



Biodegradation and leaching of polycyclic aromatic hydrocarbons in soil column

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Abstract

Polycyclic aromatic hydrocarbons are common ubiquitous compounds found in soils due to natural and anthropogenic productions, which are a threat to agricultural ecosystems. The present study aims to evaluate the efficiency of poly aromatic hydrocarbons removal in contaminated soil column. Also, prediction of the groundwater contamination as well as optimized biodegradation of these pollutants using the selective microbial consortium and various nutrients is among the crucial objectives of this study. For this purpose, four pilot bioreactors were used and filled with contaminated soil mixed with an indigenous microbial consortium and one of the following nutrients: $(\text{NH}_2)_2\text{CO}$ (T1), $(\text{NH}_4)_2\text{SO}_4$ (T2), NH_4NO_3 (T3). However, final treatment (T4) did not receive anything to estimate passive bioremediation. For each treatment, the bioremediation rates of poly aromatic hydrocarbons were estimated by measuring their concentration in effluences of the pilot and soil column with 1 m depth every 25 cm for 4-week intervals. The results showed that the highest rate of degradation of poly aromatic hydrocarbons was observed in the first two weeks in the depth of 0-25 cm soil column. The maximum percentage of biodegradation of total poly aromatic hydrocarbons after 4 weeks in the depth of 0-25 cm of the different treatments decreased in the order of (T2) 78.43% > (T3) 69.97% > (T1) 66.96% > (T4) 35.57%. After 4 weeks, the maximum percentage of total poly aromatic hydrocarbons in the drainage of soil columns was calculated as 1.56%.

Key words: Poly aromatic hydrocarbons, bioremediation, soil column, soil contamination, leaching.

Introduction

Human activities lead to release of large quantities of organic and inorganic compounds into the environment every year. Soil contamination is a typical side effect of industrial activity ¹. Polycyclic aromatic hydrocarbons (PAHs) are characterized by two or more fused aromatic rings known to be toxic to humans ². PAHs are caused due to incomplete oxidation and combustion of organic compounds. Therefore, they are produced during industrial pyrolysis and combustion processes, by residential heating, motor vehicles, etc ³. They also cause the contamination of soil and groundwater in the environment ⁴. The purposed compounds persist in the environment and regarding to their hydrophobicity, become associated with particulate matter, such as humics and clays which are deposited in soils and sediments. Accumulation of PAHs in agricultural soils may increase uptake of them by crops, and threat the quality and safety of food ⁵. PAHs are lipophilic with the potential of biomagnificity through the food chain ^{6,7}. PAHs have been demonstrated to be biodegradable under suitable conditions in soil ^{8,9}. Some fungi and bacteria are capable to oxidize PAHs. Three types of microbial PAHs degradation include complete mineralization, co-metabolic transformation and nonspecific oxidation. Some bacterial strains that belong to different phylogenetic groups can cause complete mineralization of different PAHs ¹⁰⁻¹². Bioremediation is a process based on the metabolic activity of microorganisms with certain advantages among the technologies available to deal with contaminated soils ¹. Nevertheless, to assess a biological treatment

of contaminated soil, it is appropriate to characterize every location relating to the microbial populations and the biodegradability of the contaminants. In addition, additives such as fertilizers and inocula have a wide range of effects on the overall biodegradation of PAHs ¹. One of the other difficulties of developing bioremediation strategies lies in achieving better results in the laboratory studies. Therefore, pilot experiments were carried out to evaluate the effect of inoculation of an enriched microbial consortium and various nutrient sources in remediation of PAHs into Tehran Oil Refinery contaminated soil. Tehran Oil Refinery located in the suburbs of Tehran, Iran, is the most important refinery stably supplying petroleum products throughout Tehran.

The primary objective of the present study was to isolate a high diversity of bacterial strains with the ability of degrading multiple PAHs from Tehran Oil Refinery contaminated soil, which can be confirmed by combining the use of selective enrichment. The second objective was to obtain the optimized conditions of biodegradation of PAHs in laboratory. The final objective was to optimize the ratio of PAHs bioremediation in soil column using the results of laboratory experiments and predicting the groundwater contamination as a result of the leaching of PAHs.

Materials and Methods

Soil samples and analysis of PAHs: Soil samples were collected in sterilized panels from four different geographical directions of a land next to the active units of Tehran Oil Refinery in Iran. The

samples were transported to the laboratory on ice and stored at 4°C until they were analyzed. The soil properties of samples are provided in Table 1. The presence of PAHs contamination in the soil of this land was confirmed by solvent extraction (Dichloro methane) in an ultrasonic water bath¹³ and Waters high performance liquid chromatography (HPLC) system equipped with 410 binary pumps and 470 scanning fluorescence detector. The column used was Waters PAH C18 S-5 µm packing materials at 30°C. A mixture of 65% acetonitrile and 35% water was used as the mobile phase A and pure acetonitrile as the mobile phase B at a flow rate of 1 ml/min¹⁴.

Enrichment cultures and isolation of PAHs degrading bacteria:

Five grams of soil samples were inoculated into separate Erlenmeyer flasks containing 45 ml oil broth¹⁵. One litre of oil broth contained the following composition: KH_2PO_4 , 1g; K_2HPO_4 , 1g; $(\text{NH}_4)_2\text{SO}_4$, 1g; MgSO_4 , 0.04g; FeCl_3 , 0.004g and one ml of the mixture of trace elements dissolved in double distilled deionized water (DDW). Final pH 7 was achieved by 50% NaOH titration. One litre of the mixture of trace elements has the following composition: MnCl_2 , 0.023g; MnCl_4 , 0.03g; H_3BO_3 , 0.031g; CoCl_2 , 0.036g; CuCl_2 , 0.01g; NiCl_2 , 0.02g; ZnCl_2 , 0.05g; Na_2MoO_4 , 0.02g, dissolved in DDW.

A substrate of petroleum containing 5% of vacuum bottom in crude oil of Tehran Oil Refinery was prepared and used as a source of the PAHs. Of the substrate 500 µl served as the sole carbon and energy source for bacterial growth. The enrichment cultures were incubated aerobically at 37°C on a rotary incubator at 180 r.p.m. An additional culture flask was set up for each sample site with no added carbon source to serve as a negative control. Following visible growth, aliquots of the cultures were transferred to fresh oil broth, also containing 500 µl of the substrate. To obtain PAHs degrading isolates, these enrichments were plated on oil broth agar plates and sprayed with same substrate used in enrichment. Replicate plating was done using oil broth agar plates, sprayed with substrate and oil broth agar without PAHs (control). The criterion for selection of PAHs degrading strains was to enhance the growth on plates with substrate compared to the control plates without adding substrate. Selected PAHs degrading strains were subcultured on nutrient agar plates until individual colonies were obtained^{7,12}.

Optimizing of physical and chemical conditions of PAHs biodegradation in laboratory:

Five isolated bacteria strains with approximate concentration of 10^7 cells were inoculated into separate Erlenmeyer flasks containing 10 ml of oil broth with various concentrations of substrate (1, 5, 10, 20 and 30%) and incubated aerobically at 30°C on a rotary incubator at 180 r.p.m for 24 h. The potential of PAHs biodegradation was estimated by measuring the optical density (O.D.) at 540 nm, after extracting remaining

substrate with 5 ml of n-hexane. These experiments were conducted with changing the other conditions such as pH, nutrients, and temperatures to obtain the optimal conditions of PAHs biodegradation. The limits of conditions were: pH: 6, 6.5, 7, 7.5, 8; temperature 25, 30, 37°C; nutrient NH_4NO_3 , $(\text{NH}_4)_2\text{SO}_4$, $(\text{NH}_2)_2\text{CO}$, ratio of C:N:P 100:10:1, 100:5:1, 100:10:5, 100: 1:0.2.

Bioremediation treatments in soil column: Four 8 inch, P.V.C. pipes with 1.5 m height equipped with four valves in 25 cm distance along their height were used to contain the contaminated soils and a plate which was interlaced and a container connected at the end of them as a drainage. Four treatments were carried out to evaluate the efficiency of PAHs biodegradation in contaminated soil column.

The treatments were as follows:

- (1) *Basic treatment (T4):* Water was added at the beginning of experiment until leaching it from drainage of pilot, to maintain 60% of water holding capacity of the field. These conditions were applied to all treatments. This column did not receive any nutrient and microorganisms to estimate the natural attenuation or passive bioremediation.
- (2) *Inoculated treatment 1 (T1):* $(\text{NH}_2)_2\text{CO}$ and K_2HPO_4 were added to give a concentration equivalent to a C:N:P molar ratio of 100:5:1 and a microbial consortium from five selected species isolated to reach 10^8 microorganisms g^{-1} of the soil.
- (3) *Inoculated treatment 2 (T2):* $(\text{NH}_4)_2\text{SO}_4$ and K_2HPO_4 were added to give a concentration equivalent to a C:N:P molar ratio of 100:10:1 and a microbial consortium from five selected species isolated to reach 10^8 microorganisms g^{-1} of soil.
- (4) *Inoculated treatment 3 (T3):* NH_4NO_3 and K_2HPO_4 were added to give a concentration equivalent to a C:N:P molar ratio of 100:5:1 and a microbial consortium from five selected species isolated to reach 10^8 microorganisms g^{-1} of soil.

Before being packed in columns, the contaminated soil was sprayed with the substrate in order to reach the amount of organic carbon to 2% (this process was performed for the reason of old pollution of the studied area), and then microbial consortium and calculating amount of specializing nutrients, mixed with soil samples. The contaminated soils of treatments were sampled from soil column with 1 m depth every 25 cm at 0, 1, 2, 3 and 4 weeks for determining of microbial population and concentration of PAHs (as explained above).

Microbial analysis: Microbial populations were determined using the most probable number (MPN) method. Total heterotrophic counts of bacteria were carried out on nutrient agar plates whilst population of PAHs utilizing bacteria were determined by plating on oil broth and substrate used as the carbon source. These plates were incubated at 30°C for 48 h. The resulting colonies were later counted¹⁶. PAHs utilizing bacteria strains were identified by Bergy's manual of determinative bacteriology¹⁷.

Table 1. The physico-chemical characteristics of the soil samples.

Soil sample	pH (1:1)	CEC meq/l	Organic carbon%	Total N %	Total P mg/kg	Soil texture	Saturated soil moisture %
1	7.22	52.3	0.61	0.14	20.3	Clay	27.7
2	7.25	51.5	1.12	0.13	19.3	Clay	30.5
2	7.42	51.1	0.81	0.11	18.1	Clay	30.7
4	7.49	52.1	0.84	0.08	15.6	Clay	30.8
Soil of the pilots	7.41	52.10	0.73	0.12	19.1	Clay	29.9

Statistical analysis: To determine whether the percentage of PAHs biodegradation and microbial population differ significantly upon type of the treatment in different layers of soil column, one-way ANOVA and LSD tests were applied. Statistical significance was indicated according to P value of less than 0.05¹⁸. Also, cluster analysis was carried out using MVSP software.

Results and Discussion

Enrichment and isolation: Enrichment cultures were harvested 3 weeks after inoculation. Positive growth was determined by an increase in the turbidity of the flasks containing PAHs as a sole carbon and energy source compared to the negative control flasks. Serial dilutions of enrichment cultures were plated on solid oil broth and over sprayed with the substrate. Also, this phase enables the selection of colonies surrounded by zones of clearing in comparison with the no PAHs control plate. Representative colonies of different morphotypes from each final enrichment were selected for utilization of the additional substrate in liquid oil broth. Degradation was indicated by measuring the optical density (O.D.). Five of the isolated bacteria with the highest growth rate were selected for testing the effect of the concentration of the substrate on the PAHs biodegradation and further characterization. The results showed that the O.D. increased with increasing in the concentration of the substrate and achieved the maximum at 20% substrate. The additional substrate had a negative effect on bacterial growth. Morphological and biochemical characterization revealed the following organisms: three different species of *Pseudomonas* sp., *Micrococcus* sp. and *Bacillus sphaericus*.

Optimizing of physico-chemical conditions of PAHs biodegradation in laboratory: The mean of the initial amount of the total PAHs in soil samples was 406.5 µg kg⁻¹ of soil reached to 2111 µg kg⁻¹ of soil after spraying with the substrate. The native soil approximately had a neutral pH and low concentration of N. The physico-chemical characteristics of soil samples are given in Table 1.

In this study, the effect of various pH, temperature and nutrient on PAHs biodegradation was investigated. Temperature plays a crucial role in the biodegradation of petroleum hydrocarbons by affecting the physico-chemical properties of the oil and microbial community¹⁹. In addition, the best conditions for efficient removal of PAHs were pH 7.5 and temperature of 30°C, which agree with results of the other researchers^{20,21}. They found that there is a direct correlation between the decrease of degradation percentage with temperature decreasing, where higher temperature increased the rate of hydrocarbon metabolism to maximum. The primary limiting nutrients in microbial degradation of petroleum hydrocarbons have been historically proved to be N and P²²⁻²⁴. The quantity of N and P required to convert 100% of the petroleum C to biomass may be calculated from the cellular C:N and C:P ratios²⁵. Various authors provide ratios for these nutrients. There is some disagreement on the exact ratios. Meanwhile, it was concluded that different organisms have different requirements for N and P. Thus, addition of N and P at different concentrations should be considered for different groups of organisms. The laboratory results of present study showed that the best conditions for efficient removal of PAHs are C: N: P 100:5:1, 100:10:1, and 100:5:1 for NH₄NO₃, (NH₄)₂SO₄, (NH₂)₂CO as source of N, respectively.

Biodegradation pattern of PAHs in soil column: Percentage of biodegradation of PAHs was calculated according to following equation: [(Initial PAHs concentration in pilot soil - Final PAHs concentration "effluent" PAHs concentration in treatment) / Initial PAHs concentration in pilot soil] x 100.

Figs 1-4 show the comparison of biodegradation rate of total PAHs at the different layers of soil column in the various treatments.

Based on these results, the highest biodegradation rate (78.4%) of PAHs was observed in layer of 0-25 cm of the soil column of T2 after 4 weeks. The maximum percentage of biodegradation of total PAHs after 4 weeks in the depth of 0-25 cm of the different treatments decreased in the order (T2) 78.43% > (T3) 69.97% > (T1) 66.96% > (T4) 35.57%. One-way ANOVA and LSD tests¹⁷ were

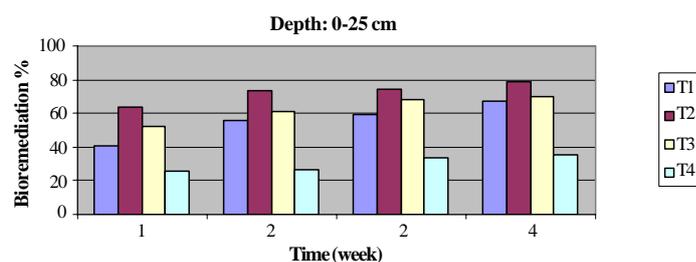


Figure 1. Comparison of biodegradation rate of total PAHs in the soil contaminated column, 0-25 cm layer.

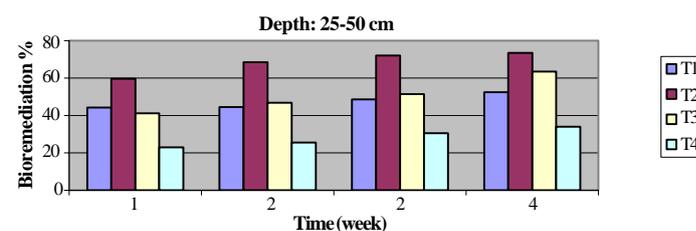


Figure 2. Comparison of biodegradation rate of total PAHs in the soil contaminated column, 25-50 cm layer.

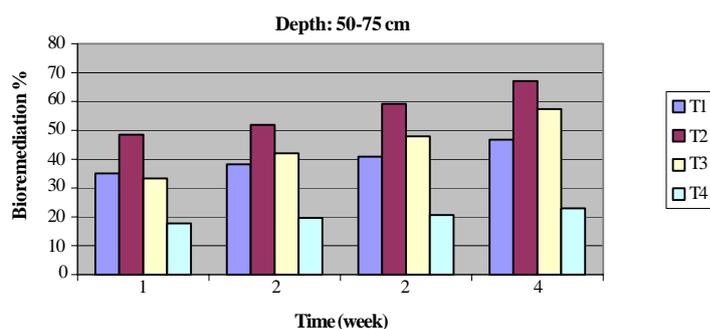


Figure 3. Comparison of biodegradation rate of total PAHs in the soil contaminated column, 50-75 cm layer.

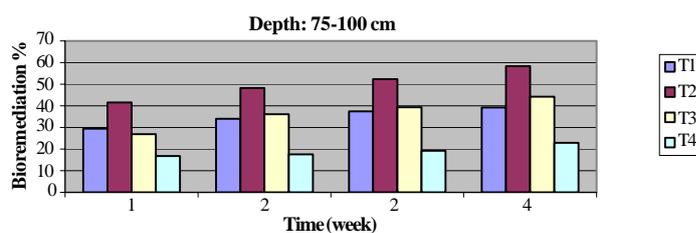


Figure 4. Comparison of biodegradation rate of total PAHs in the soil contaminated column, 75-100 cm layer.

used to determine whether the percentage of PAHs biodegradation and microbial population differ significantly according to type of the treatment in different layers of soil column. The results of statistical analysis on the 0-25 cm layer showed the significant different ($p < 0.05$) between T2 and two other treatments (T1 and T4). The results meant that the addition of $(\text{NH}_4)_2\text{SO}_4$ as a source of N (T2), could increase the bioremediation rate of PAHs in soil more than $(\text{NH}_2)_2\text{CO}$ (T1) and T4 (without adding any nutrient). Meanwhile, the average of PAHs bioremediation rate was $72.64\% > 55.68\% > 30.44\%$, respectively. Furthermore, the differences were not significant ($p < 0.05$) between T2 and T3, although the average of the percentage of PAHs biodegradation in T2 (72.64) was higher than T3 (62.84). Upon the results, it was revealed that $(\text{NH}_4)_2\text{SO}_4$ and NH_4NO_3 as a source of N had a similar effect on biodegradation of PAHs, approximately. Also, there was no significant difference ($p < 0.05$) between T1 and T3, although the effect of $(\text{NH}_2)_2\text{CO}$ as a source of N was lower than that of NH_4NO_3 for degrading PAHs in contaminated soils, with the average of PAHs biodegradation rate $55.68\% < 62.84\%$, respectively. Finally, in this layer (0-25 cm), the differences were significant ($p < 0.05$) between T4 and three other treatments (T1, T2 and T3) which meant the combination of the consortium of the selected bacteria and nutrients had positive effect on the removal of PAHs from the contaminated soil. The mean difference between T4 and other treatments were 25.2% (T1), 42.1% (T2) and 32.4% (T3). So, T2 ($(\text{NH}_4)_2\text{SO}_4$ as a source of N) was selected as the best treatment.

In three other layers (25-50, 50-75 and 75-100 cm) of different treatments, the pattern of PAHs biodegradation was similar with first layer (0-25 cm). However, the results of statistical analysis on these three layers showed a significant difference ($p < 0.05$) between T2 and T3 which shows the effect of $(\text{NH}_4)_2\text{SO}_4$ on the PAHs degrading was higher than that of NH_4NO_3 in lower layers of soil column.

The decrease of bioremediation rates of PAHs in all treatments correlated to increase in the depth of soil column. Whereas, aromatic hydrocarbons readily degraded under aerobic conditions²⁶, reduction of the biodegradation rate of PAHs is related to decreasing the aerobic conditions in the depth of soil column.

Also, Figs 1-4 show that increasing time of experiments increased the amount of the degradation of PAHs, but the highest rate of degradation was observed in the first week. This phenomenon could be concluded that in the beginning, oil-degrading microorganisms were stimulated by labile hydrocarbon sources (probably PAHs with lower molecular weight) that induced a high percentage of degradation. As those forms decreased, microbial populations had to use the more recalcitrant hydrocarbons less efficiently^{24, 27}.

Leaching of PAHs: Percentage of PAHs leaching was calculated according to the following equation:

[Final PAHs concentration "effluent" / Initial PAHs concentration in pilot soil] x 100.

Fig. 5 shows the average of the total PAHs leaching as percentage from the soil contaminated column during experiments.

The maximum percentage of total PAHs in effluences of the drainage of soil columns after 4 weeks was 1.56%. Low percentage

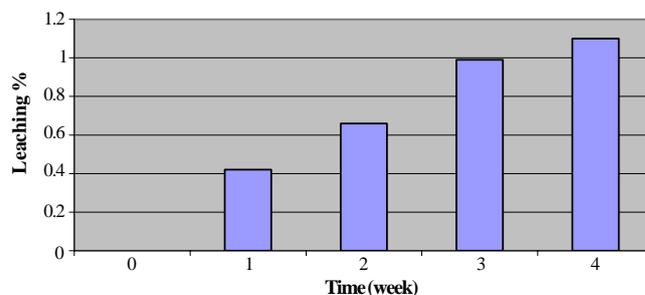


Figure 5. The average of leaching of total PAHs (%) from the soil contaminated column.

of PAHs leaching in all treatments show that the studied soil has a high potential in adsorbing of the PAHs. Although, the leaching of PAHs included the low percentage of total PAHs contamination in soil column, as they became associated in soil, they caused contamination of soil and groundwater⁴.

Microbial population: Figs 6-9 show the number of PAHs degrading microorganisms at the different layers of soil column in each treatment. The results of statistical analysis showed no significant difference ($p < 0.05$) between the population of PAHs degrading microorganisms in the different layers in each treatment. Also, there was no statistical difference ($p < 0.05$) between population of PAHs degrading microorganisms at the different layers of soil column in the various treatments, except T2 and T4.

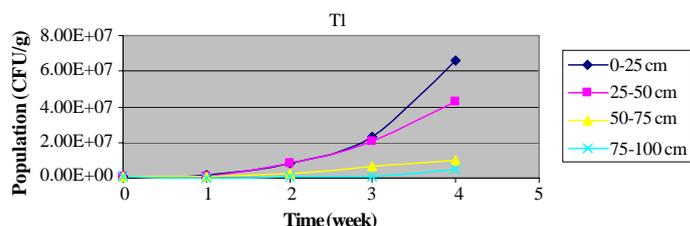


Figure 6. Comparison of the number of PAHs degrading micro-organisms in the different layers of soil contaminated column of T1.

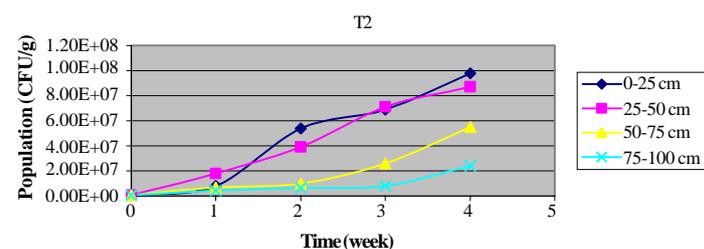


Figure 7. Comparison of the number of PAHs degrading microorganisms in the different layers of soil contaminated column of T2.

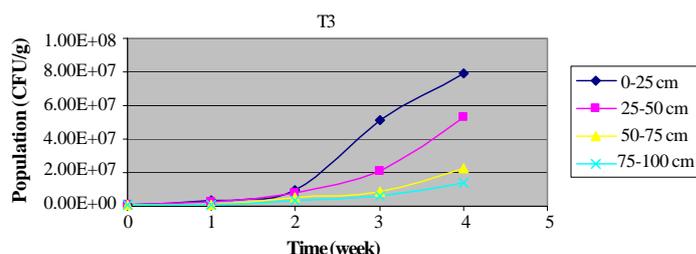


Figure 8. Comparison of the number of PAHs degrading microorganisms in the different layers of soil contaminated column of T3.

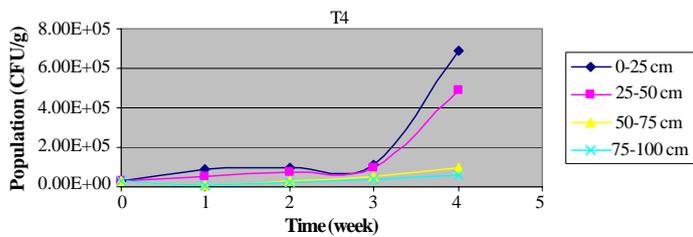


Figure 9. Comparison of the number of PAHs degrading microorganisms in the different layer of soil contaminated column of T4.

The comparison of the number of PAHs degrading microorganisms during time of experiments shows that the microbial population increased in time. Therefore, it was concluded that increasing in microbial population has a direct effect on the decrease of PAHs concentration during the same time²³.

Cluster analysis: Cluster analysis was carried out by using MVSP (Multi-Variate Statistical Package) software version 3.1 to find groups of similar parameters within the studied data and indicate the most affective parameters on the concentration of PAHs in soil column. The results shown as dendrogram (Fig. 10) classifies all of the parameters and indicates the level of similarity at which any two clusters were joined.

The dendrogram with three main clusters (A, B and C) indicates that time and depth of soil column were the most similar parameters and were clustered most closely together (A).

The number of PAHs degrading microorganisms at the different layers of soil column in different treatments were linked together with high similarity (B) and joined with Branch (A) with high correlation coefficient (0.92). Therefore, two studied parameters were the most affective ones on the number of PAHs degrading microorganisms in soil contaminated column.

Upon the cluster (C), pattern of PAHs biodegradation at the different layers of soil column in different treatments were similar. Cluster (C) joins cluster (D) with correlation coefficient of (0.7) which indicates a direct correlation between two clusters.

The high similarity between these three clusters (A, B and C) revealed that time, depth of soil column and the number of PAHs degrading microorganisms were the most affective parameters on the removal of PAHs in soil column.

Conclusions

Results showed that the highest rate of degradation of PAHs was observed in the depth of 0-25 cm of soil column at first two weeks. After 4 weeks in the depth of 0-25 cm, the maximum percentage of biodegradation of total PAHs of the different treatments decreased in the order of (T2) 78.43% > (T3) 69.97% > (T1) 66.96% > (T4) 35.57%. In all layers, T4 (passive bioremediation) had significantly lowest efficiency of PAHs degradation. The bioremediation rate of PAHs in all treatments was decreased with increasing in the depth of soil column. The differences were significant between T4 and three other treatments (T1, T2 and T3) which demonstrate that the combination of the consortium of the selected bacteria and nutrients had positive effect on the removal of PAHs from the contaminated soil column. Moreover, the mean difference between T4 and other treatments was 25.2% (T1), 42.1% (T2) and 32.4% (T3). T2 ((NH₄)₂SO₄ as a source of nitrogen was selected as the best treatment. There was no statistical difference between population of PAHs degrading microorganisms at the different layers of soil column in the various treatments, except T2 and T4. The maximum percentage of total PAHs in effluences of the drainage of soil columns after 4 weeks was 1.56%.

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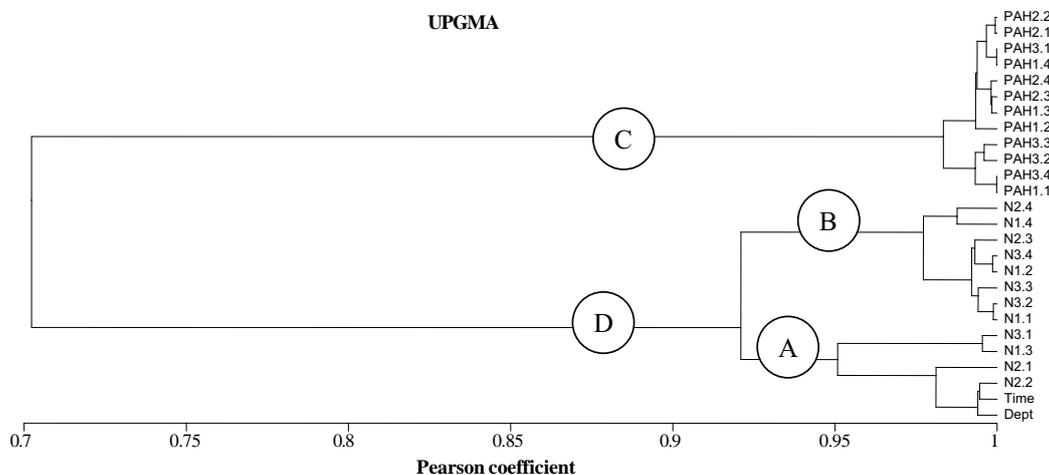


Figure 10. Dendrogram of cluster analysis of the biodegradation of PAHs in soil column.

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