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Glycerol and Phosphorus Augmentation Coupled with pH Alteration are Potential Facilitators of Petroleum Hydrocarbon Bio-diminution in Contaminated Soil

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Abstract

The combined use of glycerol, phosphorus augmentation and alteration of soil pH was investigated for application in bioremediation of petroleum hydrocarbon contaminated soil. Soil artificially contaminated with 1:1 ratio of diesel fuel and used engine oil mixture was used in this study. About 50 ml of glycerol was added to the contaminated soil, and the carbon to phosphorus ratio and pH adjusted to 16:1 and 5.5 respectively. A control which consisted of artificially contaminated soil was setup alongside the treated setup. The moisture content of the contaminated soil in the control setup and treated setups were maintained at 10%-15%. The soils in both setups were tilled intermittently during the course of the experiment to facilitate the inundation of soil pores with air. A decrease in hydrocarbon concentration of about 72% was obtained in the treated setup, while a percentage decrease of 50% was observed in the control setup. The hydrocarbon utilizing bacterial population in the treated setup ranged from 5.70×10^4 CFU.g^{-1} to 1.37×10^6 CFU.g^{-1}, while the hydrocarbon utilizing bacterial population in the control setup ranged from 6.33×10^3 CFU.g^{-1} to 9.15×10^4 CFU.g^{-1}. The extent of hydrocarbon content diminution in the treated setup was significantly ($p<0.05$) higher than that obtained in the control setup. Data from this study thus suggest that simultaneous augmentation with glycerol and phosphorus, and soil pH alteration enhanced the biodegradation of hydrocarbons in petroleum-hydrocarbon contaminated soil. Further exploration and application of this treatment method holds a promise as an innocuous intervention for sustained petroleum hydrocarbon content diminution in contaminated soils.

Keywords: Augmentation; pH; Glycerol; Phosphorus; Petroleum hydrocarbon; Bioremediation

Introduction

Bioremediation of petroleum hydrocarbon polluted soil can be achieved through the addition of easily utilizable carbon compounds that will lead to co-metabolism of the petroleum hydrocarbon molecules. Addition of glycerol, a carbon compound easily utilized by many microorganisms, has been shown to increase the biodegradation rate of high-molecular weight polycyclic aromatic hydrocarbons [1]. The degradation rates in the study suggested that the degradation of the aromatic hydrocarbons occurred through co-metabolism. High percentage removal of polycyclic aromatic hydrocarbons through the use of glycerol as a co-substrate was also obtained by the researchers in another study [2]. Addition of macronutrients and adjustment of pH can also enhance the removal of hydrocarbons in petroleum hydrocarbon polluted environment. Phosphorus as the dominant macronutrient in a nutrient mixture has been shown to enhance oxygen uptake thereby leading to enhanced bioremediation of petroleum hydrocarbon contaminated soils [3]. In another study where methane and air were initially tested for *in situ* bioremediation of a trichloroethylene-contaminated site, MPN values of methanotrophs increased only after addition of a gas mixture containing phosphorus [4]. This indicated that the gas mixture containing phosphorus stimulated methanotrophic biodegradation of the trichloroethylene contaminant.

Addition of phosphorus to a hydrocarbon contaminated environment will invariable follow the concept of CP ratio. The optimum CP ratio required for biodegradation of hydrocarbons in contaminated soil as implied from the review of Thapa et al. is 100:1. However, a CP ratio of 10:1 as implied from the work of Nwogu et al. has been shown to result in a high decrease in total petroleum hydrocarbon concentration during the bioremediation of petroleum hydrocarbon contaminated soils [5]. In a distant related study on biosurfactant, a CP ratio of 16, among other factors, was shown to optimize biosurfactant production [6]. It should be noted that biosurfactant are produced by some microorganisms to aid them in degradation of hydrocarbons [7].

pH is listed among the limiting factors for soil bioremediation [8]. Remediation treatments involving alkaline pH have been shown to result in low hydrocarbon degradation efficiency [9]. In another related study, remediation of hydrocarbon contaminated groundwater has been shown to be favoured with use of reaction mixtures having acidic pH than those having alkaline pH [10]. It has been noted that acidic pH range of 4.5-5.3 favours fungal growth, while most bacteria survive better in pH range of 6.5 to 8.5 [11].

Adjustment of the pH of a contaminated environment to a value between 5.3-6.5 may thus allow for a combined effort of both fungi and

bacteria in the biodegradation of the contaminants. In this study, the combine use of glycerol and a CP ratio of 16, followed by adjustment of the environmental pH to a slight acidic value of 5.5 were investigated as a means for bioremediation of petroleum hydrocarbon contaminated environment. The outcome of the study revealed the extent of the influence of combined effect of co-metabolism, phosphorus augmentation, and pH reduction on the success of bioremediation of hydrocarbon contaminated soil.

Materials and Methods

The bioremediation setup

The bioremediation setup consisted of about 5 kg soil artificially contaminated with about 500 ml of 1:1 diesel oil and used engine oil mixture in an amber coloured glass tank. Selected physicochemical properties of the uncontaminated soil and the subsequent contaminated soil which include total organic carbon (TOC), pH, nitrogen, phosphorus, moisture content, and total petroleum hydrocarbon (TPH) were determined.

About 50 ml glycerol was added to the contaminated soil in the bioremediation setup. A derived quantity of KH_2PO_4 was also added so as to obtain a CP ratio of 16:1. The moisture content of the contaminated soil was adjusted to about 10% using sterile warm (35°C-40°C) distilled water, and the pH was adjusted to 5.5 using 0.1 M tetraoxosulphate (VI) acid.

The moisture content was maintained between 10%-15% at weekly intervals. A control was setup alongside the bioremediation setup. The control also consisted of artificially contaminated soil. The moisture content of the contaminated soil in the control was adjusted just as in the bioremediation setup. The soils in the control and the bioremediation setup were tilled twice weekly with the aid of a disinfected hand trowel.

Monitoring of bioremediation

Soil samples were collected from the bioremediation setup and the control at weekly intervals. The samples were collected with the aid of a disinfected hand trowel, and sterile small size wide-mouth bottles of about 50 ml capacities. The samples were analysed for, Total heterotrophic bacterial (THB) population, Hydrocarbon utilizing bacterial (HUB) population, pH, and total petroleum hydrocarbon (TPH) concentration. THB and HUB were enumerated using the standard plate count method. In this method, nutrient agar (NA) plates were used for THB, while mineral salt agar (MSA) containing fluconazole were used for hydrocarbon utilizing bacteria.

Due to the insolubility of Fluconazole in water based medium, the content of a 50 mg Fluconazole capsule was used for an MSA medium volume of 300 ml so as to achieve an optimum distribution of the particles of Fluconazole in MSA plates. Petroleum hydrocarbons were supplied into inoculated MSA plates using the vapour phase transfer method, and the plates were incubated at ambient temperature (29°C-32°C) for 5-7 days. Inoculated NA plates were incubated at 37°C for 24 h.

Quantification of TPH concentration in soil samples

The TPH concentrations of the contaminated soils in the bioremediation setup and control setup were determined through spectrophotometric method. About 10 g of the soil samples were placed, separately, in a 150 ml capacity beaker, followed by the addition of 20 ml Xylene. The mixtures were agitated for about 5 min, and then filtered using a Whatman No. 1 filter paper.

The extracts from the filtration were subjected to absorbance measurement using a 721 VIS Spectrophotometer (Huanghua Faithful Instrument Co. Ltd, China) set at 420 nm. The absorbance readings of the extracts, with the aid of the equation of the straight line of the calibration graph previously obtained, were then used to calculate the TPH concentrations.

Statistical analysis

The analysis of variance (ANOVA) was used to determine if there was any significant difference between the extents of hydrocarbon degradation in the bioremediation setup and the control setup.

Results

Physicochemical properties of the soil

Selected physicochemical properties of the soil used in the bioremediation experiment are presented in Table 1. The TOC of the contaminated soil used in the experiment as determined using the Mebius method was 2.1%. Based on the TOC value and phosphorus concentration of the contaminated soil, the C/P ratio was worked out to be 1050. For the C/P ratio to be reduced to 16, a derived quantity of 35.1 g KH_2PO_4 was added.

Property	Value
pH	6.0
Electrical conductivity	18.81 mS/cm
Bulk density	1.35 $g.ml^{-1}$
Moisture content	0.4%
Porosity	53.79
TOC	0.3% (2.1%)
N	(0.56%)
P	(0.002%)
(*) – Values in bracket were results obtained for the contaminated soil	

Table 1: Selected physicochemical properties of the soil.

Determination of the quantity of KH_2PO_4 to be added so as to achieve C:P ratio of 16

Addition of 50 ml glycerol to the bioremediation setup resulted in an additional carbon concentration of 0.49%. Based on the relative molecular mass and density of glycerol, 1 ml glycerol contains 0.49 g Carbon [12]. Thus 50 ml glycerol contains $\frac{50\ ml \times\ 0.49\ g\ C}{1\ mL} = 24.5\ g\ C$. The additional Carbon content resulting from the addition of 50 ml glycerol to the soil in the bioremediation setup is thus $\frac{24.5\ g\ C\ \times\ 100}{5000\ g\ soil}\% = 0.49\%$]. The total Carbon content in the bioremediation setup is thus 0.49%+2.1% (i.e. additional carbon content from glycerol+TOC value of contaminated soil). For C:P to be

16, P would be $\frac{0.49\% + 2.1\%}{16} = 0.1619\%$. Thus the phosphorus content in the bioremediation setup needs to be increased by an additional amount of 0.1599%, i.e. 0.1619%-0.002% (worked out P value-P value of contaminated soil). The bioremediation setup contains 5 Kg of soil, and 0.1599% of 5 Kg is 7.995 g. There are 31 g of phosphorus in 136 g of KH2PO4. Thus 7.995 g of phosphorus would be contained in $\frac{136\,g \times 7.995\,g}{31\,g} KH2PO4 = 35.07\,g\,KH2PO4$. Thus an approximate value of 35.1 g KH2PO4 was added to the contaminated soil in the bioremediation setup.

Bacterial population of the experimental setup

The THB population in the control ranged from 2.01×10^6 CFU.g^{-1} to 8.03×10^6 CFU.g^{-1}, while the THB population in the bioremediation setup ranged from 1.43×10^6 CFU.g^{-1} to 2.70×10^6 CFU.g^{-1}. The HUB population in the control ranged from 6.33×10^3 CFU.g^{-1} to 9.15×10^4 CFU.g^{-1}, while the HUB population in the bioremediation setup ranged from 5.70×10^4 CFU.g^{-1} to 1.37×10^6 CFU.g^{-1}.

A comparison of the bacterial population in the control and bioremediation setup is presented in Figure 1. In Figure 1 it can be seen that the bioremediation setup had a higher THB population on day 14 and day 35. Also, it can be seen that the bioremediation setup had a high HUB population than the control setup throughout the duration of the study.

Change in pH and TPH concentration

The pH and TPH concentration of the soil samples from the bioremediation setup and the control setup at weekly intervals is presented in Figure 2 and Figure 3 respectively. Figure 2 shows that in the course of the bioremediation the pH of the contaminated soil in the two setups increased from acidic values to values close to neutral pH. Figure 3 shows a general decrease in the TPH concentration, with the decrease been more in the bioremediation setup.

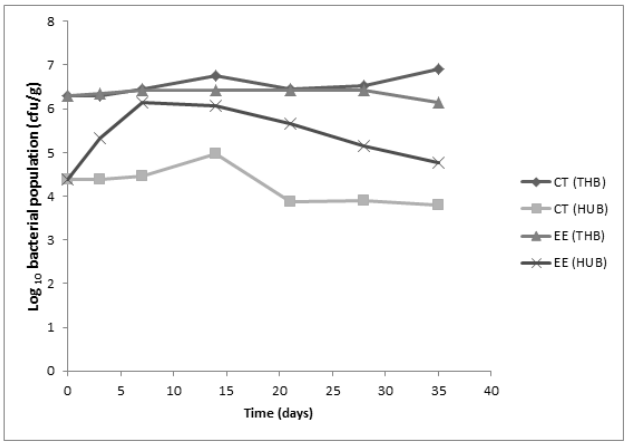

Figure 1: Total heterotrophic bacterial (THB) and hydrocarbon utilizing bacterial (HUB) population of the experimental setups.

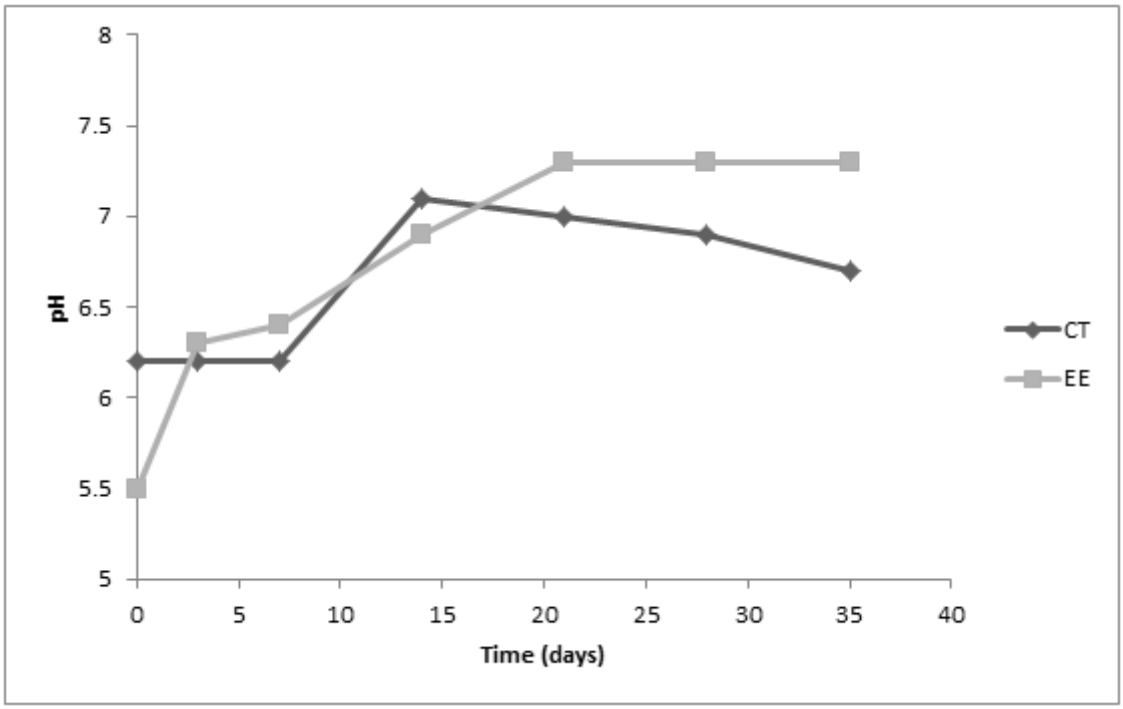

Figure 2: pH of the experimental setups.

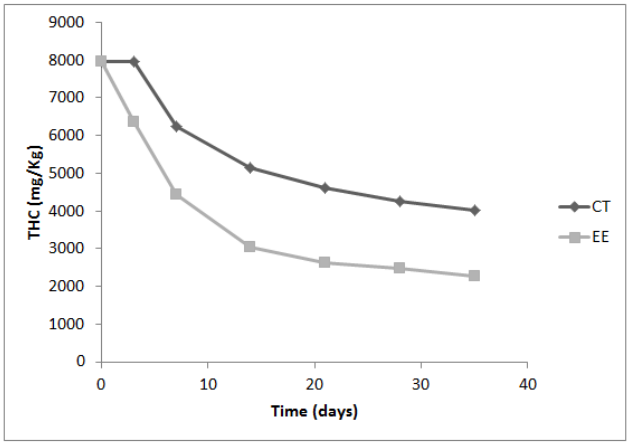

Figure 3: Reduction in the total petroleum hydrocarbon (TPH) concentration of the experimental setups.

Statistical significance of the extent of hydrocarbon degradation

The result of the analysis of variance (ANOVA) in determining if there is a significant difference between the extent of hydrocarbon degradation in the bioremediation setup and the control setup is presented in Table 2. In Table 2 it can be seen that the $F_{calculated}$ is greater than the F_{tab}. ($F_{tabulated}$). There is thus a significant difference between the extents of hydrocarbon degradation in the bioremediation setup and the control setup.

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**		
CT	3	12023.57	4007.857	139591.8		
EE	3	6773.571	2257.857	68928.41		
Source of Variation	*SS*	*Df*	*MS*	$F_{calculated}$	*P-value*	F_{tab}
Between Groups	4593751	1	4593751	44.0605	0.00267	7.7086
Within Groups	417040.5	4	104260.1			
Total	5010791	5				

Table 2: Analysis of variance of the final TPH concentrations in the bioremediation and control setups.

Discussion

Addition of certain organic compounds which are utilizable by microorganisms to petroleum hydrocarbon contaminated soils usually enhances the attenuation of the hydrocarbons through means of co-metabolism [13-15]. Glycerol, an organic compound which consist of three carbon atoms [12], is easily utilized as a carbon and energy source by many microorganisms [16-18]. The utilization of glycerol by microorganism as a co-metabolic means has been shown to lead to increase in biodegradation rate of hydrocarbons [1,2].

Apart from applying co-metabolism in the bioremediation of hydrocarbon polluted environment, the influence of other factors on the bioremediation process needs to be elucidated. Addition of glycerol and fertilizer was shown by Vasconcelos et al. to be more effective than addition of only glycerol in the removal of polycyclic hydrocarbons from hydrocarbon contaminated soil after a period of 30 days [1]. About 46% of the polycyclic hydrocarbons were removed by the combine use of glycerol and fertilizer within that period. In this study, about 72% decrease in hydrocarbon concentration was obtained (Figure 3) with the combined use of glycerol, phosphorus augmentation, and reduction of soil pH to 5.5.

The deviation between the extent of hydrocarbon reduction obtained by Vasconcelos et al. and what was obtained in this study could be attributed to the class of hydrocarbon monitored [1]. Total hydrocarbons were monitored in this study, while Vasconcelos et al. monitored a sub-class of hydrocarbons [1]. The sub-class monitored is less amenable to biodegradation compared with straight chain hydrocarbons. Thus a large extent of removal may have been observed if the total hydrocarbons were monitored. In the course of the bioremediation, the pH of the contaminated soil in the two setups increased from acidic values to values close to neutral pH (Figure 2).

From Figure 2 it can be seen that the soil already has a slight acidic pH. However, reduction of the pH of the bioremediation setup to a more acidic pH resulted in a stable value close to neutral pH, while there was a tendency in the control setup to return back to the original slight acidic pH. In some remediation studies where chemicals were used, enhanced removal of the contaminants were more effective at pH range of 3-6 [10].

This effectiveness observed at lower pH range supports the enhanced bioremediation observed at the further reduced pH used in this study. On comparing Figure 1 and Figure 2, it can be seen that in the control setup, from day 7-day 14, there was an increase in pH as well as an increase in the HUB population. The increase in the HUB population could be attributed to the improved soil moisture and tilling, which were intermittently carried out for both setups. The increase in the HUB population, which also reflects a general increase in metabolic activity, may have resulted in increasing the soil pH from an acidic value to a value close to neutral pH. In a bioremediation study in which the effect of soil pH on the biodegradation of polycyclic aromatic hydrocarbons was investigated, greatest bacterial population was observed at a soil pH of 7.5 [19]. This is in close agreement with the highest HUB population of the control setup observed at pH 7.1 in this study.

In this study, it has been revealed that the addition of glycerol, phosphorus augmentation to the tune of CP ratio of 16:1, followed by adjustment of soil pH to 5.5 resulted in about 72% decrease in hydrocarbon concentration (Figure 3). The control setup had about 50% reduction in hydrocarbon concentration. This moderate reduction in hydrocarbon concentration in the control setup could be attributed to the maintenance of soil moisture at 10%-15% and tilling, which were done for both setups. Improving soil moisture and tilling are among activities which can bring about enhanced natural attenuation of hydrocarbons in the environment [20,21].

The high reduction of hydrocarbon concentration in the bioremediation setup is supported by the relatively high population of hydrocarbon utilizing bacteria in the setup (Figure 1). Bioremediation of hydrocarbon polluted environment through means of co-metabolism can thus be further enhanced by improving phosphorus content, soil moisture, reducing soil pH to a slight acidic value, and tilling. On comparing the extent of hydrocarbon reduction in the control setup and the bioremediation setup using ANOVA (Table 2), it can be seen that there is a significant difference between the extents of hydrocarbon reduction in both setups. Thus the attenuation of hydrocarbons in the bioremediation setup is significant.

Conclusion

Addition of organic compounds such as glycerol to petroleum hydrocarbon contaminated soils can enhance the attenuation of the hydrocarbons through means of co-metabolism. However, other factors such as the availability of relevant macronutrients and soil pH can influence the outcome of the bioremediation process.

In this study, the addition of glycerol, phosphorus augmentation to the tune of C:P ratio of 16:1, followed by adjustment of soil pH to 5.5 resulted in a high reduction of the total hydrocarbon concentration. The extent of hydrocarbon reduction was significant compared to a control setup where only improve moisture content and tilling were carried out.

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