Dryland Soil Research, Vol. 1, No. 1, pp. 57-72, Summer 2024
Type of article: Original, Available online at www.dlsr.iut.ac.ir.

Republished from:
https://doi.org/10.47176/jsssi.01.01.1010

Monitoring TPH biodegradation in soil around Ray oil refinery by natural attenuation, biostimulation and bioaugmentation treatments

Maryam Doustaky[1], Soheila Ebrahimi[2†], Ali Reza Movahedi Naeini[3] and Mohsen Olamaee[3]

[1] M.Sc.Student, [2] Associate Professor and [3] Associate Professor, Department of Soil Science, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran

†Corresponding Author Email: *sohebrahimi@gmail.com*

(Received 2022/10/02, Accepted 2022/26/11)

ABSTRACT

In this study, total petroleum hydrocarbons (TPHs) decontaminating mechanisms for soils around the Rey refinery complex (South of Tehran, Iran) was investigated. Natural attenuation, biostimulation and bioaugmentation (separately and in combination) methods were evaluated TPHs and soil microbial respiration in 210 days, using laboratory treatments The modified methods were applied through 13 different treatments, including improving the environmental conditions for native bacteria (natural attenuation for treatments 1-8), adding non-native bacterial complex (bioaugmentation for treatment 9) and intensifying and stimulating growth while adding non-native bacterial complex (biostimulation-bioaugmentation for treatments 10-13). Although, overall of the treatments, a significant decreasing TPHs concentration were observed over the time, biostimulation-bioaugmentation treatments had the highest amount of TPHs decomposition, the highest rate of bio-respiration, the lowest half-life times ($t_{1/2}$), and the highest remediation efficiency and biodegradation constants rate. Among natural attenuation treatments, modifiers with manure and sawdust had the greatest effect on reducing the TPHs concentration and the highest rate of bio-respiration. The first-order kinetic model was fitted to the data related to biodegradation in a satisfactory manner. The results showed that there was a strong and positive linear correlation between decreasing TPHs concentration and microbial respiration in all modifiers. Although for the bacterial treatments and at the early stages of inoculation, the rate of total respiration was low, but as the time passed and with adaptation of the effective inoculated bacteria to contaminated soil, the respiration rate gradually was increased. Due to its low cost and low environmental risk, the proposed bioremediation technique for oil contaminated soil can be recommended to the region.

Keywords: Microorganisms, Oil-contaminated, Petroleum-contaminated soil, Remediation, Soil pollution.

1. Introduction

Petroleum consists of a mixture of various types of hydrocarbons. Analysis of total petroleum hydrocarbons (TPHs), as a common group of persistent organic pollutants, is an important indicator for monitoring their presence, amount, and changes in the environment. In general, TPHs refer to petroleum-based hydrocarbons in the environment and consist of a vast number of chemical compounds derived from crude oil (Moreira et al., 2011).

Soil pollution with petroleum hydrocarbons is a serious environmental problem, especially in oil-rich countries. Oil is leaked from pipelines and wells or improper methods of disposing of oil waste around the world during its extraction, transportation, refining or storage. Petroleum compounds negatively affect the physical, chemical, ecological and biological characteristics of the soil (Fallah et al., 2015; Ramadass et al., 2015; Tazangi et al., 2020).

The widespread use of petroleum as the main source of energy worldwide has led to the development of various refining methods. Bioremediation is a cheap, effective and environment-friendly method in which microorganisms are used to degrade contaminants, and also extensively used to treat oil pollution. Bioremediation uses the degradation of contaminants by effective microorganisms to remove contaminants from the polluted environment. It seems that this method is effective in laboratory and field scale in remediation of various oil products (Karimpoor et al., 2022; Mojarad et al., 2016; Oliveira et al., 2013). The advantages of this method are environmental compatibility, cost-effectiveness, easy maintenance, and the possibility of use on sites (Ebrahimi et al., 2011). Factors such as nutrient adequacy (N, P and K in particular), humidity, temperature, pH and oxygen concentration which are essential for microorganism survival, determine the efficiency of bioremediation. The preparation and application of these essential factors, especially on a large scale, are a relatively simple and inexpensive method (Heshmati and Ebrahimi, 2018). The researchers found that microorganisms have an essential role in the removal of hydrocarbons. Addition of nutrients and stimulants can increase the activity and diversity of decomposing microbes in the soil and ultimately reduce

soil hydrocarbon pollution. Under natural conditions, after the leakage of oil pollution, the activity of native soil microorganisms leads to the removal of pollution over time (Doustaky et al., 2013). Various bioremediation methods are used in petroleum- polluted sites, including phytoremediation, biopiles, and biostimulation and bioaugmentation (Mair et al., 2013).

Bioaugmentation and biostimulation are main bioremediation strategies for remediation of oil-contaminated soils. Biostimulation increases the metabolic activity of the native microbes in the ecosystem through the availability and modification of nutrients. Nitrogen, potassium and phosphorus are necessary for the development of microorganisms. Various research show that nitrogen, phosphorus and potassium supplements are important for enhancing oil degradation in contaminated soils (Fallah et al., 2013; John et al., 2011; Shahi et al., 2016). Adding nutrients such as carbon, taking into account the N: P: K ratio, can increase the activity and diversity of microbial species and further facilitate the degradation of hydrocarbon in soil and ultimately reduce soil hydrocarbon pollution. Due to different soil characteristics, microbial community is diverse in different contaminated soils and thus, the optimal C: N: P ratio for soil remediation may vary. The use of modifiers with adequate nutrient storage can accelerate TPH degradation by biological stimulation in contaminated soil (Khosravinodeh et al., 2013; Liao et al., 2016). Petroleum hydrocarbons can be decomposed 13-150% by bacteria and 6-82% by fungi. The efficiency of petroleum hydrolyzing bacteria on a wide range of hydrocarbons is established (Liao et al., 2016). The size of the bacterial community involved in the refining of petroleum hydrocarbons as a source of carbon supply depends on the existing biological and abiotic factors and the adaptive capacity of microorganisms (Xu and Lu, 2010). Successful bioremediation of soil by bacteria has been reported in soils polluted with TPH concentrations of up to 700000 mg kg^{-1} (70% w/w) (Poi et al., 2017).

Bioaugmentation involves the addition of exogenous microorganisms into soil. When the population of hydrocarbon degrading microbes in the polluted soil are low, this method is a very good option (Ruffini et al., 2016; Xu and Lu, 2010). Furthermore, bioaugmentation is very useful in case the pollutants negatively affect the indigenous microbes (Tyagi et al., 2011; Wu et al., 2016).

An advantage of bioaugmentation is that degrading microbes may start the remediation immediately after they are applied. Despite some research indicated that bioaugmentation technology is an efficient approach to enhance TPH degradation in polluted soils, bioaugmentation effects are case specific and inconsistent. According to some researchers, bioaugmentation effects are temporary, whereas biostimulation may be a more efficient approach to efficient petroleum degradation (Abed et al., 2014; Kauppi et al., 2011; Liu et al., 2011; Megharaj et al., 2011; Polyak et al., 2018; Sayara et al., 2011; Yang et al., 2015; Wu et al., 2019). The failure of bioaugmentation treatment in some cases is not clear and needs further research (Wu et al., 2016).

This study was carried out by applying different biological methods in the form of 13 modifier treatments applied in the contaminated soil around Rey refinery in the south of Tehran with an initial pollution of 38% TPH for 13 consecutive times. To assess the TPH biodegradation in soil around the refinery by different natural attenuation, biostimulation and bioaugmentation treatments based on microbial remediation capacity, a pollution reduction kinetics model was determined. Then, a quantitative study of the effect of treatments on the rate of TPH change and respiration of the microbial community over time was done.

2. Materials and Methods

This study was designed to evaluate the feasibility of using three categories of low-cost, accessible, and widely implemented methods for decontaminating soil around the Rey (South of Tehran, Iran) refinery complex (safe and contaminated environment in the long run). These three categories included natural attenuation, biostimulation and bioaugmentation treatments. Pots containing intact contaminated soil in addition with different modifiers were used in this study.

The soil was sampled from a petroleum-polluted region near the Rey oil refinery. The area has been polluted due to gradual oil leakage. Samples were taken in a combined manner from three different areas around the refinery, from highly contaminated areas that were visible in a black zone.

2.1. Soil properties

As mentioned, physical and chemical characteristics of soil affect hydrocarbon reduction (Tang et al., 2012). Therefore, standard methods were used to determine some of these characteristics (Table 1). The studied characteristics were soil texture using hydrometer, electrical conductivity in saturated paste, total nitrogen (N) using Kjeldahl method, available phosphorous by Olsen method (Olsen and Sommer, 1982), pH in saturated paste using a pH meter, and soil carbon content with the Walkley and Black (1934) method.

2.2. Treatments preparation and designing pot experiment

Each pot sample consisted of 700 g of contaminated soil which total of 13 treatments were applied as follows: 1- dry soil (no water and aeration), 2- moist soil (soil with water without aeration), 3- soil with a mixture of urea, potassium chloride and triple superphosphate for supplying NPK elements with 20: 5: 1 ratio, 4- soil with

Table 1. Initial physical and chemical analysis of contaminated soil around the refinery.

EC (dS/m)	pH	TPH	Potassium (ppm)	Phosphor (ppm)	Total nitrogen	Water storage capacity in soil	The initial moisture	Organic mater
5.57	6.5	38%	80	5. 44	0.45%	5.8%	22%	87%

manure with 5% ratio of soil dry weight, 5- soil with sawdust with 10% ratio of soil dry weight, 6- soil with NPK + manure, 7- soil with NPK + sawdust, 8- soil with NPK + sawdust + manure, 9- soil with complex of bacteria, 10- soil with bacteria and NPK, 11- soil with bacteria and animal manure, 12- soil with bacteria and sawdust, 13- soil with bacteria and sawdust and NPK. In fact, these 13 treatments were divided into 3 groups and included improving the environmental conditions for native bacteria (natural attenuation for treatments 1-8), adding non-native bacterial complex (bioaugmentation for treatment 9) and stimulating growth by adding non-native bacterial complex (biostimulation-bioaugmentation for treatments 10-13).

2.3. Bacterial culture and inoculation process (in order to achieve a sufficient and effective number of applications of inoculation treatments)

To achieve the desired, effective and optimal bacterial population, it is necessary to first cultivate bacteria (2×10^8 cells per kg of soil) in agar medium. This value is necessary for optimal decomposition of TPH if the total contamination rate is 5 to 10% of the soil dry weight (Doustaky et al., 2013; Thapa et al., 2012). Since the soil around the refinery south of Tehran is heavily contaminated, the initial TPH is 38% of the dry weight of the soil. With that in mind, about three times of that amount of bacteria should be added to the soil. To achieve a suitable population, bacterial count was performed by dilution method and colony count. In order to inoculate the bacteria in the corrective treatments, the target colonies (each bacterium separately) were taken with a ring under sterile conditions and inoculated in liquid nutrient medium (80 cm^3 in a flask). After 48 hours, 2 cubic centimeters (in two stages) were taken from the flask and applied to the soil in treatments 9, 10, 11, 12 and 13. After ten days of equilibrium time, 40 cm^3 (60%-70% FC) water was added to all treatments (except dry soil) throughout the study and aeration was applied every 3 days.

2.4. Determination of TPH_s

Sampling was done from 8 cm soil depth and TPH was determined using the standard method of the United States Environmental Protection Agency (EPA 4113, 1) (Hutchinson et al., 2001). TPH was measured for all treatments 13 times (every 2 weeks).

Two grams of soil was added to 10 mL of dichloromethane. The solution was shaken for 30 minutes to separate the petroleum. Then, the mixture was centrifuged at 3000 g for 10 minutes. The supernatant was moved to a glass container and placed at room temperature. After 24 h, the material remaining in the container was weighed and recorded as TPH (mg kg^{-1}). Degradation in each treatment was calculated via formula 1 (Agarry et al., 2015):

$$E\% = \frac{TPH(s) - TPH(v)}{TPH(s)} \times 100 \quad [1]$$

in which, E%, TPH(s) and TPH(v) are respectively degradation percentage, the amount of hydrocarbon elimination in treated, and untreated soil.

2.5. Determination of microbial respiration

This parameter was performed using Anderson (1982) method 7, 14, 21, 28, 35 and 60 days after treatment. 10 mL of 0.5 N NaOH was put on soil surface in a 20-mL glass container to collect the CO_2 resulted from microbial respiration. The containers were incubated at 25 ± 1 °C for a week. Then, NaOH was titrated using 0.1 N HCL. Four containers which had no soil but contained NaOH served as control to subtract the CO_2 or distilled water absorbed by NaOH from total CO_2. Thus, carbon resulted from respiration was determined via formula 2.

$$CO2 - C = \left((B - S) \times N \times E \times 1000\right) / W \quad [2]$$

in which, CO_2–C is the amount of carbon resulted from respiration in mg kg^{-1}, B and S are respectively the volume of acid used in control and sample in ml, N is acid normality, E is equivalent weight, and W is soil dry weight (Agarry et al., 2015).

2.6. Kinetics of bioremediation

Kinetic analysis is very helpful to measure the petroleum remediation efficiency by calculating the instantaneous concentration of remaining hydrocarbon in soil. Also, the time required for the remediation of soil may be estimated using this analysis. First-order kinetics are usually used to describe petroleum bioremediation (Agarry et al., 2015).

$$Ct = C_0\, e^{-Kt} \quad [3]$$

in which, C_0 is the initial TPH concentration in control soil in mg kg^{-1}, C_t is the remaining TPH at the time t in

Table 2. Analysis of variance for TPH of the contaminated soil around the refinery.

Source of variation	SS	df	MS	F	Sig.
Corrected Model	1.637[a]	155	.011	7.106	.000
Intercept	178.649	1	178.649	120160.567	0.000
treat * time	.131	144	.001	.668	.996
treat	.315	12	.026	17.678	.000
time	1.191	12	.108	72.821	.000
Error	.464	312	.001		
Total	180.750	468			
Corrected Total	2.101	467			

a. R Squared = 0.779 (Adjusted R Squared = 0.670)

mg kg^{-1}, K is degradation constant per day and t is time (days) (Agarry et al., 2013; Polyak et al., 2018; Zahed et al., 2011).

2.7. Bioremediation half-Life determination

Half-life is the time required for a material to lose half of its initial amount. This concept is used in various studies such as chemical experiments (Aronson et al., 2006), environmental fate modelling (Sinkkonen and Paasivirta, 2000), and describing pollutant transformations (Dimitrov et al., 2007), and is determined using formula 4:

$$t_{1/2}=\ln(2)/K \quad [4]$$

in which, K is biodegradation rate constant in day^{-1}. The hypothesis for this model is that hydrocarbon biodegradation rate positively correlates with the amount of hydrocarbons in soil (Yeung et al., 1997).

2.8. Statistical Analysis

Descriptive statistics, analysis of variance and comparison of mean using LSD test were conducted using SAS v.9 software. The experiment was done as split-plot factorial in time based on the completely randomized design with 3 replications.

3. Results

3.1. Evaluation of physical-chemical properties of the contaminated soil

Due to the large amount of the total primary hydrocarbon pollution harvested in the soils around the refinery south of Tehran (Rey), it can be said that so far, less attention has been paid to the issue of cleaning highly polluted soils, especially the ones with more than 10% pollution (Mukherjee et al., 2010). Therefore, clearing soils with this level of pollution (38%) in this study is very significant in a large scale for the first time in Iran, which has vast petroleum reserves. For the biodegradation of total petroleum hydrocarbons in soil by microorganisms, some environmental physicochemical factors such as soil acidity, salinity, initial concentration of total petroleum hydrocarbons, temperature, and initial moisture play important roles. Therefore, the first step in conducting biodegradation experiments is the initial analysis of contaminated soil. Table 1 shows the preliminary results of the chemical parameters.

3.2. Evaluation of TPH removal

In analyzing the effect of modification of the 13 treatments applied, their effectiveness methods were divided into three categories. The first category included the application of environmental improvement methods that were predicted in treatments 1 to 8. The second group only included the inoculation method of non-native bacterial complex, which was investigated in treatment 9. The third category of efficiency was the inoculation method of efficient non-native bacteria complex with the addition of stimulant nutrients, which was included in treatments 10 to 13. Thus, the results and discussion are examined in three categories.

The analysis of variance for TPH shows that all the treatments used in time have become significant at the level of 1% (Table 2). Therefore, they have been effective in reducing TPHs**.**

Figure 1 shows the Removal of TPH within 210 days in soil remediation process. Figure 1A shows the declining trend in dry soil (control), wet soil, chemical fertilizers and animal manures. Figure 1B shows the amount of oil in sawdust treatments, a mixture of animal and chemical fertilizers, a mixture of sawdust and chemical fertilizers, as well as a mixture of sawdust and animal and chemical fertilizers. Figure 1C relates to bacterial treatments, a mixture of bacterial and chemical fertilizers, a mixture of bacterial and livestock manure, a mixture of bacterial and sawdust, a mixture of bacterial and livestock manure, chemical fertilizers and sawdust. As can be seen, with the use of different types of modifiers, a decreasing trend in TPH is evident over time.

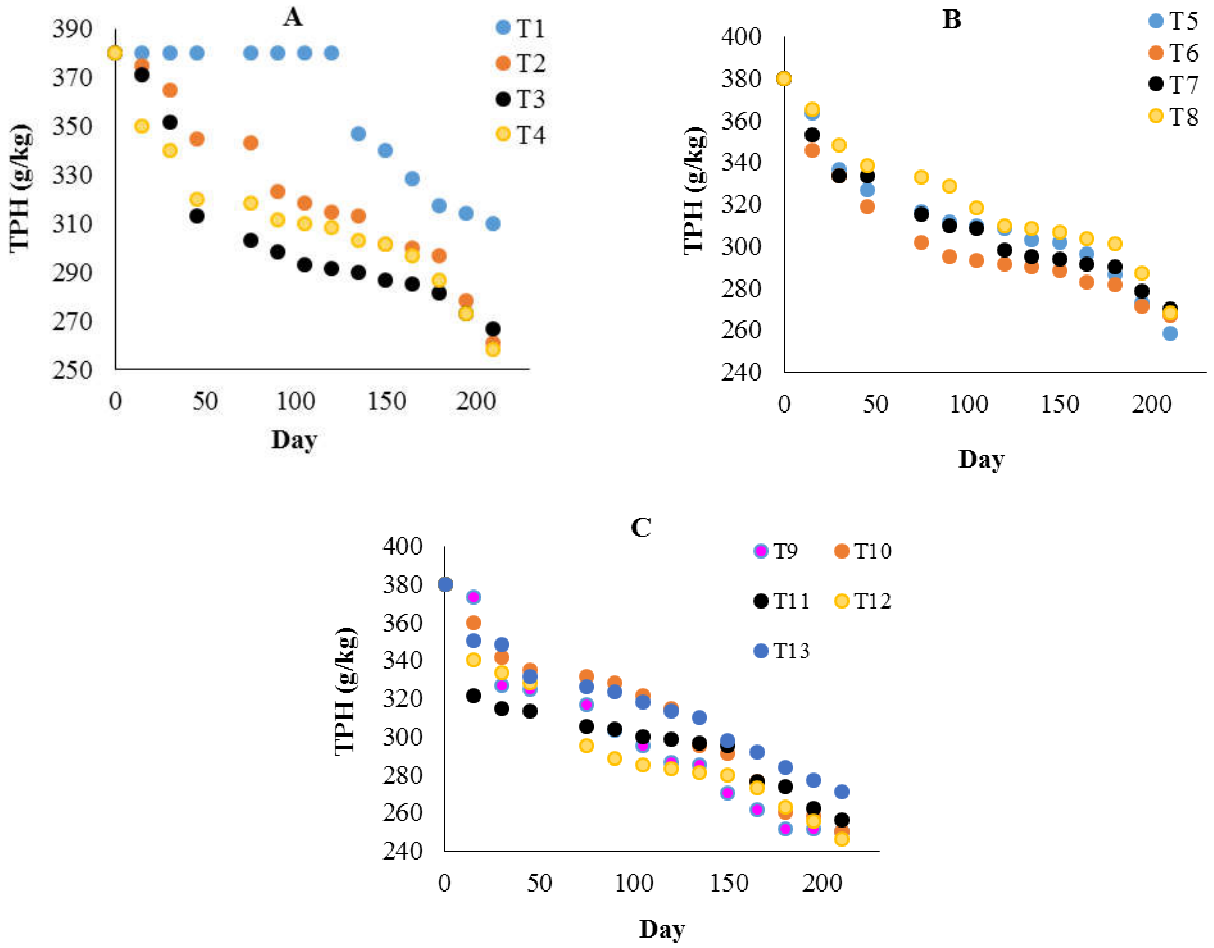

Fig. 1. The trends of removing TPH from the contaminated soil by applying 13 modifier treatments during 210 days

The results of the test, comparing the mean of the interactions of time and treatment with Duncan's method, are given in Table 3. Comparing the mean with the treatments, compared to the first treatment at all times, there was a significant difference. At all times, the highest amount of oil was observed in treatment 1. The lowest amount of oil was observed in the treatments in which the bacteria were inoculated (except treatment 13). At 45, 135, 195 and 210 days, there was no significant difference between all treatments except treatment 1.

3.3. Kinetics of biodegradation and half-life

The results of constant values of decomposition rate (K) and half-life (t1 / 2) are presented in Table 4. As can be seen, among the first 8 modifiers (related to the study of improving environmental conditions for hydrocarbon decomposition), treatments 1, 3 and 8 had the lowest decomposition rate constant and the longest half-life (493 days), and treatment 6 had the highest decomposition rate constant and the lowest half Omar (363 days). Among the bacterial inoculation treatments with nutrient stimulants, treatment 13 had the lowest decomposition rate constant and the highest half-life (575 days) and treatments 12 and 9 had the highest decomposition rates and the lowest half-life (287.5 days). Based on these results, it can be said that the half-life was reduced in modifiers 12, 9 and 10, which had bacterial inoculation with nutrient stimulants.

3.4. Results of the remediation efficiency

According to the results of Fig. 2, it seems that treatments 12, 9 and 10 had the highest remediate Efficiency (35.2, 34.3 and 34.2, respectively). However, as shown in Fig. 2, TPH reduction in T2 (moist soil) was 31.2%.

It seems that by providing moisture conditions for microorganisms in oil-contaminated soils, it is possible to improve the decomposition of hydrocarbon pollution. The results of evaluating the efficiency of environmental improvement method (treatments 1 to 8) in reducing TPH during the mentioned period showed a significant difference in dry and wet treatments. Wet soil treatment reduced TPH by 31% and dry treatment by 18.4%. The two main factors in reducing TPH in soil can be weathering (evaporation and light effects) and

Table 3. Comparison of the mean TPH with the use of modifiers at different times based on Duncan test

Treatments	Time 15	Time 30	Time 45	Time 75	Time 90	Time 105	Time 120	Time 135	Time 150	Time 165	Time 180	Time 195	Time 210
Treat1	0.76^{c}	0.76^{d}	0.76^{b}	0.6867^{d}	0.6567^{d}	0.6933^{c}	0.69^{d}	0.6933^{b}	0.68^{d}	0.6633^{b}	0.6567^{c}	0.6^{b}	0.58^{b}
Treat2	0.726^{bc}	0.683^{abc}	0.656^{a}	0.6367^{abcde}	0.6067^{abcd}	0.63^{abc}	0.61^{abc}	0.5933^{a}	0.5867^{ab}	0.5667^{a}	0.5633^{ab}	0.52^{a}	0.49^{a}
Treat3	0.73^{bc}	0.7033^{bc}	0.666^{a}	0.6533^{bcde}	0.6467^{cd}	0.6367^{bc}	0.62^{abc}	0.6167^{a}	0.59^{ab}	0.5833^{ab}	0.5933^{bc}	0.53^{a}	0.5^{a}
Treat4	0.7^{abc}	0.68^{abc}	0.653^{a}	0.6333^{abcde}	0.62^{abcd}	0.6^{ab}	0.5967^{abc}	0.5833^{a}	0.5767^{ab}	0.5767^{ab}	0.55^{ab}	0.52^{a}	0.49^{a}
Treat5	0.7^{abc}	0.6733^{abc}	0.65^{a}	0.6333^{abcde}	0.6233^{abcd}	0.6167^{ab}	0.5967^{abc}	0.59^{a}	0.58^{ab}	0.57^{ab}	0.5633^{ab}	0.52^{a}	0.49^{a}
Treat6	0.72^{bc}	0.666^{ab}	0.663^{a}	0.6667^{de}	0.6467^{cd}	0.62^{ab}	0.6167^{abc}	0.59^{a}	0.5967^{b}	0.6^{ab}	0.5667^{ab}	0.53^{a}	0.5^{a}
Treat7	0.75^{bc}	0.73^{cd}	0.676^{a}	0.63^{abc}	0.6567^{d}	0.6433^{bc}	0.63^{c}	0.6267^{a}	0.6033^{b}	0.6067^{ab}	0.58^{abc}	0.5433^{a}	0.5^{a}
Treat8	0.743^{bc}	0.696^{bc}	0.67^{a}	0.6633^{cde}	0.6333^{bcd}	0.6367^{bc}	0.6267^{bc}	0.6067^{a}	0.6033^{b}	0.5833^{ab}	0.5733^{ab}	0.53^{a}	0.5^{a}
Treat9	0.7^{abc}	0.653^{ab}	0.63^{a}	0.6067^{ab}	0.6067^{abcd}	0.5867^{ab}	0.5733^{ab}	0.57^{a}	0.54^{a}	0.5533^{a}	0.5033^{a}	0.51^{a}	0.48^{a}
Treat10	0.68^{ab}	0.666^{ab}	0.62^{a}	0.61^{abc}	0.5767^{a}	0.5867^{ab}	0.5833^{abc}	0.5633^{a}	0.56^{ab}	0.5467^{a}	0.52^{ab}	0.5^{a}	0.49^{a}
Treat11	0.64^{a}	0.63^{a}	0.64^{a}	0.6033^{ab}	0.5967^{abc}	0.59^{ab}	0.5833^{abc}	0.58^{a}	0.5833^{ab}	0.55^{a}	0.5467^{ab}	0.51^{a}	0.49^{a}
Treat12	0.7^{abc}	0.666^{ab}	0.62^{a}	0.59^{a}	0.59^{ab}	0.57^{a}	0.5667^{a}	0.58^{a}	0.5733^{ab}	0.5233^{a}	0.51^{ab}	0.49^{a}	0.47^{a}
Treat13	0.746^{bc}	0.696^{bc}	0.69^{a}	0.67^{de}	0.6467^{cd}	0.6367^{bc}	0.63^{c}	0.62^{a}	0.6133^{b}	0.5933^{ab}	0.6033^{bc}	0.54^{a}	0.5^{a}

Table 4. First-order kinetic function, degradation constant (K), and half-life (t1/2) (R^2 is coefficient of determination).

Treatment	First-order kinetic function	K (day^{-1})	R^2	t1/2 (days)
1	C = -0.0014t - 0.2737	0.0014	0.62	492.9
2	C = -0.0017x - 0.4078	0.0017	0.88	405.9
3	C = -0.0014x - 0.3767	0.0014	0.92	492.9
4	C= -0.0017x - 0.3755	0.0017	0.78	405.9
5	C = -0.0019t - 0.2963	0.0019	0.92	363.2
6	C= -0.0017x - 0.363	0.0017	0.95	405.9
7	C = -0.0016x - 0.3597	0.0016	0.85	431.3
8	C = -0.0014x - 0.4058	0.0014	0.85	492.9
9	C = -0.0024x - 0.349	0.0024	0.95	287.5
10	C = -0.0023x - 0.3131	0.0023	0.95	300.0
11	C = -0.0016x - 0.3471	0.0016	0.97	431.3
12	C = -0.0024x - 0.349	0.0024	0.95	287.5
13	C = -0.0012x - 0.4308	0.0012	0.90	575.0

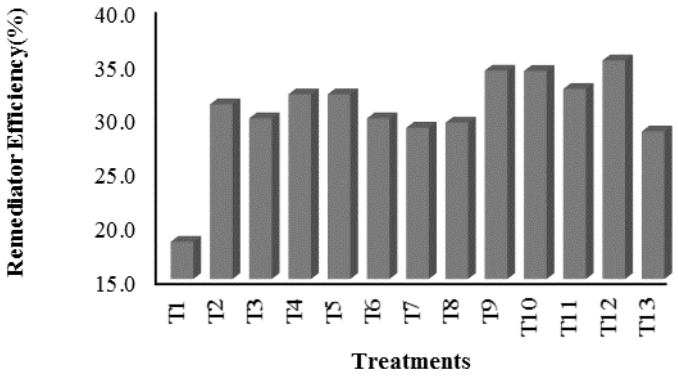

Fig. 2. Results of remediation efficiency of 13 treatments during 210 days (T1, T2, …, T13, are treatment 1-13 respectively)

biodegradation (Nicodem et al., 1997). In the control (dry) treatment, the biodegradation factor was minimized; therefore, the most important factor in reducing TPH can be considered the evaporation of petroleum products. Significant changes in TPH reduction in this treatment began after 120 days. Wet soil treatment reduced the total oil petroleum hydrocarbon by 31%. Animal manure and sawdust treatments (treatments 4 and 5) had the highest reduction of total petroleum hydrocarbons (32%) compared to other treatments that were considered to improve the environmental conditions (32%).

3.5. Estimation of the time required for complete degradation of TPH

The results of fitting the regression equation obtained from the data related to the percentage of complete decomposition of TPH in different treatments during 210 days, as line equation, are provided in Table 5. The highest and lowest times required for complete decomposition of TPH was related to the treatment one (759 days) and treatments 4 and 5 (548 -590 days), respectively. These times in bacterial inoculation treatments were 12, 9 and 10, and also were 481, 494 and 495 days, respectively.

The highest time required for complete decomposition of TPH in environmental modifiers was related to treatment one (759 days) and the lowest time for 100% decomposition of total petroleum hydrocarbons was related to treatments 4 and 5 (584 and 590 days, respectively). Also, treatments 1, 3, and 8 led to the lowest decomposition rate constant and the highest half-

Table 5. Number of days required for different decomposition percentages in 13 modifier treatments.

Treatments/ reduction percentage			0	10	20	30	40	50	60	70	80	90	100
T1	y = 0.112x + 15.043	R^2 = 0.8315	0	24	44	134	223	312	401	491	580	669	759
T2	y = 0.135x + 10.583	R^2 = 0.8804	0	35	70	144	218	292	366	440	514	588	662
T3	y = 0.1352x + 12.416	R^2 = 0.7203	0	20	58	135	212	289	366	443	520	597	674
T4	y = 0.1465x + 14.876	R^2 = 0.8837	0	24	36	104	173	241	310	378	447	515	584
T5	y = 0.149x + 12.148	R^2 = 0.9056	0	32	53	120	187	254	321	388	455	523	590
T6	y = 0.1254x + 11.579	R^2 = 0.9023	0	32	67	147	227	307	387	467	547	627	707
T7	y = 0.147x - 3.619	R^2 = 0.8693	0	97	169	240	311	383	454	526	597	669	740
T8	y = 0.1328x + 8.0659	R^2 = 0.9251	0	15	90	166	242	318	393	469	545	621	697
T9	y = 0.1855x + 11.166	R^2 = 0.8979	0	23	49	105	160	216	271	327	382	438	494
T10	y = 0.1872x + 7.4678	R^2 = 0.9738	0	32	67	120	174	227	281	334	388	441	495
T11	y = 0.1343x + 9.5495	R^2 = 0.9775	0	38	78	153	228	302	377	451	526	601	675
T12	y = 0.2005x + 3.9091	R^2 = 0.908	0	31	81	131	181	231	281	331	381	431	481
T13	y = 0.1055x + 16.439	R^2 = 0.9357	0	25	36	136	236	336	436	536	636	736	836

life and treatment 5 had the highest decomposition rate constant and the lowest half-life. According to the results, all treatments used for TPH analysis had a significant effect, but treatments 4 and 5 had the highest performance compared to other treatments used. In treatment 9, only a complex of *Bacillus megatrium, Bacillus subtilis* and *Pseudomonas putida* (active TPH-degrading bacteria) was inoculated without adding any nutrients. The results of 34% reduction of total petroleum hydrocarbons were observed. And a faster reduction rate was observed in this treatment than other treatments in which the bacterial complex was added with chemical fertilizer. This treatment was one of the treatments that had the highest decomposition rate constant and the lowest half-life. Also, 494 days are required for complete decomposition of TPH from this treatment. The results of bacterial complex inoculation with the addition of nutrients (treatments 10-13) showed that the treatment of bacterial complex with soil with 35% reduction of total petroleum hydrocarbons, the highest reduction rate and treatment of bacterial complex with chemical fertilizers, soil Saws and manure had the lowest reduction rates with a 28% reduction. Among the bacterial inoculation treatments with nutrients, treatment 13 had the lowest rate of decomposition rate and the highest half-life and treatment 12 had the highest rate of decomposition and the lowest half-life. Also, the minimum time required to decompose 38% of petroleum hydrocarbon contamination in bacterial inoculation treatments containing nutrients was observed in T12, T9 and T10 treatments (481, 494 and 495 days, respectively). Based on the results of bacterial inoculation treatments, bacterial complex treatments with sawdust and chemical fertilizer had the highest performance in reducing TPH.

3.6. Decomposition percentage data of the modifier treatments

According to Fig. 3A, the control treatment reduced the total amount of petroleum hydrocarbons by 19.4% and among the first 7 treatments, the highest percentage reduction was related to treatments 5 and 2 (33.8%). In Fig. 3B, treatments 8-13 were compared with treatment 1, where treatments 12, 9 and 10 had the highest percentage of TPH reduction compared to the dry soil.

3.7. Evaluation of microbial respiration results

The microbial respiration criterion is used to measure the rate and intensity of decomposition of organic matter, including petroleum hydrocarbons. This method shows the level of microbial activity in the presence of hydrocarbon pollutants by measuring microbial respiration (measurement of carbon dioxide). In this study, changes in TPH and microbial respiration rate were measured in parallel and complementarily. As shown in Fig. 4, microbial respiration increased within 210 days of the start of the modifiers.

Interaction of time and treatment and the simple effects of time and treatment were significant at 1% level (Table 6). Figure 5 shows the comparison of treatments at different times by Duncan test. Over time, the amount of microbial respiration in all modifiers increased. Based on the comparison of the mean, Duncan method grouped the lowest value into group (a) and with increasing

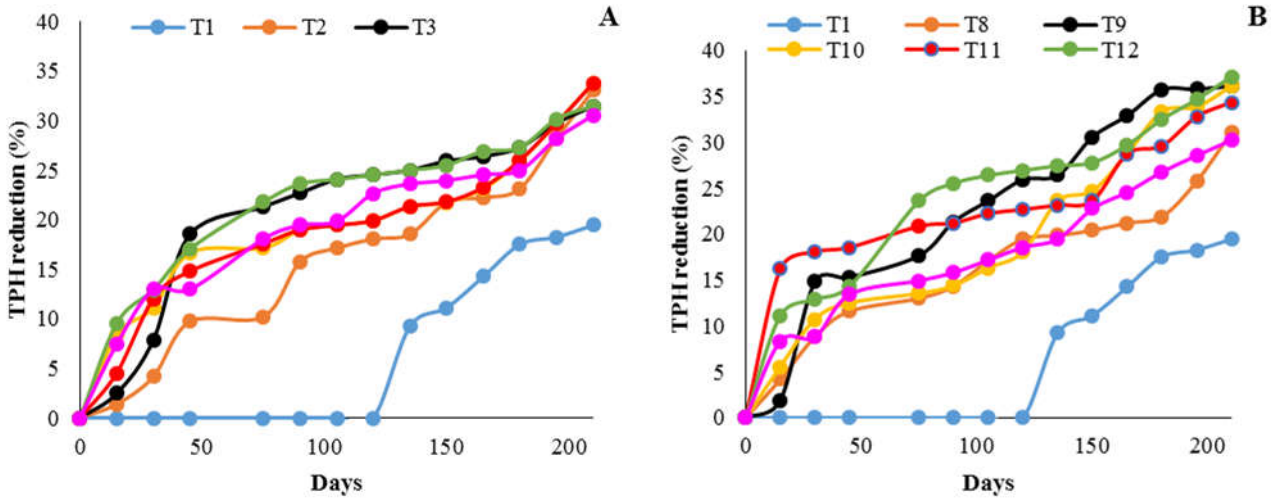

Fig. 3. Trends of TPHs reduction during the 210 days of applying treatments

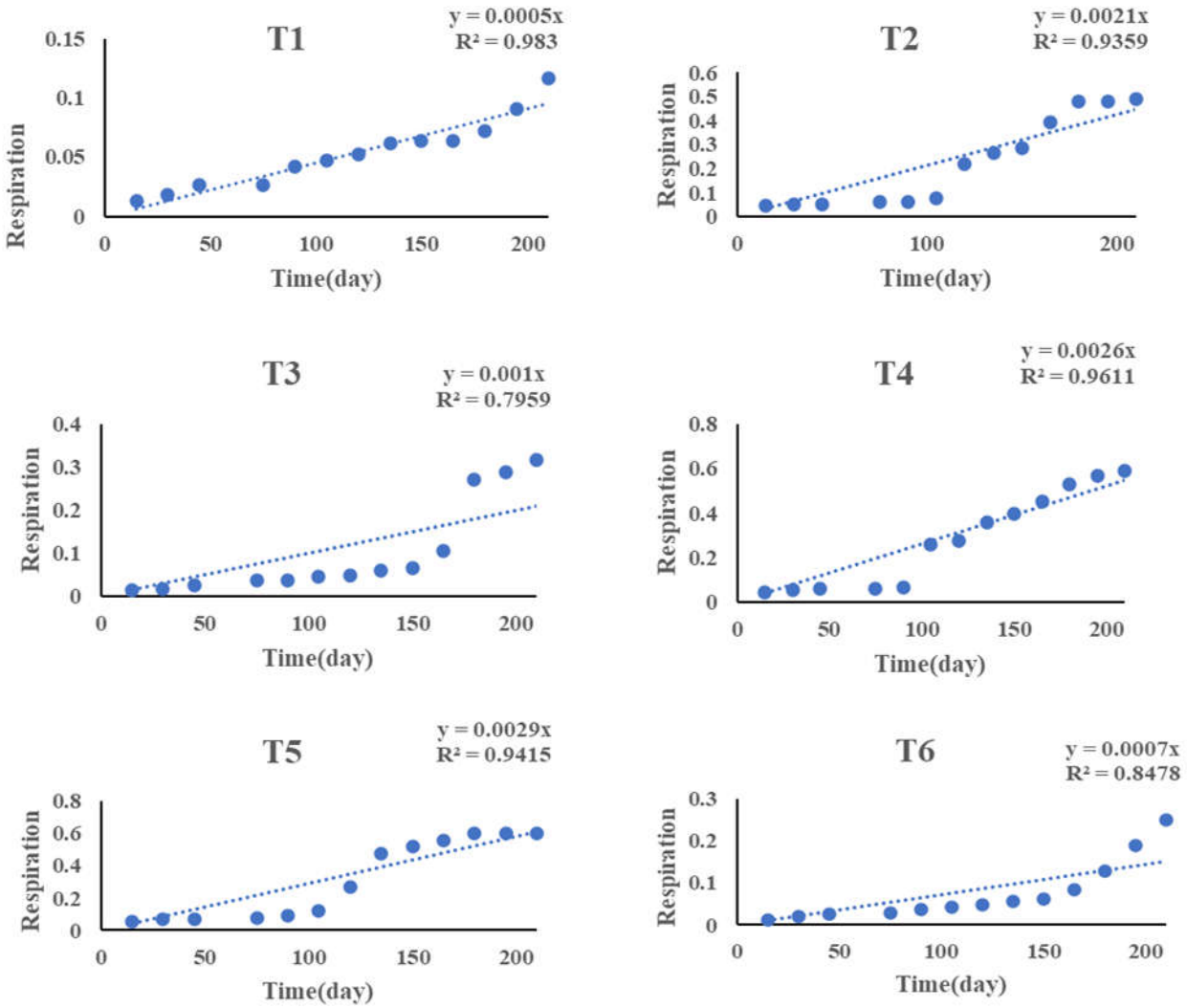

Fig. 4. Microbial respiration changes during 210 days by applying 13 different treatments (T=Treatment, T1, T2, T3…, T3)

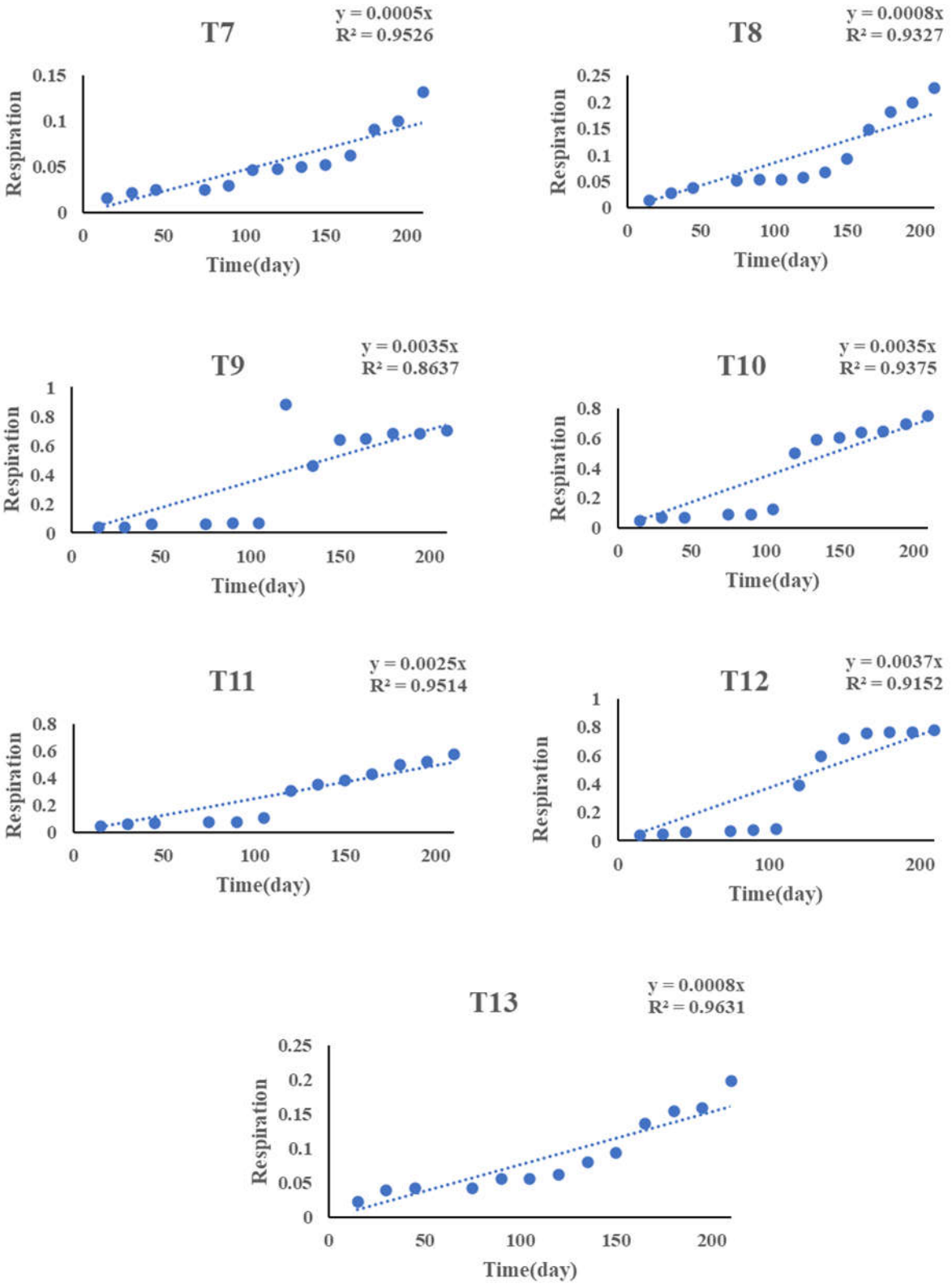

Fig. 4. (continued)

Table 6. Analysis of microbial respiration variance for all treatments over time

Source of variation	SS	df	MS	F	Sig.
Corrected Model	26.127[a]	168	.156	63.514	.000
Intercept	22.473	1	22.473	9177.821	.000
time * treat	5.656	144	.039	16.041	.000
time	12.272	12	1.023	417.668	.000
treat	8.199	12	.683	279.027	.000
Error	.828	338	.002		
Total	49.427	507			
Corrected Total	26.955	506			

a. R Squared = .969 (Adjusted R Squared = .954)

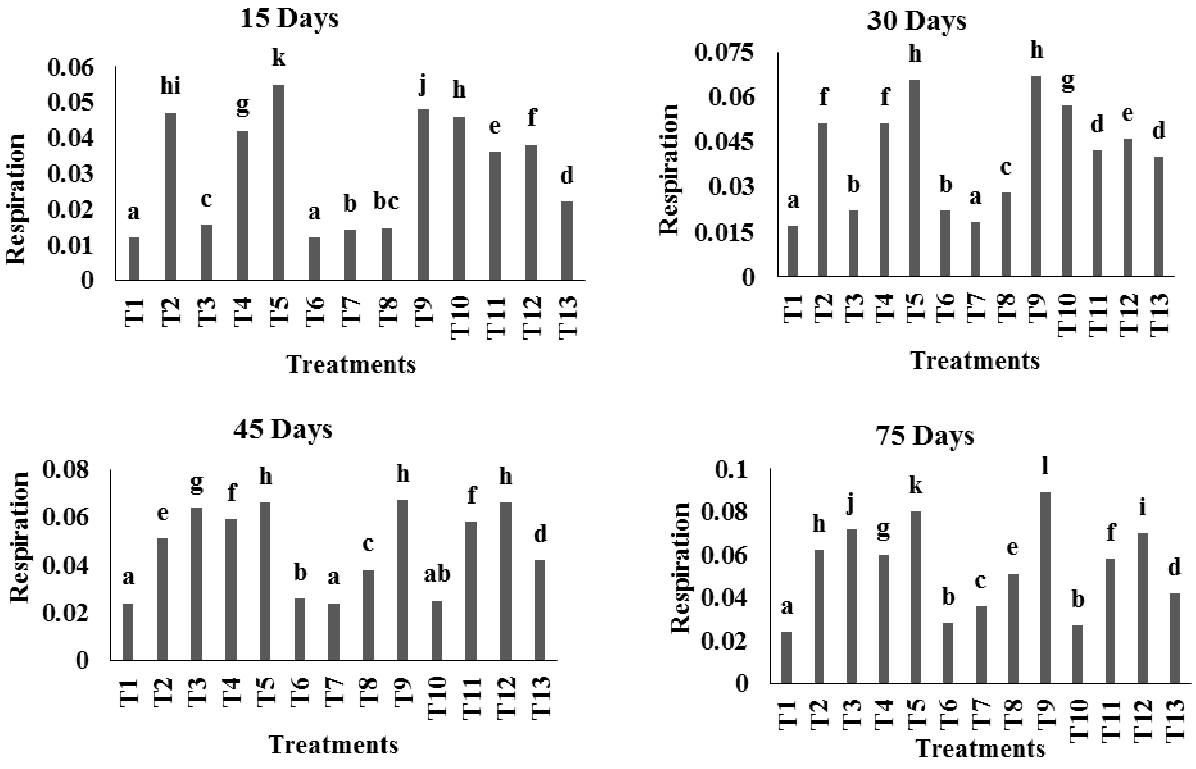

Fig. 5. Comparison of microbial respiration (mg /day soil) during 210 days with application of 13 different treatments (T1, T2, T3, …, T13)

respiration in the modifiers, respectively. Treatment 1 had the lowest amount of microbial respiration and was placed in group (a). After 15 days from the start of the experiment, treatments 1 and 6 had the lowest amounts of microbial respirations and treatment 5 had the highest amount of respiration. After 30 days, treatments 1 and 7 had the lowest values and treatments 5 and 9 had the highest amounts of microbial respirations. After 45 days, the treatments were divided into 9 groups, with treatments 1 and 7 in group a (minimum respiration) and treatments 5, 9 and 12 in group h (highest microbial respiration). After 75 and 90 days from the start of experiments, treatments 5 and 9 had the highest amount of respiration. After 105 days, the amount of respiration increased from 0.05 to 0.38 in the treatments and treatment 12 had the highest value (group b) and treatments 4, 5 and 9 did not differ significantly in group ab and other treatments. They were in group a. At 120 and 135 days, treatments 9 and 12 and at 150 days, treatments 10 and 12 had the highest amounts of respirations. After 165 days, all of the treatments in group a, and treatment 12 in group i, were included.

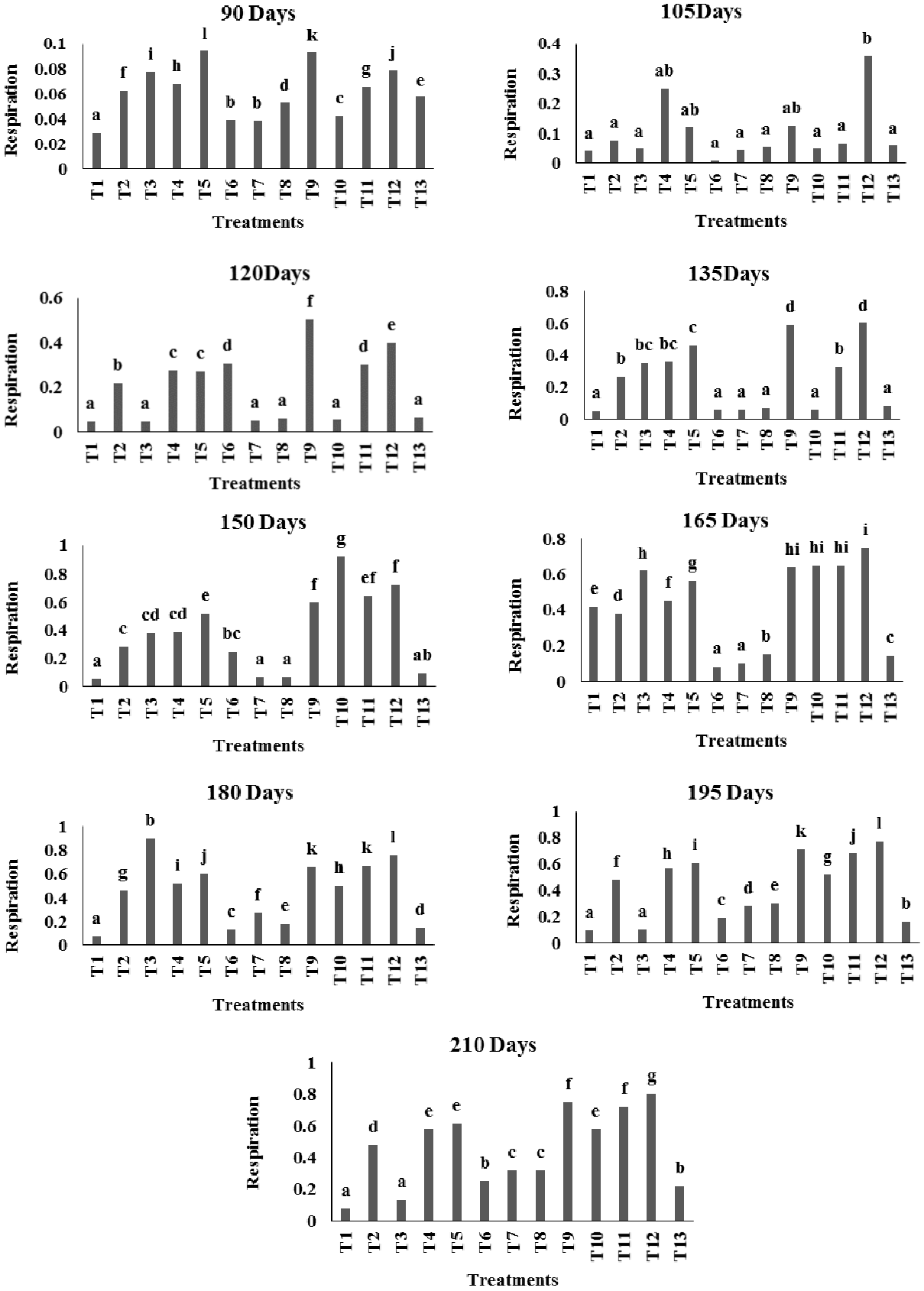

Fig. 5. (continued)

After 180 and 195 days, among the bacterial inoculation treatments, except for treatment 13, the rest had more microbial respirations. However, at 180 days, treatment 3 had the highest value compared to other treatments. Finally, at the end of the experiment and after 210 bacterial inoculation treatments except treatment 13, they had the highest amount of microbial respiration. However, after 210 days, the amount of respiration in all treatments had increased compared to the first days.

As can be seen in Fig. 5, the highest rate of microbial respiration was observed in treatments 12, 10 and 9 and the lowest in treatment 1. Modifiers 4 and 5 also significantly increased respiration. In bacterial treatments in the early stages of inoculation, respiration was low, but over time and the adaptation of inoculated bacteria to contaminated soil, respiration gradually increased. This indicates the high potency of these bacteria in the decomposition of total petroleum hydrocarbons. It can be seen that the highest rate of respiration was in treatments 12, 10, 9, which also had the lowest level of TPH after 210 days. On the other hand, the lowest respiration was in treatments 1, 7 and 13. According to Figs. 3 and 4, treatments 7, 8, 13, 6, 3 and 10 had the lowest respiration rates, which had chemical fertilizers. Comparison of microbial respiration in different methods used in most of the 13 treatments, at 210 days, showed that the bacterial complex treatment with sawdust had the highest respiration rate and the control treatment had the lowest microbial respiration.

Table 6 shows the correlation between soil TPH and microbial respiration based on Pearson coefficient at the level of one percent. As can be seen, there is a strong and negative linear correlation between oil content and microbial respiration in all modifiers. It also can be seen that in all treatments, the amount of microbial respiration increased with decreasing oil content.

4. Discussion

This study was conducted on the soil around Rey refinery (Southern Tehran), with an initial contamination of 38%. It seems that due to the long time since the emission of pollution, the native microorganisms degrading TPH were well adapted to the soil located in the environment (Ruberto et al., 2003). By applying three categories of solutions including improving the environmental conditions for degraded native bacteria (treatment 1-8), adding effective bacterial complex (treatment 9) and intensifying and stimulating growth while adding non-native bacterial complex (treatment 10-13), the decomposition process of TPH was investigated and at the same time soil microbial respiration was monitored for 210 days.

The results showed that in each of the 13 treatments applied, a significant downward trend was observed in reducing the total amount of petroleum hydrocarbons over time.

According to the results of LSD test, T12, T9 and T10 treatments (treatments containing bacteria) had the highest TPH decomposition. Among the environmental treatments, Modifiers 5 and 4 also had the highest oil reduction. Modifier 1 (control) also showed the lowest reduction. After 210 days, modifiers 12, 9, and 10 reduced the initial amount of petroleum hydrocarbons by approximately 38%, while modifier 1 reduced TPH by only 19% compared to the initial time.

Based on the results obtained from fitting the regression equation for complete decomposition of soil with very high pollution (38%), in case of not using nutrients and applying only soil aeration, approximately 759 days of purification time was predicted. This time was estimated to be 560 days if animal and chemical fertilizers were used and 485 days for complete soil clearing by bacterial inoculation (Yaman, 2020).

Based on the results of the first-degree kinetic equation, the half-life in the application of modifiers 12, 9 and 10, which had bacterial inoculation with nutrients, had the highest reduction of total soil hydrocarbon contamination compared to other modifiers. Treatments 12, 9 and 10 had the highest remediate efficiency. Interestingly, treatment 2, due to the fact that it had no modifiers and only moisture was added to the treatments, increased the remediate efficiency (31.1% compared to the control). It seems that a significant amount of TPH degradation can be expected in hydrocarbon contaminated soils by providing optimal moisture conditions for native microorganisms (Kauppi et al., 2011).

The results showed that there was a strong and negative linear correlation between oil content and microbial respiration in all modifiers. Microbial respiration increased as pollutants decreased over time, which serves as an indicator to assess the amount of TPH in the soil. The results showed that among the 13 available treatments, three modifiers, 12, 10 and 9, had the highest rate of increased respiration over 210 days. But after these three modifiers, treatments 4 and 5 had a significant increase in microbial respiration. In bacterial treatments in the early stages of inoculation, respiration was low, but over time and the adaptation of inoculated bacteria to contaminated soil, respiration gradually increased, which shows the high strength and ability of these bacteria to decompose TPH. Comparison of microbial respiration in different methods used in most of the 13 treatments, at 210 days, showed that the bacterial complex treatment with sawdust had the highest respiration rate and the control treatment had the lowest microbial respiration rate.

Summarizing the results showed that finally, in the category of environmental improvement methods, along with bacterial inoculation, three treatments (12, 10 and 9) were able to cause the most TPH reduction and the most increase in microbial respiration and had the shortest half-life. However, if only the environmental

improvement method is used to stimulate native microorganisms, animal manure and chemical fertilizers, along with aeration of contaminated soil, can also have significant and acceptable results in improving hydrocarbon contaminated soils. The method proposed in this paper for petroleum-polluted soils is recommended because of being cheap and environment-friendly. It must be noted that although the results of this study are specific to the studied site and may not be extended to other conditions with certainty, the method used may serve as a supportive tool to devise biological remediation plans.

5. Conclusion

Since the soil in this study area had been under high initial TPHs concentration (38%) and for a long time, the microorganisms present in this soil were adapted to this polluted environment. This condition resulted that microorganisms not to be to remove the TPHs. Therefore, inoculation of the soil with non-indigenous bacteria (*Bacillus megatrium, Bacillus subtilis* and *Pseudomonas putida*) significantly increased TPH removal over 6.5 months. Bacterial complex treatment+ sawdust led to the highest TPH removal, respiration and bacteria count increase. Apparently, inoculation of TPH degrading bacteria, along with organic fertilizers (sawdust and livestock manure), enhances the remediation of soils polluted with TPH.

References

Abed, R. M., Al-Sabahi, J., Al-Maqrashi, F., Al-Habsi, A., & Al-Hinai, M. (2014). Characterization of hydrocarbon-degrading bacteria isolated from oil-contaminated sediments in the Sultanate of Oman and evaluation of bioaugmentation and biostimulation approaches in microcosm experiments. *International Biodeterioration & Biodegradation, 89,* 58-66. https://doi.org/10.1016/j.ibiod.2014.01.006

Agarry, S. E., Aremu, M. O., Aworanti, O. A. 2013. Biodegradation of 2, 6-dichlorophenol wastewater in soilcolumn reactor in the presence of pineapple peels-derived activated carbon, palm kernel oil and inorganic fertilizer. *Journal of Environmental Protection, 4(6),* 537.

Agarry, S. E., Oghenejoboh, K. M., & Solomon, B. O. (2015). Kinetic modelling and half-life study of adsorptive bioremediation of soil artificially contaminated with bonny light crude oil. *Journal of Ecological Engineering, 16(3),* 1-13.

Anderson, J.P.E. (1982). Soil respiration. In: A.L., Page, R.H., Miller, & D.R., Keeney, (Eds.), *Methods of soil analysis*. (Part 2, pp 831–871). Wisconsin: Soil Science Society of America.

Aronson, D., Boethling, R., Howard, P., & Stiteler, W. (2006). Estimating biodegradation half-lives for use in chemical screening. *Chemosphere, 63(11), 1953*-1960. https://doi.org/10.1016/j.chemosphere.2005.09.044

Dimitrov, S., Pavlov, T., Nedelcheva, D., Reuschenbach, P., Silvani, M., Bias, R., Comber, M., Low, L., Lee, C., Parkerton, T., & Mekenyan, O. (2007). A kinetic model for predicting biodegradation. *SAR and QSAR in Environmental Research, 18(5-6),* 443-457.

Doustaky, M, Ebrahimi, S, Movahedi Naeini, S.A.R., & Olamaei, M. (2013). Optimization of petroleum hydrocarbon biodegradation by indigenous and non-indigenous microorganisms. *Journal of Water and Soil Conservation, 20(4),*165-181.

Ebrahimi, S., Shayegan, J., Malakouti, M., & Akbari, A. (2011). Environmental Evaluation and Assessment of Some Important Factors of Oil Contamination in Soil around Sarkhoun Gas Refinery of Bandar Abbas. *Journal of Environmental Studies, 37 (57),* 9-26.

Fallah, M., Ebrahimi, S., & Shabanpour, M. (2013). Hydrocarbon pollution emission in the pilot and pulse condition in saturated porous media of soil. *Journal of Water and Soil Conservation, 20(3),* 227-240.

Fallah, M., Shabanpor, M., Zakerinia, M., & Ebrahimi, S. (2015). Risk assessment of gas oil and kerosene contamination on some properties of silty clay soil. *Environmental Monitoring and Assessment, 187(7),* 1-13. https://doi.org/10.1007/s10661-015-4633-0

Heshmati, G., & Ebrahimi, S. (2018). Evaluation of Petroleum-Degrading Bacteria in phytoremediation of soil contaminated with petroleum (Case study: Soils surrounding Tehran Oil Refinery). *Journal of Plant Ecosystem Conservation, 5(11),* 131-144.

Hutchinson, S. L., Banks, M. K., & Schwab, A. P. (2001). Phytoremediation of aged petroleum sludge: effect of inorganic fertilizer. *Journal of Environmental Quality, 30(2),* 395-403. https://doi.org/10.2134/jeq2001.302395x

John, R. C., Itah, A. Y., Essien, J. P., & Ikpe, D. I. (2011). Fate of nitrogen-fixing bacteria in crude oil contaminated wetland ultisol. *Bulletin of Environmental Contamination and Toxicology, 87(3),* 343-353. https://doi.org/10.1007/s00128-011-0320-1

Karimpoor, R., Ebrahimi, S., Malekzadeh, E., & Hassanpour-bourkheili, S. (2022). Bioremediation of total petroleum hydrocarbons in oil sludge-polluted soil using active carbon remediator. International *Journal of Environmental Science and Technology, 19,* 7649–7660. https://doi.org/10.1007/s13762-022-03964-9

Kauppi, S., Sinkkonen, A., & Romantschuk, M. (2011). Enhancing bioremediation of diesel-fuel-contaminated soil in a boreal climate: comparison of biostimulation and bioaugmentation. *International Biodeterioration & Biodegradation, 65(2),* 359-368. https://doi.org/10.1016/j.ibiod.2010.10.011

Khosravinodeh, M., Abbaspour, A., Ebrahimi, S.S., & Asghari, H.R. (2013). Phytoremediation of a fuel oil-contaminated soil using alfalfa and grass with pseudomonas putida bacterium. *Journal of Water and*

Soil Conservation, 20, 219-234.

Liao, C., Xu, W., Lu, G., Deng, F., Liang, X., Guo, C., & Dang, Z. (2016). Biosurfactant-enhanced phytoremediation of soils contaminated by crude oil using maize (*Zea mays* L.). *Ecological Engineering, 92,* 10-17. https://doi.org/10.1016/j.ecoleng.2016.03.041.

Liu, P. W. G., Chang, T. C., Whang, L. M., Kao, C. H., Pan, P. T., & Cheng, S. S. (2011). Bioremediation of petroleum hydrocarbon contaminated soil: effects of strategies and microbial community shift. *International Biodeterioration & Biodegradation, 65(8),* 1119-1127. https://doi.org/10.1016/j.ibiod.2011.09.002

Mair, J., Schinner, F., & Margesin, R. (2013). A feasibility study on the bioremediation of hydrocarbon-contaminated soil from an Alpine former military site: effects of temperature and biostimulation. *Cold Regions Science and Technology, 96,* 122-128. https://doi.org/10.1016/j.coldregions.2013.07.006.

Megharaj, M., Ramakrishnan, B., Venkateswarlu, K., Sethunathan, N., & Naidu, R. (2011). Bioremediation approaches for organic pollutants: a critical perspective. *Environment International, 37(8),* 1362-1375. https://doi.org/10.1016/j.envint.2011.06.003

Mojarad, M., Alemzadeh, A., Ghoreishi, G., & Javaheri, M. (2016). Kerosene biodegradation ability and characterization of bacteria isolated from oil-polluted soil and water. *Journal of Environmental Chemical Engineering, 4(4),* 4323-4329. https://doi.org/10.1016/j.jece.2016.09.035.

Moreira, I. T., Oliveira, O. M., Triguis, J. A., dos Santos, A. M., Queiroz, A. F., Martins, C. M., Silva, C. S., & Jesus, R. S. (2011). *Phytoremediation* using *Rizophora* mangle L. in mangrove sediments contaminated by persistent total petroleum hydrocarbons (TPH's). *Microchemical Journal, 99(2)*, 376-382. doi: https://doi.org/10.1016/j.microc.2011.06.011

Mukherjee, S., Bardolui, N. K., Karim, S., Patnaik, V. V., Nandy, R. K., & Bag, P. K. (2010). Isolation and characterization of a monoaromatic hydrocarbon degrading bacterium, *Pseudomonas aeruginosa* from crude oil. *Journal of Environmental Science and Health, Part A, 45,* 1048–1053. https://doi.org/10.1080/10934529.2010.486328

Nicodem, D. E., Fernandes, M. C., Guedes, C. L. B., & Correa, R. J. (1997). Photochemical processes and the environmental impact of petroleum spills. *Biogeochemistry, 39,* 121–138. https://doi.org/10.1023/A:1005802027380

Oliveira, F. J. S., da Rocha Calixto, R. O., Felippe, C. E. C., & de Franca, F. P. (2013). Waste management and contaminated site remediation practices after oil spill: a case study. *Waste Management & Research, 31(12),* 1190-1194. https://doi.org/10.1177/0734242x13507309

Olsen, S. R., & Sommer, L. E. (1982). Phosphorus. In A. L. Page, R. H. Miller, & D. R. Keeney (Eds.), *Methods of Soil Analysis.* (vol. 9, Part II, pp. 403–430). Wisconsin: Soil Science Society of America.

Poi, G., Aburto-Medina, A., Mok, P. C., Ball, A. S., & Shahsavari, E. (2017). Large scale bioaugmentation of soil contaminated with petroleum hydrocarbons using a mixed microbial consortium. *Ecological Engineering, 102,* 64-71. https://doi.org/10.1016/ j.ecoleng.2017.01.048

Polyak, Y. M., Bakina, L. G., Chugunova, M. V., Mayachkina, N. V., Gerasimov, A. O., & Bure, V. M. (2018). Effect of remediation strategies on biological activity of oil-contaminated soil-A field study. *International Biodeterioration & Biodegradation, 126,* 57-68. https://doi.org/10.1016/j.ibiod.2017.10.004

Ramadass, K., Megharaj, M., Venkateswarlu, K., & Naidu, R. (2015). Ecological implications of motor oil pollution: earthworm survival and soil health. *Soil Biology and Biochemistry, 85,* 72-81. https://doi.org/10.1016/j.soilbio.2015.02.026

Ruberto, L., Vazquez, S. C., & Mac Cormack, W. P. (2003). Effectiveness of the natural bacterial flora, biostimulation and bioaugmentation on the bioremediation of a hydrocarbon contaminated Antarctic soil. *International Biodeterioration & Biodegradation, 52(2),* 115-125. https://doi.org/10.1016/S0964-8305(03)00048-9

Ruffini Castiglione, M., Giorgetti, L., Becarelli, S., Siracusa, G., Lorenzi, R., & Di Gregorio, S. (2016). Polycyclic aromatic hydrocarbon-contaminated soils: bioaugmentation of autochthonous bacteria and toxicological assessment of the bioremediation process by means of *Vicia faba* L. *Environmental Science and Pollution Research, 23(8),* 7930-7941. https://doi.org/10.1007/s11356-016-6049-y

Sayara, T., Borràs, E., Caminal, G., Sarrà, M., & Sánchez, A. (2011). Bioremediation of PAHs-contaminated soil through composting: Influence of bioaugmentation and biostimulation on contaminant biodegradation. *International Biodeterioration & Biodegradation, 65(6),* 859-865. https://doi.org/10.1016/j.ibiod.2011.05.006

Shahi, A., Aydin, S., Ince, B., & Ince, O. (2016). Evaluation of microbial population and functional genes during the bioremediation of petroleum-contaminated soil as an effective monitoring approach. *Ecotoxicology and Environmental Safety, 125,* 153-160. https://doi.org/10.1016/j.ecoenv.2015.11.029

Sinkkonen, S., & Paasivirta, J. (2000). Degradation half-life times of PCDDs, PCDFs and PCBs for environmental fate modeling. *Chemosphere, 40(9-11),* 943-949. https://doi.org/10.1016/S0045-6535(99)00337-9

Tang, J., Lu, X., Sun, Q., & Zhu, W. (2012). Aging effect of petroleum hydrocarbons in soil under different attenuation conditions. *Agriculture, Ecosystems & Environment, 149,* 109-117. https://doi.org/10.1016/ j.agee.2011.12.020

Tazangi, M. H., Ebrahimi, S., Nasrabadi, R. G., & Naeeni, S. A. M. (2020). Kinetic monitoring of bioremediators for biodegradation of gasoil-polluted soil. *Water, Air, and Soil Pollution, 231,* 418.

https://doi.org/10.1007/s11270-020-04794-6

Thapa, B., Kc, A. K., & Ghimire, A. (2012). A review on bioremediation of petroleum hydrocarbon contaminants in soil. *Kathmandu University Journal of Science, Engineering and Technology, 8(1),* 164-170.

Tyagi, M., Da, F. M., & de Carvalho, C. C. (2011). Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. *Biodegradation, 22(2),* 231-241. https://doi.org/ 10.1007/s10532-010-9394-4

Walkley, A., & Black, I. A. (1934). An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. *Soil Science, 37(1),* 29–38.

Wu, M., Dick, W. A., Li, W., Wang, X., Yang, Q., Wang, T., Xu, L., Zhang, M. & Chen, L. (2016). Bioaugmentation and biostimulation of hydrocarbon degradation and the microbial community in a petroleum-contaminated soil. *International Biodeterioration & Biodegradation, 107,* 158-164. https://doi.org/10.1016/j.ibiod.2015.11.019

Wu, M., Wu, J., Zhang, X., & Ye, X. (2019). Effect of bioaugmentation and biostimulation on hydrocarbon degradation and microbial community composition in petroleum-contaminated loessal soil. *Chemosphere, 237,* 124456. https://doi.org/10.1016/j.chemosphere. 2019.124456.

Xu, Y., & Lu, M. (2010). Bioremediation of crude oil-contaminated soil: comparison of different biostimulation and bioaugmentation treatments. *Journal of Hazardous Materials, 183(1-3),* 395-401. https://doi.org/10.1016/j.jhazmat.2010.07.038

Yaman, C. (2020). Performance and kinetics of bioaugmentation, biostimulation, and natural attenuation processes for bioremediation of crude oil-contaminated soils. *Processes, 8(8),* 883. https://doi.org/10.3390/pr8080883.

Yang, Q., Wu, M. L., Nie, M. Q., Wang, T. T., & Zhang, M. H. (2015). Effects and Biological Response on Bioremediation of Petroleum Contaminated Soil. *Huanjing Kexue, 36(5),* 1856-1863.

Yeung, P. Y., Johnson, R. L., & Xu, J. G. (1997). Biodegradation of petroleum hydrocarbons in soil as affected by heating and forced aeration. *Journal of Environmental Quality, 26(6),* 1511–1516. https://doi.org/10.2134/jeq1997.00472425002600060009x

Zahed, M. A., Aziz, H. A., Isa, M. H., Mohajeri, L., Mohajeri, S., & Kutty, S. R. M. (2011). Kinetic modeling and half life study on bioremediation of crude oil dispersed by Corexit 9500. *Journal of Hazardous Materials, 185(2-3),* 1027-1031. https://doi.org/10.1016/j.jhazmat.2010.10.009