., 2011; Nwaichi *et al*., 2011; Teccari *et al*., 2012), they are consistent with that of Chagas-spinelli *et al*. (2012) and Pontes *et al*. (2013). The rate of degradation in the present study was higher compared to that of the previous studies. An explanation to this finding is probably because the concentration of the diesel spiked in the microcosms was comparatively lower.

There were an interesting growth of microbes in sterilised samples and effective bioremediation in NA. It is thought that indigenous microbes may have resisted sterilisation process and recovered as quickly as possible upon restoration of favourable conditions. This assertion has been supported by Trevors (1996) noting that microbial pores are more resistant to dry heat which needs to be moistened 1-2 days prior sterilisation to allow the pores to be hatched for ease of sterilisation. The recommended sterilisation temperature by this author is 200^{o}C for minimum of 24 hours. The temperature and time used in samples for this study were far below the descriptions of Trevors (1996) for sterilising 50g of soil which was precisely the same soil quantity used in this study.

Conclusion

1. Particle size distribution has affected both the physical and the chemical properties of the soils. Modification of the particle sizes led to significant changes in sample's pH which is a factor of chemical composition. Similarly, change in porosity due to particles manipulation has caused significant changes in permeability, soil aeration, water holding capacity and volume of voids. A change in pH seems insignificant but it is apparent that its implications were found to be significant especially when there is wide variation in soil texture. The pH difference affects the bioremediation and microbial optimum pH requirements. It was found that chemical content of soil is a function of soil particle sizes and there is significant variation in bacterial growths and activities within the different soil textures and treatments.

2. Natural attenuation was the best treatment in both soils, and amendment with zeolite has improve the microcosms and provided the best environment for the inoculated bacteria with obviously no indigenous microorganisms surviving. However, the bacteria from the stock performed less efficient than the indigenous microorganisms.

3. Finally, no significant difference was observed between the use of organic amendment (poultry manure) and inorganic amendment (NPK), but an abiotic amendment (zeolite) has greatly improved the bioremediation efficiency.

4. Significant mortality of indigenous microorganisms especially fungi was noticed and this indicated that they are not compatible with the organisms from the stock.

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Table 1.1

Particle Distribution of Samples

Sieve size	Particle sizes	Mass of particles (g)
4 mm	>4 mm	117.4
2 mm	2-4 mm	268.2
1 mm	1-2 mm	1908.3
500 µm	500 µm-1 mm	2500.5
250 µm	250-500 µm	523.9
125 µm	125-250 µm	119.1
63 µm	63-125 µm	127.1
PAN	<63 µm	219.1
		Total = 5783.6

*PAN: Container at the bottom of the set of sieves which holds the finest particles less than 63 µm.

Table 1.2

Sample Ph, Porosity and Permeability

Soil Types	pH of Samples			Average pH	Porosity (%)	Permeability (ml/s)
	Sample 1	Sample 2	Sample 3			
Original soil sample	6.89	6.86	6.77	6.84	-	0.1099
Sample A	6.04	6.06	6.00	6.03	0.40	0.0442
Sample B	7.05	7.07	7.00	7.04	0.54	0.3480

Table 1.3

Viable Count at 7 Days after Treatments

Treatments	Soil Types	
	Growth on Sample A (CFU)	Growth on Sample B (CFU)
Sterilised	14×10^{-7}	15×10^{-3}
NA	98×10^{-6}	70×10^{-2}
BS with NPK	23×10^{-4}	1×10^{-3}
BS (NPK) + BA (bacteria)	39×10^{-3}	44×10^{-1}
BS (NPK) + BA (bacteria) + Zeolite	34×10^{-6}	25×10^{-8}
BS (PM) + BA (bacteria)	7×10^{-4}	1×10^{-3}
F test of variance		
P value	< 0.0001	
P value summary	****	
Significantly different? (P < 0.05)	Yes	

BS = Biostimulation, BA = Bioaugmentation, NA = Natural attenuation, PM = Poultry Manure

Table 1.4

Bacterial Growth at 21 Days after Treatment

Treatments	Soil Types	
	Growth on Sample A (CFU)	Growth on Sample B (CFU)
Sterilised	4×10^{-5}	33×10^{-5}
NA	52×10^{-3}	55×10^{-4}
BS with NPK	6×10^{-2}	11×10^{-5}
BS (NPK) + BA (bacteria)	4×10^{-2}	10×10^{-5}
BS (NPK) + BA (bacteria) + Zeolite	22×10^{-6}	58×10^{-5}
BS (PM) + BA (bacteria)	2×10^{-2}	15×10^{-4}
F test of variance		
P value	< 0.0001	
P value summary	****	
Significantly different? (P < 0.05)	Yes	

Figure 1.1

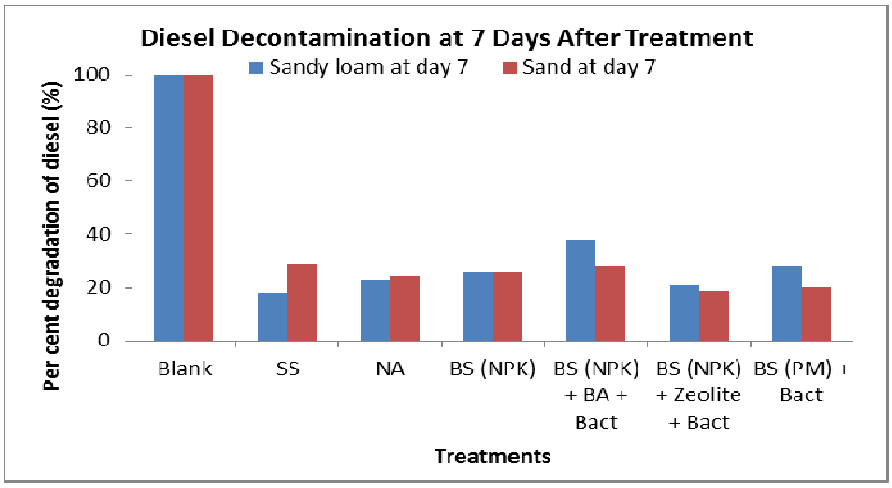

Figure 1.2