Iranian J Publ Health, Vol. 32, No. 3, pp.28-32, 2003

Biodegradation of Petroleum Hydrocarbons in Soil

*MR Mehrasbi[1], B Haghighi[2], M Shariat[1], S Naseri[1], K Naddafi[1]

[1] School of Public, Tehran University of Medical Sciences, Iran
[2] Institute for Advanced Studies in Basic Sciences, Gava Zang, Zanjan, Iran

Abstract

Biodegradation of petroleum hydrocarbons (20 g/kg dw soil) was investigated in 3 media, differing in the kind of petroleum fractions. In the laboratory experiments, during 5 months, the activities of petroleum hydrocarbon-degrading microorganisms and dehydrogenase activity of soil was determined. Gas chromatographic analysis showed the biological decontaminations for gas oil, kerosene and synthetic mixture (gas oil, kerosene and furnace oil) are 60 %, 36 % and 55 %, respectively. Dehydrogenase activity which was assessed by TTC technique, correlated significantly positive with the numbers of microorganisms. The Spearman rank correlation coefficients(r) in contaminated soils with gas oil, kerosene and synthetic mixture were 0.79, 0.80 and 0.69, respectively.

Keywords: *Petroleum hydrocarbon, Biodegradation, Soil, Zanjan*

Introduction

Hydrocarbon compounds such as petroleum are essential elements of life. Since they do not naturally occur in the forms most useful to humans, they can be hazardous. Fuel and lubricating oil spills have become a major environmental hazard to-date. The contamination of the environment with petroleum hydrocarbons provides serious problems for many countries. Scientists have conducted research on cost-effective clean-up techniques with minimal long-term damage to the environment. Biodegradation of hydrocarbon-contaminated soils, which exploits the ability of microorganisms to degrade and/or detoxify organic contamination, has been established as an efficient, economic, versatile and environmentally sound treatment (1). The extent of hydrocarbon biodegradation in contaminated soils is critically dependent upon three factors: a) the creation of optimal environmental conditions to stimulate biodegradative activity, b) the predominant Petroleum hydrocarbon types in the contaminated matrix and c) the bioavailability of the contaminants to microorganisms. Additionally, the petroleum hydrocarbon degradation is also affected by the molecular composition of the hydrocarbons (2). The effectiveness of bioremediation often is a function of the microbial population and how they can be enriched and maintained in an environment. Microorganisms with the ability to degrade crude oil are ubiquitously distributed in soil and mine environments (3). However, when few or no indigenous degradative microorganisms exist in a contaminated area or when there is no time for the natural enrichment of a suitable population; inoculation (bioaugmentation) can be realistic option (2). Microbial enzyme activity assay is a simple and rapid method to indicate the cycling of nutrients in soil (4). Enzymes may originate from biotic (viable cells) or abiotic (extracellular) components and their very specific reactions may only allow a small fraction of the total population being detected in the test. Therefore, soil enzyme activities may not always show a strong correlation with other soil biological parameters (5). Dehydrogenase involves in the

***Corresponding author:** P.O. BOX: 45157-86349, Fax: +98 241 7273153, E-mail: mehrasbi@yahoo.com

electron transport system to remove the oxidative substrate, and has been found is in correlation with the oxygen uptake and organic substrate removal rates in aerobic systems (6).The aims of this study were to evaluate the biodegradation of petroleum hydrocarbons by inoculum addition to the soil where the indigenous population of hydrocarbon-degrading microorganisms is small, and to evaluate the soil dehydrogenase activity within the biodegradation period.

Materials and Methods

Soil samples were obtained from depths of 0.5 and 1 m as well as from ground surface in a contaminated area close to the Storage and Distribution Center of Oily Products in Zanjan-Tehran road and gasoline stations of Zanjan. Processing on soils began immediately upon arrival at the laboratory. Soil samples were sieved moist using a 2 mm mesh screen and thoroughly mixed. 10 g of soil was added to 95 ml deionized water containing 2 drops of Tween 80 and then was incubated and shaked (150 rpm) for 30 min at room temperature. The mixture prepared is called soil solution. A 100 ml Erlenmeyer (flask 1) was prepared containing 2.5 ml soil solution and 95 ml of mineral salt medium (MSM)(KH_2PO_4, 1.52 g; Na_2HPO_4, 2.44 g; $CaCl_2.2H_2O$, 0.50 g; $MgSO_4.7H_2O$, 0.20 g; $(NH_4)_2SO_4$, 0.50 g; trace element, 10ml) and 2.5ml of synthetic mixture of petroleum hydrocarbons (gas oil, kerosene, furnace oil; 1:1:0.1) as sole source of carbon (1). The flask was incubated at 22 ± 2 ^oC. After 15 days, 2.5 ml of flask1 was transferred to a second flask (flask 2) with the same condition as flask 1.These incubating -transferring were repeated 4 times and at the final stage (fourth period) the amount of cfu (colony forming units) in flask 4 was 20×10^8 per ml. The experiments were carried out with soil from an uncontaminated area around Zanjan. The soil samples were sieved by a 2 mm screen and were sterilized three times by autoclaving at 200 ^oC for 30 min followed by incubation for 24 h at 37 ^oC (7). Six 250 ml Erlenmeyer flasks were prepared for each of the soils investigated. In each flask 200 g dw of soil was contaminated and mixed with 4 g of petroleum hydrocarbons. Flasks A and Ab were contaminated with gas oil, flasks B and Bb with kerosene, flasks C and Cb with the mixture of petroleum hydrocarbons (gas oil, 2 g; kerosene, 1g; furnace oil, 1g). For the determination of abiotic hydrocarbon losses, poisoned controls (flasks Ab, Bb and Cb) were contaminated with 0.3 % (w/w) sodium aside (8). Flasks A, B and C were amended with inoculum (20 ml/g dw soil). Controls received no inoculum. Ratios of C/N (Carbon/Nitrogen), N/P (Nitrogen/Phosphor) and P/K (Phosphor/Potassium) were adjusted to 10/1, 5/1 and 0.5/1, respectively by agricultural, water-soluble inorganic fertilizer (9). The water content was adjusted with sterile water to 45 % of the maximum water holding capacity in all flasks (10). Water losses during inoculation were compensated for regularly by the addition of sterile water. The pH of the soil during incubation was adjusted at 7 ± 0.2 by adding CaO. The flasks were closed with cotton wool stoppers and incubated in the dark at 22 ± 1 ^oC for 5 months. In order to avoid anaerobic conditions, the contents of flasks were mixed thoroughly every second day. The experiments were performed for 5 months and samples for analysis were taken every 10 days (i.e. 15 samples). Soil microbial counts were determined by the plate-count method. Soil suspensions were prepared as described previously (8). Triptose soy agar (TSA) (Pronadisa cat.1068.00) was used for counting (11). For the determination of soil dehydrogenase activity, 5 g of soil was weighted into test tubes and mixed with 5 ml of 3 % sterile triphenyltetrazolium chloride (TTC) solution. The tubs were sealed with rubber stoppers and incubated for 24 h at 30 ^oC. After incubation, 40 ml acetone was added to each

tube and the tubes were shaken thoroughly and re-incubated at room temperature for 2 h in the dark. The soil suspension was then filtered (15 ml) and the absorbance of the extracted triphenylformazan (TPF) were measured at 546 nm using a spectrophotometer (12). Enzyme activity and cfu measurements were performed in three replicates. The mean results of three replicates were used for statistical data treatment. Normal distribution of the data was tested by Kolmogrov-Smirnov test. All of data were nonparametric, therefore correlation between the measured parameters (cfu and enzyme activity) were analyzed by Spearman rank order correlation method. TPH was analyzed using a gas chromatograph (Varian model 3800), which was equipped with a flame ionization detector (FID) and a capillary column (25 m, long; 0.25 mm, ID; 0.25 μm, film thickness). Temperatures of the injection port and detector were 260 and 390 ^{o}C, respectively. Oven temperature was kept constant at 40 ^{o}C for 5 min and then programmed with the rate of 15 ^{o}C/min to reach 240 ^{o}C. Samples were prepared by extraction 5 g soil with 5 ml n-hexane. The method of extraction has been described in TNRCC methods (13).

Results

The number of microorganisms, as assessed by plate counts on TSA in the soils contaminated with gas oil, kerosene and synthetic mixture at the start of the experiment were 20×10^7, 15×10^7 and 20×10^7 cfu/g dw, respectively and at the end of incubation period these numbers were reduced to 10×10^7, 2×10^7, 0.8×10^7 cfu/g dw soil. The numbers of microorganisms were reduced significantly after almost 3 months of incubation (Fig. 1). The activities of dehydrogenase enzyme in the flasks A, B and C after starting the experiment were 120, 100 and 112 μg TPF/g dw.2h soil, respectively. The enzyme activity increased after about 25 days of incubation (Fig. 2). Correlation between the soil enzyme activity and numbers of microorganisms was analyzed according to the Spearman rank order correlation method. Microbial counts and enzyme activity correlated significantly positively to each other. The Spearman rank correlation coefficients were 0.79, 0.80 and 0.69 for flasks A, B and C, respectively. The initial concentrations of gas oil, kerosene and synthetic mixture immediately after starting the experiment were 24.9, 22.5 and 24.4 mg/g soil dw, respectively. These concentrations were reduced to 1.95, 6.24, and 4.99 mg/g soil dw, respectively in the main flasks (non-poisoned) within 5 months. The reductions of hydrocarbons concentrations are shown in Table 1.

Table 1: Decontamination of petroleum hydrocarbons in soil.

Petroleum fraction	Initial concentration mg/g dw soil	Final concentration mg/g dw soil	Total reduction (%)	Biological reduction (%)
Gas oil				
Poisoned	24.9	2.28	31	
Non-poisoned	24.9	17.2	91	60
Kerosene				
Poisoned	22.5	14.4	36	
Non-poisoned	22.5	6.29	72	36
Mixture[a]				
Poisoned	24.4	18.5	24	
Non-poisoned	24.4	4.99	79	55

[a] Mixture: gas oil, kerosene and furnace oil.

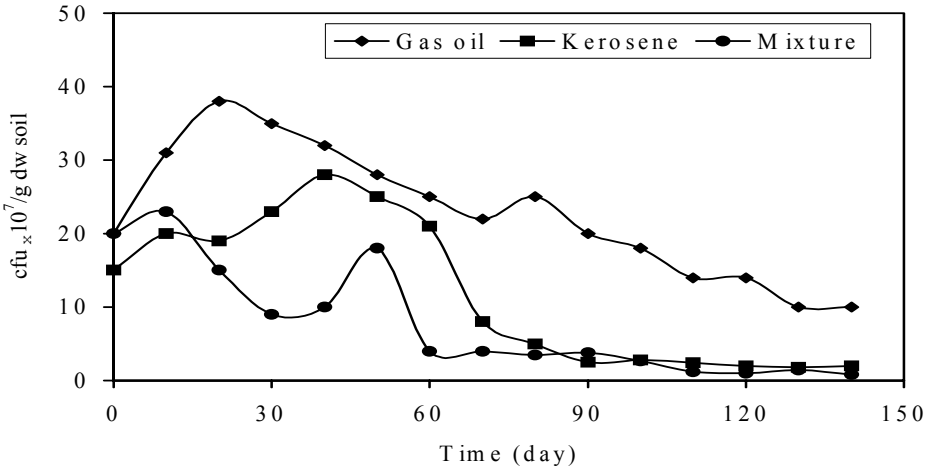

Fig. 1: Time course of bacterial growth on petroleum hydrocarbon.

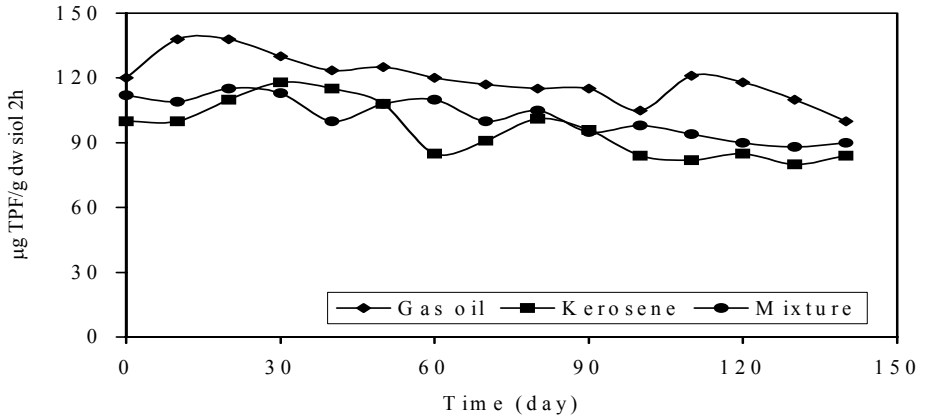

Fig. 2: Time course of dehydrogenase activity during the growth period.

Discussion

In the artificially contaminated soils, abiotic processes such as volatilization and adsorption on soil colloids (clay minerals and humus particles) play an important role in the decontamination of petroleum hydrocarbons. A part of the mineral oil hydrocarbons added to the soils remains undetectable (14). In the present study a considerable loss of hydrocarbons can be attributed to abiotic processes. Hydrocarbon loss by biological processes is the difference between the eliminated amounts of hydrocarbons in Poisoned control soils and non-poisoned soils. In our study the amounts of hydrocarbons lost by biological processes (biodegradation) are significant within 5 months. It was shown that the inoculum addition to soil could stimulate bioremediation of the TPH that are used in Iran. In this study microbial inoculation for removal of TPH achieved better results than those previously cited (9, 14). N-alkenes with the intermediate chain length (C10 - C24) are degraded most rapidly. Short chain alkenes are toxic for many microorganisms but they

generally evaporate rapidly. Very long chain alkenes are increasingly resistant to biodegradation (15). In this study, biodegradation of gas oil with intermediate number of carbon was greater than others. Kerosene contains of light hydrocarbons, so its abiotic reduction is greater than gas oil. In mixture we added, furnace oil contains long chain hydrocarbons so its biodegradation is smaller than gas oil. Soil dehydrogenase activity reflects a broad range of microbial oxidative activities. In our study, dehydrogenase activity was strongly related to the number of microorganisms in all cases, so dehydrogenase activity can be considered as an indicator for aerobic biodegradation of TPH.

Acknowledgements

The authors are grateful to all the staff of the School of Public, Tehran University of Medical Sciences, Iran who sincerely cooperate in performing this research.

References

1. Margesin R, Schinner F (1997). Efficiency of indigenous and inoculated cold adapted soil microorganisms for biodegradation of diesel oil in Alpine soils. *Appl Environ Microbiol,* 63 (7): 2660-64.
2. Marques-Rocha FJ, Hernandez-Rodrigues V, Lamela MAT (2000). Biodegradation of diesel oil by microbial consortium. *Water, soil and air pollution,* 128: 313-20.
3. Venkateswaran K, Harayama S (1995*). Cand J Microbiol*, 41: 767.
4. Pepper IL, Gerba CP (1995). *Environmental Microbiology*, Academic Press. New York.
5. Anderson JPE (1990). Principles of and assay systems for biodegradation. *Adv Appl Technol*, 4: 129-45.
6. Tiwari SC, Tiwari BK, Mishra RR (1989). Microbial population and enzyme activities. *Biol Fertil Soils,* 8: 178-82.
7. Kastner M, Breuer-Jammali M, Mahro B (1998). Impact of inoculation protocols, salinity and pH on the degradation of PAHs and survival of PAH-degrading bacteria introduced into soil. *Appl Environ Microbiol,* 64: 359-62.
8. Margesin R, Schinner F (1994). Properties of cold adapted microorganisms and their potential role in biotechnology. *Biotechnol,* 33: 1-14.
9. Margesin R, Schinner F (2000). The impact of hydrocarbon remediation on enzyme activities and microbial properties of soil. *Acta biotechnol*, 20: 313-33.
10. Baldrian P, Wiesche C, Gabriel J, Nerud F, Zadrazil F (2000). Influence of Cd and Hg on activities of ligninolytic enzymes and degradation of PAHs by *Pleurotus ostreatus* in soil. *Appl. Environ Microbiol,* 66(6): 2471-78.
11. Kleinheinz GT, Bagely ST (1997). A comprehensive approach to laboratory two phase liquid biodegradation study, gtkleinh@mtu.edu
12. Alef K, Paoplo N (1995). *Methods in Applied Soil Microbiology and Biochemistry.* Academic Press, New York.
13. Texas Natural Recourse Conservation Commission (TNRCC), method 1005 (2001). *Total petroleum hydrocarbons*, Revision 03
14. Venosa AD, Haines JR, Allen DM (1992). Efficiency of commercial inocula in enhancing biodegradation of withered crude oil contaminating. *J Ind Microbiol*, 10: 1-11.
15. Atlas B (1998). *Microbial Ecology*, Benjamin Cummings, California.