



The synergistic effect of oil-degrading bacteria *Bacillus megaterium* and *Bacillus subtilis* on phytoremediation of oil-contaminated soils by *Agropyron cristatum* and *Achillea millefolium*, Case study: Soils around Ray refinery in Tehran

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Article Info	Abstract
Article type: Research Article Article history: Received: November 2023 Accepted: October 2025 Corresponding author: sohebrahimi@gmail.com Keywords: petroleum-degrading bacteria rangeland species Tehran oil refinery TPH	The objective of the present research is to assess the application of petroleum-degrading bacteria and the phytoremediation of petroleum-contaminated soils in a greenhouse experiment. For this purpose, we used rangeland species <i>Agropyron cristatum</i> and <i>Achillea millefolium</i> in treatments with and without inoculation with petroleum-degrading bacteria <i>Bacillus megaterium</i> and <i>Bacillus subtilis</i> for petroleum contaminated soil surrounding Tehran refinery with 5.3 and 7 wt% pollution. Another objective of this research was to monitor the TPH changes in the greenhouse condition with and without inoculation of bacteria for <i>Agropyron cristatum</i> and <i>Achillea millefolium</i>. The results indicated the best TPHs removal efficiency for <i>Agropyron cristatum</i> at the end of the growth period in the presence of <i>Bacillus megaterium</i> and <i>Bacillus subtilis</i> treatment in 5.3 wt% contamination level, was 72.18 and 64.08% respectively. For <i>Achillea millefolium</i> this rate was 60.59 and 58.12% respectively. Also, the percentage of EC reduction in <i>Agropyron cristatum</i> at the end of the growth period in the presence of <i>Bacillus megaterium</i> and <i>Bacillus subtilis</i> treatment at 5.3 wt% contamination level was 42.37% and 39.27% respectively and as for <i>Achillea millefolium</i> it was 33.41 and 31.48% respectively. In the presence of bacteria, the highest pH reductions were recorded in <i>Bacillus subtilis</i> treatment for <i>Agropyron cristatum</i> with 7.25%. Finally, it can be noted that <i>Agropyron cristatum</i> and <i>Achillea millefolium</i> cultivated in TPH-contaminated soil are resistant to oil contamination and reduce some oil contamination. However, <i>Agropyron cristatum</i> was superior in removing petroleum contaminants.

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Introduction

Environmental pollution has become an adverse health issue affecting human sustainability and survival throughout the world (Pazhmaan et al., 2021). Pollution of soil and groundwater by various pollutants, including hydrocarbons and chemical solvents, has various adverse environmental impacts (Shahrie and Ebrahimi., 2025). The pollution of the subterranean environment with petroleum hydrocarbons around refineries, refueling stations, oil tanks and petroleum products, and the passage point of fuel pipelines is particularly important, and the pollution caused by industrial activities is always a cause of concern for industry practitioners and environment conservations authorities (Ebrahimi et al., 2009; Akhavan et al., 2018). Today, the increasing development of petroleum and related industries in Iran has placed petroleum hydrocarbons in the ranks of the most important environmental pollutants. Most of this contamination has resulted from oil exploration, transport, and refining as well as from the absence of waste oil recycling and the disposal of hazardous oil wastes into landfill areas without sufficient management (Khudur et al., 2019; Grifoni et al., 2020). Given the increasing intensity of environmental pollution over the last two decades, the control, elimination, or reduction of these pollutions using various technologies has received a great deal of attention on the part of researchers (Chaillan et al., 2004; Seyed Alikhani et al., 2011). Many methods are used today to remove pollutants from petroleum. In the meantime, biological degradation by microorganisms plays a fundamental role in removing oil substances, especially non-volatile components of oil from the environment (Diaz, 2008). So far, a large number of bacteria that decompose and degrade petroleum materials have been isolated, but few of them seem to be important in the biodegradation of petroleum in natural environments. The genera of *Pseudomonas*, *Acinetobacter*, *Bacillus*, *Vibrio*, and *Marinobacter* are among the most important bacteria that can decompose such substances (Jain et al., 2011). The microorganisms that degrade petroleum substances first emulsify the crude oil by producing and secreting biosurfactants, which increases the surface-

to-volume ratio of oil droplets and thus increases the access of bacterial cells to petroleum compounds hence, their efficiency in oil consumption increases substantially (Vyas and Dave, 2011). Therefore, bacteria that have a high mineralization potential mainly produce strong emulsifying compounds and have a high ability to remove oil hydrocarbon compounds (Ferhat et al., 2011). Studies have shown that the microorganisms isolated from natural environments are the most compatible with the environment. Therefore, the best option is to use endemic microorganisms in the process of biological remediation of oil patches in polluted environments (Milić et al., 2009; Hashemi Tazangi et al., 2021; Ebrahimi et al., 2024).

Bioremediation includes biological methods and the use of microorganisms, especially bacteria, to reduce or remove pollution from the environment, and is superior to other soil rehabilitation methods (Alexander, 2000; Shamloo et al., 2025). Microorganisms need suitable conditions for their growth and survival, which include suitable pH, temperature, oxygen, salinity, and nutrients (Sparks, 2003; Parrish, 2005; Taghdisi et al., 2025). Bioremediation technology is effective in the treatment of oil pollutants since the majority of molecules in petroleum hydrocarbons are biodegradable. Because petroleum components are so widely distributed in the environment, oil-degrading microorganisms are ubiquitous (Fiorenza et al., 2000).

One of the most essential roles of plants in the phytoremediation process is attributed to the rhizosphere, as soil quality is not only dependent on its physical and chemical properties but also closely related to its biological properties (Ebhin Masto et al., 2006). Therefore, this area is a place where interaction between soil, plant and microorganisms occurs, and its microbial communities are qualitatively and quantitatively different from non-rhizosphere soil microbial communities (Pilon-Smits, 2005.).

So far, few studies investigated the potential application of phytoremediation of rangeland

plants and the use of degrading bacteria in cleaning organic and inorganic contaminants. Baneshi et al. (2014) investigated the potential of phytoremediation of phenanthrene and pyrene by rangeland plants sorghum and spruce (*Onobrychissativa*) and found that both sorghum and spruce are effective in phytoremediation of pyrene and phenanthrene from contaminated soils to 22 and 16% respectively. Lu et al. (2009) also investigated the effect of grass in the bioremediation of oil-contaminated soil and reported that after 5 months, the contaminant concentration decreased by 47% in the soil containing vegetation and by 11% in the control soil, and the bacterial population in the rhizosphere was 72 times more than the control soil. Doustaki et al. (2022) while evaluating the effect of endemic and exotic microorganisms on the degradation of petroleum hydrocarbons, found that bacteria degraded petroleum contaminants by 35%. Lin et al. (2006) showed that the combination of PCP and copper has a toxic antagonistic effect on the growth of *Raphanus sativus* and *Lolium perenne*.

Finally, various laboratory scale researchers have evaluated in various methods the potential of such technologies in well-controlled environments however its field applications are still not common and are considered less known than physic-chemical approaches (Hoang et al., 2021; Hashemi Tazangi et al., 2023).

To the best of our knowledge, there has not been a comprehensive study on the combined effect of bacteria and endemic plants in rangelands, especially in Iran. It should be noted that one of the most important roles of rangeland plants, which has received little attention, is their purification ability in polluted environments. These plants are more successful than imported and cultivated ones due to their developed root system, adaptation to the environment and rainfall fluctuations, high biomass, and symbiotic communities present in their roots (Devanny et al., 2005). For this purpose, in this research, the remediation of all petroleum hydrocarbons, as well as some physical and chemical properties of oil-contaminated soil, with the rangeland species *Agropyron cristatum* and

Achillea millefolium and the combination of two bacteria *Bacillus megaterium* and *Bacillus subtilis* at the end of the growth period of the plant was investigated in pilot conditions.

Materials and Methods

Study area

The studied area is contaminated soils around Tehran Refinery located near the city of Ray, District 20 of Tehran Municipality with the coordinates 51° 35' 25" E and 35° 32' 26" N. The study area is part of Tehran plain aquifer with an area of 900 hectares. Tehran plain is located in the south of the middle Alborz slopes. The southern region of Tehran has dry desert climate. The average annual precipitation and evaporation of this area are 200 and 2500 mm, respectively. The maximum temperature is around 44 and the minimum temperature is -10 C, the average temperature is 17 c and the average wind speed is 37.8 km/h.

Plant species

Agropyron cristatum

Agropyron cristatum is one of the important grasses of perennial pasture, which is used to create pastures and produce dry fodder. This species grows in a wide range of cold steppe and semi-arid pastures and can be seen at an altitude between 800 and 1900 meters. This species has a good resistance to drought and grows quickly after a period of drought and heat with minimal humidity (Rahmani et al., 2014).

Achillea millefolium

Achillea millefolium (yarrow) is a perennial plant, a broad-leaved herbaceous species of the chicory family. In terms of ecological needs, yarrow does not need special climatic conditions during its growth and grows in almost any climate. It is a long-day plant, the most suitable temperature for its growth and flowering is 18-26 C. Therefore, it grows better in hot and sunny areas and produces more flowers (Beigi, 2014; Kahraman, 2016).

Soil sampling

In this research, the soil contaminated with TPHs was collected from the waste disposal site of the Tehran Refinery in randomized

manner and the uncontaminated soil was collected from the lands around the refinery. Samples of soil with different levels of pollution were taken randomly at specific intervals from Tehran Refinery. Given the requirement of 10 liters of soil for each pot, soil samples were collected from 1 x 1 m plots using an auger with a 10 cm radius, in 10 replicates, at a depth of 0-30 cm. (Parsadoost et al., 2007). The soil sample was transferred to the greenhouse of Karaj Technical and Engineering Agricultural Research Institute. After the soil was air-dried, it was passed through a 10 mm sieve and the remains of plant roots and stones and pebbles were separated. Then, completely contaminated and non-contaminated soil were mixed in a ratio of 1 to 1 (completely contaminated soil: non-contaminated soil, soil treatment with moderate contamination) and a ratio of 1 to 3 (completely contaminated soil: non-contaminated soil, soil treatment with low contamination).

To prepare the inoculum, *Bacillus* bacteria isolate with petroleum degrading potential was used. The bacteria were obtained from the collection of the herbal medicine department of the Research and Education Center of Agriculture and Natural Resources of Tehran province. According to the research, for degrading bacteria to be able to degrade petroleum hydrocarbons, their minimum number should be 2×10^8 per kilogram of soil (Thapa et al., 2012). This value is for the optimal conditions of degradation of petroleum materials, in which the amount of contamination is 5-10% of the dry weight of the soil (Thapa et al., 2012). Since the soil in this research was tested at two levels of contamination of about 3.5 and 7 wt%, the number of bacteria used in both levels of contamination is considered to be the same as the optimum number (2×10^8). In order to count the number of bacteria, dilution and colony-counting methods were used. First, each of these bacteria was cultured in a Nutrient Agar culture medium, then under completely sterile conditions and by a loop, the colonies of each of the bacteria were removed and inoculated to separate test tubes with 5 cc of nutrient broth medium. After the calculations, the number of bacteria reached

the desired number in 24 hours. Then the bacteria were added to the soil in a mixture with perlite. It should be noted that the degrading bacteria were added to the contaminated soil sample two weeks before planting (Heshmati et al., 2018).

Greenhouse experiments

The seeds of the plant species were prepared by the Research Institute of Forests and the Rangelands. The purpose of selecting these two plant species was their phytoremediation potential and resistance to polluted soils. The plant species were sowed in pots in the greenhouse. The greenhouse had standard conditions and its ambient temperature was $25 \pm 5^\circ\text{C}$ during the day and $17 \pm 5^\circ\text{C}$ during the night. Then, the seeds of the species were planted in pots at a depth of 20 mm and a depth of 18 cm according to the size of the seeds. Irrigation was done regularly and once every 4 days. The humidity is maintained at FC (field capacity). Finally, at the end of the growing season, the soil of each pot was sampled to measure the physical and chemical properties of the soil and TPHs.

Laboratory tests

The soil samples were transferred to the soil science laboratory of the Faculty of Natural Resources of Tehran University and after air drying and passing through a 2 mm sieve, they were uniformly mixed and some physical and chemical parameters of the soil including electrical conductivity (Halushak, 2006), pH (Haloshak, 2006), the percentage of organic matter (Walkley and Black method) and the TPHs (EPA standard method) were measured.

Total Petroleum Hydrocarbons (TPH): To measure the concentration of petroleum hydrocarbons in the soil, the modified method of Marquez Roca et al. (2000) was adopted. First, the soil was pounded in a mortar, one gram of dry soil was weighed, then it was poured into centrifuge tubes, 10 ml of dichloromethane + acetone solution was added and the tubes were shaken for 4 minutes and then were centrifuged at 3000 rpm until sediments settlement. After centrifugation, one milliliter of the supernatant was taken from the supernatant solution and used to measure the number of

hydrocarbon compounds (Minai Tehrani et al., 2006; Hutchinson et al., 2001). Then, a gas chromatograph equipped with an FID detector was used to determine petroleum hydrocarbons according to the standard method of the EPA (Method No. 831) (Ebrahimi et al., 2010; Ebrahimi et al., 2011). It should be noted that to increase the accuracy of TPH measurement (one sample from each treatment after extracting the solution), an Agilent 7890A flame ionization-type gas chromatography device was used. The GC conditions were as follows: Injector temperature: 300 °C, the initial oven temperature was 40 °C for two minutes and then increased to 300 °C by 10 °C/min. The detector temperature was 300 degrees. Helium was used as a carrier gas at a rate of 1 ml/min.

Data analysis

First Shapiro–Wilk normality test was considered for normalization. Finally, for the statistical analysis of the measured data, Duncan's multi-range mean comparison test was used at the probability level of 5% ($P < 0.05$) in the SPSS software and to draw the graphs Excel software was used.

Results

TPHs removal trends during the phytoremediation process

In this research, the level of TPH removal in the treatments of degrading bacteria in two concentrations of oil pollution (3.5% and 7% wt) for the two rangeland species *Agropyron cristatum* and *Achillea millefolium* at the end of the plant vegetative period were investigated. According to Table 1, the best TPHs removal efficiency for both studied species at the end of the growth period of plants was observed, in the treatment of *Bacillus megatrium* bacteria at a concentration of 3.5% oil pollution, and the lowest efficiency of TPHs removal in the treatment without bacterial inoculation at a concentration of 7% oil pollution. In this way, the inoculation of non-endemic bacteria (*Bacillus megatrium* and *Bacillus subtilis*) into the contaminated soil increased the rate of oil degradation in the degrading bacteria treatments and reduced the soil contamination. As it can be seen, as the contamination levels increased from 3.5% to 7% wt, the removal efficiency of TPHs decreased in both studied species, which seems that the increase in contamination level led to a decline in the growth of shoots and roots of the studied species and subsequently water resulted in a decrease in the concentration of total petroleum hydrocarbons in the soil.

Table 1. The level of variations in TPHs in the treatments of degrading bacteria in the presence of two species *Agropyron cristatum* and *Achillea millefolium* in two concentrations of 3.5 and 7% wt of oil pollution in mg/kg

Species	<i>Agropyron cristatum</i>		<i>Achillea millefolium</i>	
	3.5%	7%	3.5%	7%
The percentage of pollution				
Initial TPH	36340	71152	36340	71152
No bacterial inoculation	26013	58014	27431	61862
<i>Bacillus megaterium</i>	10107	36679	14321	40805
<i>Bacillus subtilis</i>	13051	38218	15217	42970

The comparison of the mean percentages of TPHs reduction for the studied species showed that the highest percentage of soil pollution reduction was observed in the treatment of *Agropyron cristatum* species along with the degrading *B. megaterium* at the pollution level of 3.5% by 72.18% and the lowest percentage reduction of TPH in the

soil was observed in *Achillea millefolium* species and the control treatment at the pollution level of 7% by 13.05% (Figure 1). The results of the present research indicated that the *Agropyron cristatum* species have more potential to reduce the TPH of the soil than the *Achillea millefolium* species in all the treatments.

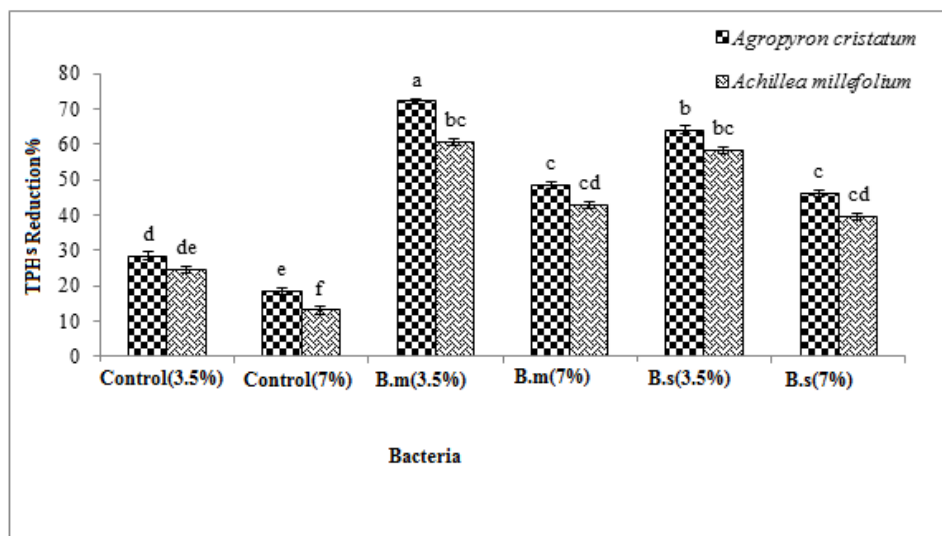


Figure 1. The comparison of the mean percentage of TPH reduction in the treatments of degrading bacteria in the pollution level of 3.5 and 7% wt for the two studied species, each number is the mean of 4 repetitions and the lines on the columns are the standard error ($\pm$ SE). The treatments shown with different letters are significantly different from each other in Duncan's test ($P \leq 0.05$).

pH changes during the phytoremediation process

Changes in soil pH during the phytoremediation process at the end of the plant vegetative period were measured at concentrations of 3.5 and 7% wt of oil pollution. According to Table 2, it can be seen that the pH increased during the

phytoremediation process in the control treatment and decreased in the degrading bacteria treatments. The most changes in pH were in the presence of degrading bacteria treatments, and the most changes in the concentration of 3.5% wt oil pollution and the treatment of *Bacillus subtilis* bacteria for *Agropyron cristatum* species was 7.25%.

Table 2. The level of variations in pH in the treatments of degrading bacteria in the presence of two species *Agropyron cristatum* and *Achillea millefolium* in two concentrations of 3.5 and 7% wt of oil pollution

Species	<i>Agropyron cristatum</i>		<i>Achillea millefolium</i>	
The percentage of pollution	3.5%	7%	3.5%	7%
Initial pH	7.58	7.06	7.58	7.06
No bacterial inoculation	7.69	7.17	7.72	7.21
<i>Bacillus megaterium</i>	7.14	6.72	7.28	6.83
<i>Bacillus subtilis</i>	7.03	6.69	7.24	6.88

The variations in soil pH during the phytoremediation process at the end of the growth period of plants were measured at concentrations of 3.5 and 7% wt of oil pollution. The results of the analysis of variance (Table 3) showed that the effect of plant species and different treatments on soil pH is significant at the 5% level of significance ($p < 0.05$).

According to Figure 3, it can be seen that the pH increased during the phytoremediation process in the treatment without bacterial inoculation and decreased in the treatments of oil-degrading bacteria. The highest changes in pH were in the presence of oil-degrading bacteria treatments and the highest changes in the concentration of 3.5% wt of oil pollution and the treatment of *Bacillus subtilis* bacteria for *Agropyron cristatum* species was 7.25%.

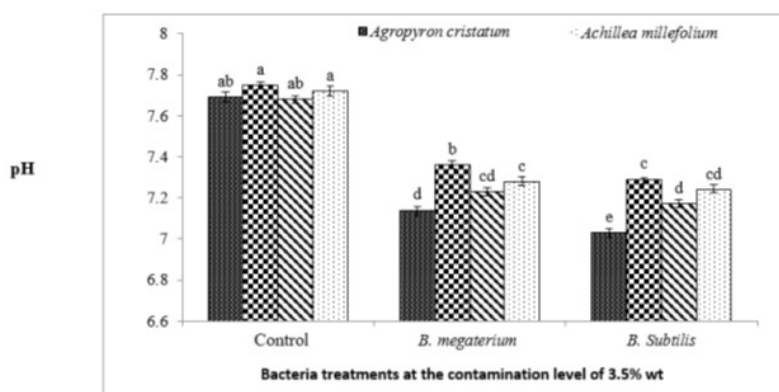


Figure 3. The comparison of the mean for the interaction of treatment plant species and degrading bacteria in the pollution level of 3.5 % wt. For the four studied species, each number is the mean of 4 repetitions and the lines on the columns are the standard error ($\pm$ SE). The treatments shown with different letters are significantly different from each other in Duncan's test ($P \leq 0.05$).

The variation of soil pH at the pollution level of 7% wt for different treatments is presented in Figure 4. The comparison of means showed that the highest amount of soil pH reduction

was related to *Agropyron cristatum* along with oil-degrading bacteria *B. megaterium* and *B. subtilis*.

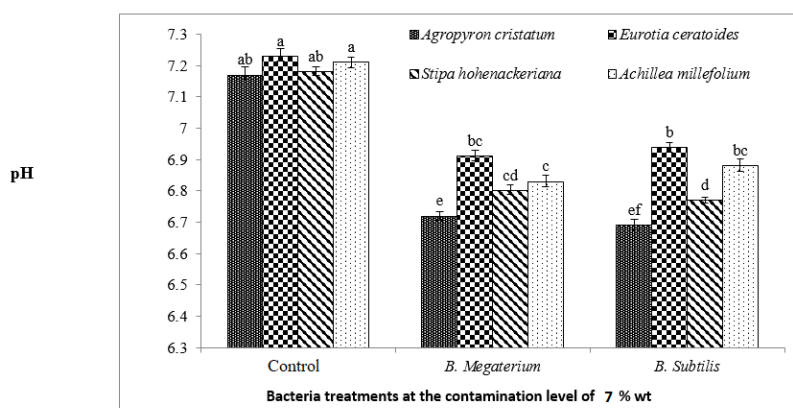


Figure 4. The comparison of the mean for the interaction of treatment plant species and degrading bacteria on pH the pollution level of 7 % wt for the four studied species, each number is the mean of 4 repetitions and the lines on the columns are the standard error ($\pm$ SE). The treatments shown with different letters are significantly different from each other in Duncan's test ($P \leq 0.05$).

The results of variance analysis of the effect of plant species on soil pH are shown in Table 3. A comparison of means showed that the pH value in *Eurotia ceratoides* was significantly

higher than *Agropyron cristatum*, *Stipa hohenackeriana*, and *Achillea millefolium* (Figure 5).

Table 3. Analysis of variance for the interaction of species and treatment on soil pH

Variable	Source of variation	df	RMS
pH	Plant species	3	0.127*
	Treatment	5	0.362*
	species*treatment	15	0.026*
	(% CV)	0.81	

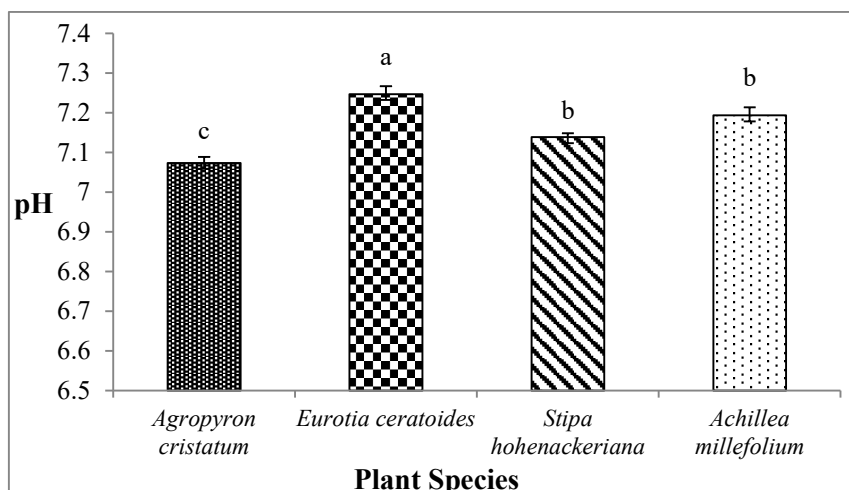


Figure 5. The comparison of the mean for the effect of plant species on soil pH. The lines on the columns represents the standard error ($\pm$ SE). The treatments shown with different letters are significantly differed from each other in Duncan's test ($P \leq 0.05$).

Salinity changes during the phytoremediation process

The salinity changes were measured at the end of the plant vegetative period. During the process of phytoremediation, the salinity level decreased more in the inoculated bacteria treatment than in the control group. According to Table 3, it can be seen that the most changes in salinity were observed in the presence of degrading bacteria treatments and the

concentration of 3.5% for both studied species. Also, in the treatment of the *Agropyron cristatum* plant with *Bacillus subtilis* bacteria, the most reduction of soil salinity was observed by 42.37%. It should be noted that the level of salinity reduction in the concentration of 3.5% oil pollution was higher than the concentration of 7% oil pollution in all treatments.

Table 4. The level of variations in EC in the treatments of degrading bacteria in the presence of two species *Agropyron cristatum* and *Achillea millefolium* in two concentrations of 3.5 and 7% wt of oil pollution in ds/m

Species	<i>Agropyron cristatum</i>		<i>Achillea millefolium</i>	
	The percentage of pollution			
	3.5%	7%	3.5%	7%
Initial pH	4.13	5.1	4.13	5.1
No bacterial inoculation	3.18	4.11	3.31	4.25
<i>Bacillus megaterium</i>	2.51	3.52	2.83	3.68
<i>Bacillus subtilis</i>	2.38	3.41	2.75	3.59

The percentage of organic carbon changes during the phytoremediation process
EC variations during the phytoremediation process

In this research, the amount of EC variations at the end of the plant growth period was measured. The results of analysis of variance (Table 5), showed that the effect of plant species and different treatments on soil electrical conductivity is significant at the five percent level ($p < 0.05$). During the process of phytoremediation, the amount of EC decreased and this decrease was more in the treatment of oil-degrading bacteria

inoculation than in the treatment with no bacteria inoculation. Figure 6 shows that the highest variations in EC were observed in the presence of oil-degrading bacteria treatments and at a concentration of 3.5% for all four studied species. Also, in the treatment of *Agropyron cristatum* plant along with *Bacillus subtilis* bacteria, the highest decrease in soil EC was 42.37%.

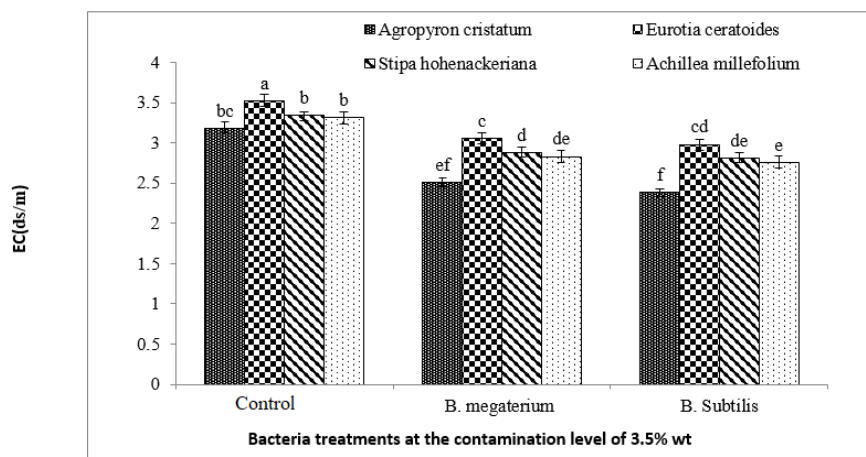


Figure 6. The comparison of the mean for the interaction of treatment plant species and degrading bacteria on pH the pollution level of 3.5 % wt for the four studied species, each number is the mean of 4 repetitions and the lines on the columns are the standard error ($\pm$ SE). The treatments shown with different letters are significantly different from each other in Duncan's test ($P \leq 0.05$).

Changes in the electrical conductivity of soil at the pollution level of 7% wt for different treatments are presented in Figure 7. As can be seen, the amount of salinity reduction in the concentration of 3.5% oil pollution was higher than the concentration of 7% oil pollution in all treatments.

of plant species on soil electrical conductivity are shown in Table 5. The comparison of means showed that the value of electrical conductivity in *Eurotia ceratoides* species is significantly higher than *Agropyron cristatum*, *Stipa hohenackeriana*, and *Achillea millefolium* species (Figure 9).

The results of variance analysis of the effect

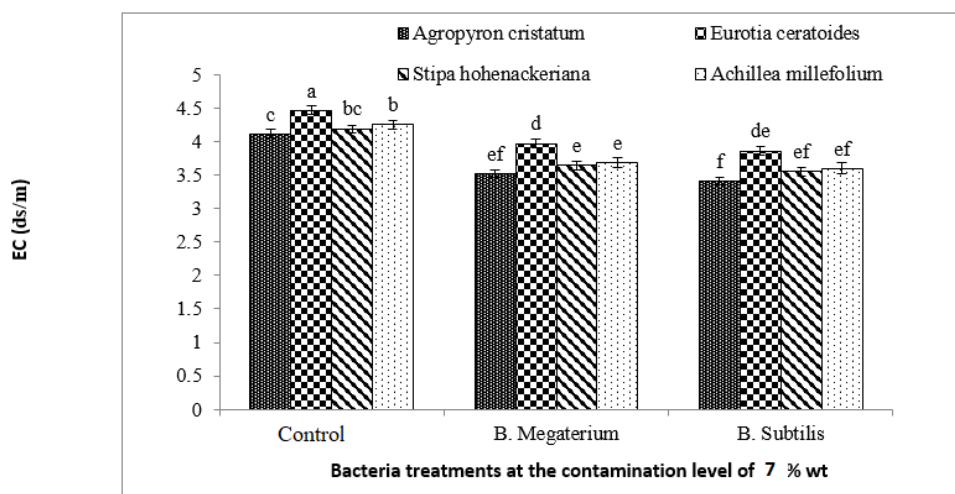


Figure 8. The comparison of the mean for the interaction of treatment plant species and degrading bacteria on soil EC at a level of 3.5 % wt for the four studied species, each number is the mean of 4 repetitions and the lines on the columns are the standard error ($\pm$ SE). The treatments shown with different letters are significantly different from each other in Duncan's test ($P \leq 0.05$).

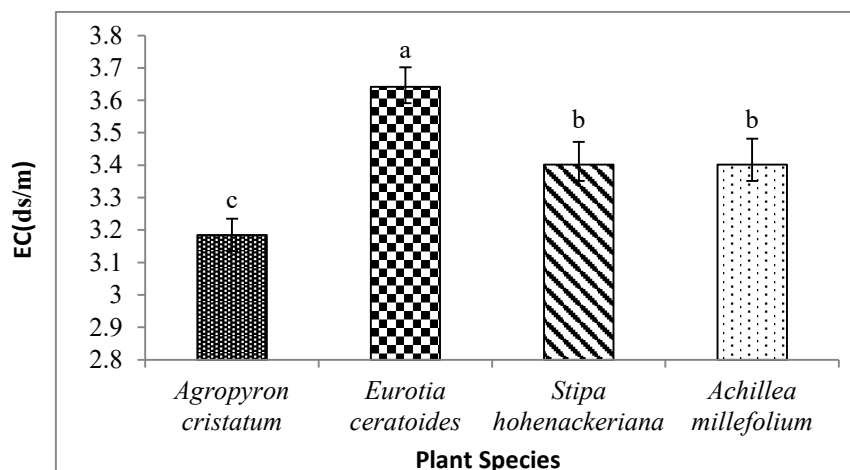


Figure 9. The comparison of the mean for the effect of plant species on soil EC. The treatments shown with different letters are significantly different from each other in Duncan's test ($P \leq 0.05$).

Table 5. Analysis of variance for the interaction effect of species and treatment on soil electrical conductivity

Variable	Source of variation	df	RMS
EC	Plant species	3	0.253*
	Treatment	5	0.211*
	species*treatment	15	0.021*
	(% CV)	2.73	

Soil organic carbon changes during the phytoremediation process

The changes in the percentage of soil organic carbon at the end of the vegetative period of plants were measured in concentrations of 3.5 and 7% wt of oil pollution. During the process of phytoremediation, the organic carbon decreased and this decrease was higher in the

Bactria inoculation treatments than in the control. Therefore, degrading bacteria treatments reduced soil organic carbon. According to Table 6, it can be seen that the most changes in soil organic carbon were observed in the treatments of degrading bacteria and the concentration of 3.5 in both studied species.

Table 6. The level of variations in organic carbon in the treatments of degrading bacteria in the presence of two species *Agropyron cristatum* and *Achillea millefolium* in two concentrations of 3.5 and 7% wt of oil pollution

Species	<i>Agropyron cristatum</i>		<i>Achillea millefolium</i>	
The percentage of pollution	3.5%	7%	3.5%	7%
Initial organic carbon	2.21	3.44	2.21	3.44
No bacterial inoculation	1.89	3.05	2.01	3.17
<i>Bacillus megaterium</i>	1.69	2.78	1.79	2.91
<i>Bacillus subtilis</i>	1.73	2.84	1.82	2.96

Changes in the percentage of organic carbon during the phytoremediation process

The results of ANOVA (Table 7), showed that the effect of plant species and different treatments on soil organic carbon percentage is significant ($p < 0.05$). The amount of changes in soil organic carbon percentage at the end of the growth period of plants was

measured in concentrations of 3.5 and 7% wt of oil pollution. During the phytoremediation process, the amount of organic carbon decreased and this decrease was more in the treatments inoculated with oil-degrading bacteria than in the treatment without bacteria inoculation. Therefore, oil-degrading bacteria treatments have reduced soil organic carbon.

According to Figure 10, it can be seen that the highest changes in soil organic carbon were observed in the treatments of bacteria at a level of 3.5 in the studied species. Variations in soil organic carbon percentage at the pollution level of 7% wt for different

treatments are presented in Figure 11. The comparison of means showed that the highest decrease in soil organic carbon percentage was related to *Agropyron cristatum* species along with oil-degrading bacteria *B. megaterium*.

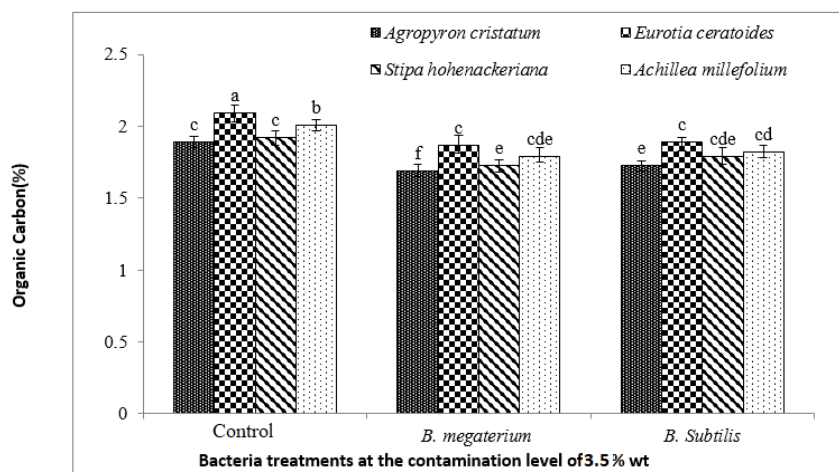


Figure 10. The comparison of the mean for the interaction of treatment plant species and degrading bacteria on soil organic carbon at a level of 3.5 % wt for the four studied species, each number is the mean of 4 repetitions and the lines on the columns are the standard error ($\pm$ SE). The treatments shown with different letters are significantly different from each other in Duncan's test ($P \leq 0.05$).

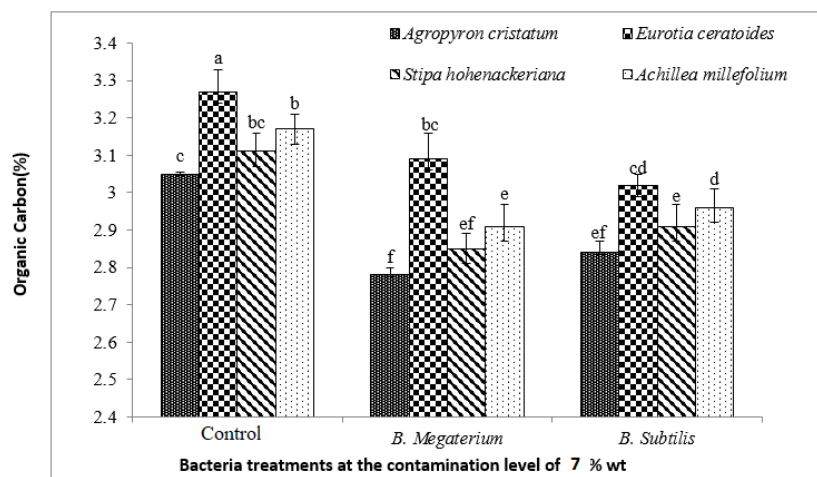


Figure 11. The comparison of the mean for treatment plant species and degrading bacteria interaction on soil organic carbon at a level of 7 % wt for the four studied species, each number is the mean of 4 repetitions and the lines on the columns are the standard error ($\pm$ SE). The treatments shown with different letters are significantly different from each other in Duncan's test ($P \leq 0.05$).

The results of variance analysis of the effect of plant species on soil organic carbon percentage are shown in Table 7. The comparison of means showed that the amount

of organic carbon percentage in *Eurotia ceratoides* species is significantly higher than *Agropyron cristatum*, *Stipa hohenackeriana*, and *Achillea millefolium* species (Figure 12).

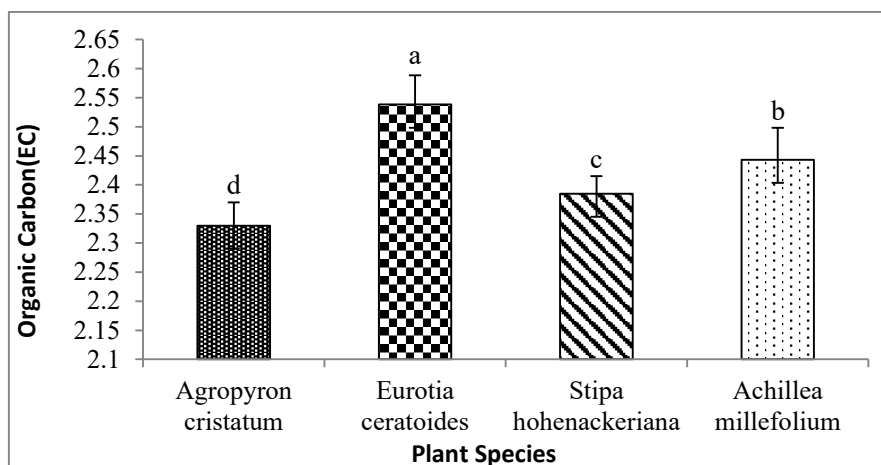


Figure 12. The comparison of the mean for the effect of plant species on soil organic carbon and the lines on the columns are the standard error ($\pm$ SE). The treatments shown with different letters are significantly different from each other in Duncan's test ($P \leq 0.05$).

Table 7. Variance analysis of the interaction of species and treatment on soil organic carbon percentage

Variable	Source of variation	df	RMS
Organic Carbon (%)	Plant species	3	0.241*
	Treatment	5	0.682*
	species*treatment	15	0.034*
	CV (%)	1.72	

Discussion

In this research, the potential of two plant species, *Agropyron cristatum* and *Achillea millefolium*, along with two petroleum-degrading bacteria, *B. megaterium* and *B. subtilis*, to remove oil pollution and changes in physical and chemical parameters of soil in two concentrations of 3.5 and 7% wt of oil pollution were investigated.

The results showed that in the presence of *Bacillus megaterium* and *Bacillus subtilis* bacteria treatments, the concentration of petroleum hydrocarbons in the soil was significantly lower than in the control. The addition of degrading bacteria increases the solubility of the unavailable part of the pollutants due to the release of biosurfactants, and of course, it increases the pollutant degradation. In this regard, Brooijmans et al. (2009), Chikere et al. (2012), Tahhan et al. (2011) and Asadi Rad et al. (2016) evaluated the effect of *Bacillus subtilis*, *Bacillus megaterium* and *Pseudomonas aeruginosa* strains in the degradation of petroleum hydrocarbons in soil. The results of these researchers are in line with those of the

present research on the significant increase in the degradation of soil petroleum hydrocarbons in the presence of degrading bacteria.

In this research, in the presence of *B. megaterium* and *B. subtilis*, the concentration of soil petroleum hydrocarbons was significantly lower than in the control treatment. The interaction between plant roots, micro-organisms and *B. megaterium* and *B. subtilis* bacteria added to rhizosphere soils can facilitate bioavailability of petroleum hydrocarbons and thus improve its reduction. On the other hand, the addition of oil-degrading bacteria increases the solubility of the inaccessible part of pollutants due to the secretion of biosurfactants, and hence, it increases the degradation of pollutants.

The results showed that in the presence of *Bacillus megaterium* and *Bacillus subtilis* bacteria treatments, the concentration of petroleum hydrocarbons in the soil was significantly lower than in the treatment without bacterial inoculation. Among the

reasons for the removal of more total petroleum hydrocarbon in the presence of bacteria treatments, it can be said that the addition of oil-degrading bacteria increases the solubility of the unavailable part of pollutants due to the secretion of biosurfactants, and hence, it increases the degradation of total petroleum hydrocarbon. In this regard, Brooijmans et al. (2009) isolated a strain of *Bacillus subtilis* that could break down hexadecanaphthalene hydrocarbons by 98% and 75%, respectively. Chickere et al. (2012) investigated the microbial degradation of oil hydrocarbons in a tropical oil-contaminated area in Lagos, Nigeria. In this study, several isolates including the *Bacillus subtilis* bacterial strain with the ability to break down crude oil hydrocarbons (TPH) were isolated. Also, the ability to break down diesel fuel by a strain of *Bacillus subtilis* has been proven (Adebusoye et al., 2007). Foods, especially nitrogen, phosphorus and in some cases iron, play a very important role in the biodegradation of hydrocarbon pollutants. Also, Asadi Rad et al. (2015) showed that the biodegradation rate of crude oil was 65.23% and 48.95% for *Bacillus licheniformis* and *Bacillus megaterium* in sterile conditions, respectively. The results of the studies of Alarcon et al. (2008) showed that among the four investigated treatments, the treatment receiving the liquid of bacteria and mycorrhizal fungi with 59% degradation of hydrocarbon pollutants had the highest efficiency among the treatments and among the degradation rates of all four treatments compared to Significant differences were reported in the control treatment. Das et al. (2006) used *Bacillus subtilis* and *Pseudomonas aeruginosa* strains for biodegradation of petroleum compounds. In their study, the TPH level was measured in the control soil and the test sample, and it was found that the TPH level had decreased significantly at the end of the experiment. Also, Tahhan et al. (2011), by using two bacteria isolated from soil and inoculating them with oil sludge, increased the rate of degradation of total oil hydrocarbons. In their investigation, inoculation was done in two stages in the periods of 62 days and 198 days, which finally reduced more than 30% of TPH.

The results of these researchers are consistent with the results of this research regarding the significant increase in the degradation of soil petroleum hydrocarbons in the presence of bacteria.

The results of the present research showed that *Agropyron cristatum* species have much more potential in removing TPHs in the soil than *Achillea millefolium* species. In this regard, Muratova et al. (2012) showed that several wild types of grass including *Lolium perenne* (46%), *Agropyron cristatum* (45%), and *Agropyrum tanrum* (44%) were able to remediate and remove pollutants well, which is in line with the results of this research that implying the high potential of *Agropyron cristatum* species to reduce petroleum hydrocarbons in the soil. the potential of this species in phytoremediation of TPHs in the soil is higher due to the well-developed root system compared to shoots and the extensive fibrous root system.

As a whole, the results showed that two species *Agropyron cristatum* and *Achillea millefolium* in soils contaminated with petroleum hydrocarbons were resistant to soil oil pollution and reduced the amount of oil pollution in the soil. Of course, the *Agropyron cristatum* was superior in reducing soil petroleum pollution. Therefore, the results of the present study imply the positive effect of the plant in removing petroleum hydrocarbons. Also, in the presence of *Bacillus bacteria* treatments, the concentration of soil petroleum hydrocarbons was significantly lower than in the bacterial inoculation treatment. Two non-endemic bacteria are recommended for bioremediation and restoration of polluted environments, especially soils contaminated with petroleum hydrocarbons, taking into account the environmental conditions and feasibility studies. It is recommended that the use of these hyperaccumulator rangeland plants, together with effective non-endemic bacteria, should be carefully monitored and investigated to clean up oil pollution

The results showed that the plant species studied in the current research succeeded in reducing the concentration of total petroleum hydrocarbons in the soil and can be used as

leading plants in reducing petroleum pollution. Plants can stimulate and increase the activity of the microbial community that destroys oil pollutants through the release of nutrients and their secretions in the soil and the transfer of oxygen to their root area. Schwab and Banks (1994) also pointed to a greater decrease in the total concentration of petroleum hydrocarbons in the presence of vegetation compared to the control treatment without vegetation. There are other similar results to prove that plants are a suitable means to accelerate the degradation of pollutants in soil (Anderson et al., 2003). Studies have shown that vegetation can reduce soil contamination with petroleum compounds (Cunningham, 1996). Also, Rajai et al. (2013) during a research, showed that the presence of plants in soil contaminated with old crude oil causes the biological activities of the soil to intensify and then increase the biological degradation of hydrocarbons. This is because the roots of plants by secreting organic compounds such as glucose, enzymes and complex carbohydrates, provide a suitable source of carbon and energy for the microorganisms in the root area, and thus break down petroleum compounds. (Shim et al., 2000). Many studies showed that there is a direct relationship between the increase in the degradation of petroleum hydrocarbons and the microbial population in contaminated soil under cultivation compared to uncultivated soil. Because the roots of plants provide a more suitable environment for the activity and development of the microbial population, and a larger microbial population is formed especially in the area of their roots, which leads to further degradation and destruction of petroleum compounds in this area (Moreira et al., 2011). Tang et al. (2012) also showed that the population of microorganisms in the root area is significantly higher than in the soil without the presence of plant roots.

In this research, two species from the Poacea family were selected among the studied species, and the results show their high ability to reduce total petroleum hydrocarbons. Bruce and Pivetz (2001) also showed during a research that among the grasses, the wheat family is considered the most effective and

well-known cleansing plant. Many wheat species from the genera *Avena*, *Hordeum*, *Poa*, *Lolium*, *Bromus*, *Sorghum*, *Zea*, *Festuca* and *Agropyron* are used to clean organic pollutants including aliphatic and polyaromatic oil compounds. Also, Merkl (2005) showed during a research that plants from the legume and wheat families have the ability to significantly reduce oil pollution, which is consistent with the results of this research that the wheat family has a high ability to reduce total petroleum hydrocarbons. It seems that grassy plants, due to having fibrous roots, have stimulated microbial activity and increased the decomposition and destruction of these pollutants in their rhizosphere environment. Compared to other roots, fibrous roots provide a more suitable environment with a higher specific level for the activity and development of the microbial population and a larger microbial population is formed in their rhizosphere environment (April and Sims, 1990).

The results showed that the highest amount of total petroleum hydrocarbons remaining in the soil is related to *Eurotia ceratoides* species and the lowest amount is related to *Agropyron cristatum* species. The results of the present research show that *Agropyron cristatum* species have a better effect in reducing total petroleum hydrocarbons in the soil than other plant species. In other words, the highest amount of reduction of total petroleum hydrocarbons in the soil is related to *Agropyron cristatum* species. Muratova et al. (2012) during a research on 7 crops, 5 wild species of grass including *Lolium*, *Bromus inermis*, *Agropyron cristatum*, *Agropyrum tenrum* and *Festuca pratensis* and 3 legumes including alfalfa, three-leaf clover and spruce showed that some of the grasses Wild including *Lolium* (46%), *Agropyron cristatum* (45%) and *Agropyrum tenrum* (44%) were able to purify and remove pollutants well, which is consistent with the results of this research on the high ability of *Agropyron cristatum* species to reduce soil petroleum hydrocarbons. has it. Due to the greater amount of roots produced compared to aerial parts and the production of extensive fibrous root system by *Agropyron cristatum*

plant, the ability of this plant to reduce total petroleum hydrocarbons in the soil is greater and the use of this plant in studies of phytoremediation of oil pollution Refinery is more suitable.

The results showed that using degrading bacteria treatments reduced the pH of the soil compared to the control treatment. Soil pH affects plant growth directly or indirectly. The most important role of soil pH is to control the solubility of nutrients in the soil. In other words, the potential to take up nutrients is highly dependent on soil pH (Moreira et al., 2011). Nutrient elements have different solubility at various pHs. Usually, with increasing pH, the solubility of nutrients, except molybdenum is declined. In this research, *Bacillus* bacteria increased phytoremediation of phosphorus and micronutrients by reducing soil pH and leading to an increase in plant growth. Also, since the pH factor is important in the growth and biodegradation of crude oil by bacteria, and the growth of bacteria and the production of biosurfactants lead to the acidification of the soil. Hence, controlling the pH of the culture medium is very important. The results of this research are consistent with the results of Alahdadi et al. (2011) which showed the reduction of soil EC and pH at the end of the plant growth period.

The results showed that during the plant treatment process, the electrical conductivity was reduced and this reduction was more in the bacteria inoculation treatment than in control. Therefore, it can be noted that *Bacillus* bacteria isolates caused a decrease in soil EC. *Bacillus subtilis* has been studied by many researchers as a salinity-resistant bacterium; Sarcheshmepour et al. (2008) isolated *Bacillus subtilis* isolates that can colonize at a high level of salinity (64 ds/m). This strain has also been considered by many authors including Tilak et al. (2005) as a rhizosphere bacterium that stimulates plant growth, playing a great role in dissolving insoluble phosphates and siderophore production. Ward and Brock (1978) also observed the biodegradation of hydrocarbons in salinity conditions and found that the mineralization of hydrocarbons decreases with the addition of salt, which is related to

the decrease in oxygen levels or the bioavailability of nutrients by existing microorganisms that can use oil as a source of carbon and energy, but due to very high salinity, biodegradation decreased significantly, and they found that the rate of hydrocarbon metabolism decreases with increasing salinity levels. Shiaris (1989), while studying the effect of salinity on the degradation of hydrocarbons, showed that there is a positive relationship between salinity and the rate of mineralization of phenanthrene and naphthalene. In low salinities, the mineralization of these two compounds increases. Several studies on the effect of salinity on the degradation of hydrocarbons have shown that the rate of metallization of oil increases at low salinity values, usually high salt concentrations prevent the growth of bacteria and the degradation of petroleum hydrocarbons. Because it leads to the absence of water from the bacterial cells and thus leads to the death of the bacterial cells (Leahy and Colwell, 1990).

The results showed that during the plant treatment process, the organic carbon decreased and this decrease was more in the bacteria treatment than in the control. The high amount of organic carbon in the soil is due to the presence of petroleum hydrocarbons in the soil. Comparing the data related to organic carbon with those related to TPHs, it was found that there is a direct relationship between the amount of organic carbon and the concentration of petroleum hydrocarbons (a significant source of carbon).

Given the results obtained in this study, it can be noted that the bio and phytoremediation mechanisms which include the vegetation and its microbial activities in the rhizosphere have great potential to remove TPHs from the soil. In this vegetation–bacteria symbiosis, vegetation provides the bacteria with a special carbon source that triggers them to degrade organic pollutants in the soil. On the other side, bacteria isolates can improve the vegetation's potential to cope with pollution and enhance vegetation growth and development. As well, plants take advantage of the hydrocarbon-degrading bacteria, in turn resulting in high hydrocarbon

mineralization and reducing both phytotoxicity and evapotranspiration of volatile hydrocarbons (Khan et al., 2013) and enhancing the effectiveness of phytoremediation (Weyens et al., 2009). Su and Zhu (2008) at the same time demonstrated the role of plant uptake and microbial biodegradation of TPHs in various environments (Su and Zhu, 2008). The limitations of this research are the high cost of experiments, preparing pure bacteria and the desired species, and keeping the greenhouse conditions constant.

Conclusion

The phytoremediation process is a suitable method for eliminating TPH from the soil. In this study, two hyperaccumulator species

Agropyron cristatum and *Achille amillefolium* along with two bacteria *Bacillus megatrium* and *Bacillus subtilis* displayed a positive effect on TPH bioremediation. This may be a good approach to combine the benefits of microbe-plant symbiosis into an efficient biodegradation technology. This approach may be a promising strategy for the restoration of more contaminated areas. Hence, this method is feasible in developing countries as it is cost-effective and requires little management.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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