Advances in Bioresearch
Adv. Biores., Vol 7 (2) March 2016: 73-81
©2015 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
ICV Value 8.21 [2014]

Advances in Bioresearch

ORIGINAL ARTICLE

Ability of White Clover and Alfalfa to Grow in Petroleum Hydrocarbon-Contaminated soil in Iran and their Phytoremediation Potential

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ABSTRACT
Over the past decades rapid growth in industrial and agricultural activities, waste disposal and etc., have significantly contributed to extensive soil contamination. Total petroleum hydrocarbons (TPHs) are one of the most common groups of persistent organic contaminants. To date, many developing countries like Iran have almost completely relinquished remediation of oil-polluted soils due to the high costs of conventional soil remediation methods. The main objectives of this study were to investigate the influence of petroleum hydrocarbons on growth characteristics of White Clover (Trifolium repens L.) and Alfalfa (Medicago sativa L.) as well as to evaluate the phytoremediation capability of the these plant species in aged hydrocarbon contaminated soil in Iran. Impact of organic fertilization on plant growth in contaminated soil and phytoremediation effectiveness was also evaluated. Soil samples were analyzed for TPHs content by GC-FID. Adverse effect of petroleum hydrocarbon contamination on growth parameters of both studied plant species was observed in this research; however, hydrocarbon contamination could not inhibit their germination and growth, especially in organic amended soil. The highest removal of TPHs was obtained for white clover in presence of peat (88.1% removal). Results demonstrated that white clover and alfalfa are promising species for phytoremediation of aged, petroleum hydrocarbon-contaminated soils. Phytoremediation as a novel technology to remediate hydrocarbon contaminated sites has been received increasing attention in recent years; however, there is still a significant need to pursue both fundamental and applied research to provide comprehensive insight into its effectiveness under various environmental conditions.
Keywords: *Alfalfa; Phytoremediation; Soil pollution; Total Petroleum hydrocarbons; White Clover*

Received 11/11/2015 Accepted 18/02/2016

How to cite this article:
Ali Daryabeigi Z, Gholamreza Nabi B, Hassan H. Ability of White Clover and Alfalfa to Grow in Petroleum Hydrocarbon-Contaminated soil in Iran and their Phytoremediation Potential. Adv. Biores., Vol 7 [2] March 2016: 73-81. DOI: 10.15515/abr.0976-4585.7.2.7381

INTRODUCTION

Soil contamination with petroleum hydrocarbons as an important environmental problem has been attracting significant attention over the past years [1-4]. Oil products have been disposed of in the environment for hundreds of years assuming that the environment will adequately absorb them; however, this is no longer the case and accumulating pollutants are now affecting the health of living organisms [5,6]. Although, various physical, chemical and biological processes have been employed for effective remediation of contaminated soil, to date, many developing countries like Iran have almost completely relinquished remediation of oil-polluted soils due to the high costs of conventional (physical/chemical) soil remediation methods. Phytoremediation is an emerging green technology that can be a promising solution to remediate hydrocarbon-polluted soils [7, 8], not only in developed countries but also in developing countries like Iran in which uncontrolled disposal of oil industry wastes has polluted soil resources over the past decades. Synergistic cooperation of plant roots and soil microorganisms promotes the degradation of persistent organic contaminants in phytoremediation. Removal of petroleum hydrocarbons from soil in phytoremediation is often attributed to microorganisms living in rhizosphere under the influence of plant roots [9, 10]. Microorganisms in rhizosphere benefit from the root exudates and plants, in turn, from the metabolic detoxification of potentially toxic compounds brought about by microbial communities. Some author's report a higher degradation of petroleum contaminants in soil vegetated with white clover, sorghum, cowpea, alfalfa and black rush compared to unvegetated soil [11]. Many plant species are sensitive to petroleum contaminants [12]. Chaineau et al. (1997) found a growth rate reduction of beans and wheat by more than 80% [13]. Significant reduction of plant biomass by the presence of petroleum hydrocarbons has also been reported by Merkl *et al*. (2004) [11]. Additionally, germination reduction and delay was observed by Adam and Duncan [14]. Eemploying native plant species that are tolerant to high concentrations of TPHs in soil can be a key factor in the success of phytoremediation.. In addition, the influence of organic fertilization upon plant growth and phytoremediation effectiveness was also evaluated since nutrient addition to soil through fertilization may also increase the plant biomass thereby promoting pollutant removal as suggested by some researchers [15-17]. The main objectives of the present study were to evaluate the phytoremediation potential of alfalfa and white clover, as well as their growth behavior in weathered hydrocarbon contaminated soil.

MATERIALS AND METHODS

Weathered contaminated soil was prepared from contaminated lands around Oil Refinery of Tehran. This site had been used for dumping of petroleum wastes for many years and it has been abandoned in a few recent years. Therefore, the contamination of the soil was not fresh. The clean soil which is also called control soil in this study was used to compare plant growth in hydrocarbon-contaminated soil with plant growth in clean (control) soil. The results of this comparison reveal the impact of weathered hydrocarbon pollution on plant growth. The soil prepared from the site was sieved through a 10 mm sieve and mixed thoroughly. In most studies soil is sieved by 2 mm sieve which according to AASHTO and Massachusetts's Technology Institute standards is the boundary limit between sand and gravel particles [18]. However, this leads to a considerable loss of coarse grain portion of real soil and lack of accordance between real soil from contaminated site and soil used in phytoremediation experiment. Some physical and chemical properties of the experimental soil are presented in Table 1. After a relatively homogeneous mixture of soil was obtained, the soil was weighed and transferred to PVC pots (1 kg of soil per pot).

In order to study the effect of fertilization on plant growth in hydrocarbon polluted soil as well as phytoremediation efficiency, peat fertilizer was used. Characteristics of the utilized peat fertilizer were as follows: pH= 5.5, total nitrogen= 1.1 percent, existing phosphorus= 32.7 mg Kg^{-1}, potassium= 2280 mg Kg^{-1} and organic carbon= 30.9 percent. The soil composition in the pots was as follows:

T1: clean soil of lands surrounding Oil Refinery of Tehran without any kinds of contamination background (control soil), T2: contaminated soil, T3: Soil used in treatment T2 (90%) + peat fertilizer (10%). The initial concentration of TPHs in the soil used in treatment T2 was 30438±1056 mg Kg^{-1} which demonstrates rather high level of contamination in soil. A natural attenuation treatment (without plant) in which petroleum hydrocarbons were naturally attenuated was also considered. Alfalfa and white clover were cultivated over a four-month period in a greenhouse. Two grams of alfalfa as well as white clover seeds were planted in the 1.5-2.0 cm depth of the surface soil in each pot. The pots were placed inside the greenhouse under sunlight. The temperature was between 24 °C and 29 °C. Monitoring of plant growth was done every 10 day. The pots were watered twice a week to maintain a constant and sufficient moisture level and to minimize the generation of leachate. PVC pans were placed under each pot to collect leachate. Leached water was collected and included in the next watering to avoid petroleum hydrocarbons loss. However, Hutchinson et al. (2001) showed that only 0.02% of the TPH in aged soil was leached from the pots with irrigation water [15]. Germination rate in the initial weeks was studied by surface density observation. The shoot height was measured and monitored too. Destructive pots were destroyed after 30, 60, and 120 days. For this purpose, first the plants were carefully removed from their soil and carefully washed with running water avoiding breaking of roots. Then using a ruler, root length and shoot height were measured. In order to measure dry biomass plants were placed in an oven in 70 °C for 48 hours and then weighed. Soil samples were taken by a core sampler (inner diameter = 8 mm) from the whole height of the pots every month.

For TPHs analysis, soil samples were air dried at room temperature and passed through a 2 mm sieve. The samples were stored at 4 °C prior to extraction and analysis. Ultrasonic extraction was performed using dichloromethane solvent. Ten cc of dichloromethane was added to about 5 grams of contaminated soil and then it was placed in an ultrasonic water bath for three minutes at room temperature. All of these operations were repeated three times. Then the obtained extracts were concentrated to 1 ml under a gentle stream of nitrogen gas. Two μL of the sample was injected into a gas chromatograph (GC) equipped with a flame ionization detector (FID). The column used for analysis was DB-5 with 30 m length, 0.25 mm internal diameter and 0.2 μm thickness of film. The injector and FID detector temperatures were adjusted on 280 °C and 340 °C, respectively. Initial column temperature was adjusted at 50 °C for 5 minutes, and then increased to 250 °C with 10°C/min slope and remained at 250 °C for 40 minutes. The difference between soil treatments was tested by one-way ANOVA. Significance level was considered at 0.05. All statistical analyses were performed using the software, Statistical Package for Social Sciences (SPSS) 11.0 for Window, SPSS Inc., IL, USA.

RESULTS AND DISCUSSION

Alfalfa and white clover showed promising behaviour in petroleum hydrocarbon-contaminated soil. However, oil pollution depressed plant growth to some extent. Final seedling emergence of studied plants and shoot height monitoring results are presented in Figures 1 and 2, respectively. Root length, root biomass, and shoot biomass measurement results for destructive pot are presented in Table 2. In addition, Figs. 3 and 4 show residual amounts of TPHs in soil in applied treatments. Germination is one of the most important stages in plant establishment. Sensitivity of germination as well as initial growth steps of plant species can influence the phytoremediation efficiency. Some studies have suggested a link between poor germination and subsequent poor growth in hydrocarbon contaminated soil [13, 19]. In this study, petroleum hydrocarbon pollution did not have significant adverse effect on germination of studied plant species ($P>0.05$); however, the subsequent growth was depressed significantly in most cases by petroleum hydrocarbon pollution ($P<0.05$). In the current study, delay in seedling emergence was recorded in some cases. Alfalfa germination has been also reported to be retarded in presence of 100 mg Kg^{-1} of diesel [20]. Peat fertilizer increased seedling emergence of white clover significantly ($P<0.05$). Germination of white clover reached up to 85% in presence of peat. Peat fertilizer couldn't increase alfalfa seed germination significantly ($P>0.05$). Generally seedling emergence was considerably lower in contaminated soil than clean soil which demonstrates the influence of oil presence in soil as obtained by Carmen et al, (2009) [21]; however the seedling emergence was considerable in both plant species. Lower germination rate of alfalfa in presence of diesel was obtained by Adam and Duncan (1999) [22]. This may be attributed to the fact that they used fresh hydrocarbons while in the current study weathered, aged contaminated soil was used. Aged soils contain less toxic compounds to plants (e.g. low molecular weight hydrocarbons). Seedling emergence can be inhibited or delayed by toxic effects of the oil. Salanitro et al. (1997) reported seedling emergence reduction of corn, wheat, and oat in soil contaminated with heavy crude oil [23]. A reduction of germination by 30% to 90% for some native species of Mexico in petroleum-polluted soil was also observed by Gallego-Martinez et al. (2000) [24]. Oil components can enter into the seed and disturb metabolic reactions or even kill the embryo. Hydrocarbons may act as a physical barrier preventing seeds from access to water and oxygen or delaying their access [14]. The soil used in this study was aged soil and mainly contained high molecular weight hydrocarbons. Therefore a probable reason for germination delay of studied plants may be attributed to the water repellent property of hydrocarbons.

Remarkable reduction of shoot height by the presence of petroleum hydrocarbons was observed for both alfalfa and white clover. Peat fertilizer positively affected both plant growths. In most cases, plant growth was diminished after 80 days cultivation probably due to nutrient depletion in confined soil of pots. Maximum root length, shoot biomass, and root biomass in contaminated treatments were achieved in white clover (T3) (Table 2). In none of the cases peat fertilizer increased plant biomass compared with control treatment. Although usage of fertilizer may not have important impact on plant tolerance or sensitivity to petroleum contamination, it can have a positive effect on plant growth even in contaminated soils through biostimulation. Considerable reduction of plant biomass as well as root length by the presence of petroleum hydrocarbons was found in some cases. Chaineau et al. (1997) reported a growth rate reduction of beans and wheat by more than 80 percent [13]. Jagtap et al. (2014) also found a reduction of biomass for three plant species [25]. Inhibition of plant growth can be caused by toxic effects of petroleum hydrocarbons. Small molecules of hydrocarbons can enter and pass cell membranes leading to reduced membrane integrity or even to death of the cell [11]. Plant height and shoot biomass are good indicators of plant health; however, greater shoot biomass measurements are not necessarily indicative of enhanced remediation efficiency [26]. Greater root biomass is likely to be associated with more extensive root exploration of the soil and, subsequently, higher microbial biomass and activity. On-site observations also showed that white clover posses more extensive and dense root system compared to alfalfa in contaminated soil. Promising tolerance of white clover and developing a dense fibrous root system in presence of hydrocarbons was also observed in other studies [27]. Alfalfa also had dense root system which has been reflected in their root biomass. With regard to their root system and also their remarkable tolerance in petroleum contaminated soil, it seems that these plant species may be a promising in phytoremediation of TPHs contaminated soils, particularly for aged contaminated soils. Generally the adverse effect of petroleum hydrocarbon on plant growth parameters was not very high in the current study compared to some other researches. For instance, a high reduction of biomass of *B. brizantha* and *P. maximum* by 85% and 99%, respectively, compared to the control, was found in soil contaminated with light crude oil which has a large fraction of small molecular compounds [11]. The difference between the results of this study and related researches can be originated from the fact that aged soils has less content of low molecular weight hydrocarbons leading to less toxic effects on plant tissues. In general, the effectiveness of phytoremediation relies on the establishment of plants with sufficient biomass growth and active root proliferation which can support a flourishing microbial consortium assisting contaminant dissipation in the rhizosphere [28].

Table 1: Physical and chemical characteristics of the soil used in the current study

Parameter	Value	Analytical method
Clay (%)	31	Hydrometer
Silt (%)	29	Hydrometer
Sand (%)	28	Hydrometer
Gravel (%)	12	Sieve
OM[a] (%)	4.12	Walkley-Black
Organic C (%)	3.05	-
Soil pH	7.5	1:1 soil/water slurry
EC[b] (dS/m)	3.35	1:2 soil/water slurry
Total N (%)	0.14	Kjeldahl
Phosphorus (mg Kg^{-1})	29.4	Olsen

[a] Organic Matter
[b] Electrical Conductivity

Table 2: Measurement results of root length, root biomass, and shoot biomass for destructive pots

Plant species	Parameter	Time (day)	Soil treatment		
			T1	T2	T3
Alfalfa	Root length (cm)	30	12	8 (-33)[a]	9 (-25)
		60	25	16 (-36)	19 (-24)
		120	29	18 (-38)	23 (-21)
	Root biomass (g)	30	2.0	1.4 (-30)	1.6 (-20)
		60	4.3	2.8 (-35)	3.3 (-23)
		120	6.8	4.4 (-35)	5.1 (-25)
	Shoot biomass (g)	30	2.2	1.7 (-22)	1.9 (-14)
		60	4.5	3.1 (-31)	3.5 (-22)
		120	6.2	4.0 (-35)	5.1 (-18)
White clover	Root length (cm)	30	15	11(-27)	12 (-20)
		60	29	23 (-21)	25 (-14)
		120	34	29 (-17)	30 (-12)
	Root biomass (g)	30	2.6	2.1 (-19)	2.2 (-15)
		60	5.6	4.5 (-20)	5.2 (-7)
		120	8.9	7.5 (-16)	8.2 (-8)
	Shoot biomass (g)	30	2.5	1.9 (-24)	1.9 (-24)
		60	5.0	3.1 (-38)	3.5 (-30)
		120	7.4	5.0 (-32)	6.6 (-11)

[a] Values in parentheses represent changes in comparison with control (%)

Figures 3 and 4 show TPHs concentrations decreased in all treatments. Significant influence of studied plants on petroleum hydrocarbon removal at different sampling times was obtained ($P<0.05$). The studied plant species caused a significantly higher petroleum hydrocarbon dissipation compared to unplanted soil ($P<0.05$), i.e. reduction by natural attenuation. Natural attenuation could reduce TPHs level in soil by 38.04% at the end of the experiment. The highest phytoremediation efficiency in unfertilized treatments obtained for white clover in which plant presence could reduce TPHs level by 43.99% in comparison with unplanted treatment.

Since the most important mechanism of phytoremediation is based on the stimulation of soil microorganism it can be assumed that higher root biomass, as obtained for white clover in this study, means a larger rhizosphere for microbial population and it is correlated with a higher degradation of hydrocarbons in soil [29]. Phytoremediation efficiency of alfalfa and white clover was rather close. Alfalfa could remove petroleum hydrocarbons by 35.12% compared to the unplanted treatment. The obtained results are in agreement with those of Escalante-Espinosa [30]. Peat fertilizer didn't have significant influence on phytoremediation efficiency of alfalfa and white clover ($P>0.05$), however, phytoremediation effectiveness of both studied plant species enhanced by around 5 percent in presence of peat. Relatively high reduction of TPHs in the rhizosphere of surveyed plants may be attributed to suitable plant growth in hydrocarbon contaminated soil used in the current study. Reduced plant height and biomass production may be considered as a basis for unsuccessful phytoremediation. When evaluating plant species for phytoremediation, the decrease in plant growth and specially root biomass should be as low as possible [29], as obtained in the current research.

Obtained results demonstrate that white clover and alfalfa are promising species for phytoremediation of aged, petroleum hydrocarbon-contaminated soils. However, petroleum hydrocarbon contamination depressed growth of the studied plants. White clover showed best root biomass production and caused highest hydrocarbon dissipation compared to unplanted soil suggests that greater root biomass is likely to be associated with higher microbial population and activity in the rhizosphere and, subsequently, higher phytoremediation efficiency. Petroleum hydrocarbon dissipation in unfertilized contaminated soil in presence of alfalfa and white clover were 73.16 and 82.03 percent, respectively. The maximum removal was obtained for white clover in presence of peat (88.1% removal). Peat fertilizer had positive effect on plant growth in most cases, but it couldn't enhance phytoremediation efficiency significantly. Alfalfa and white clover are well-known and easy to access plant species in many countries. Based on the obtained results, plant-aid remediation with alfalfa and white clover plant species can be a promising way to manage weathered hydrocarbon polluted sites. The investigation of microbial population in the rhizosphere as well as studying the influence of other organic and inorganic fertilizers on hydrocarbon removal through biostimulation would furthermore increase understandings about plant-based remediation of hydrocarbon contaminated soils.

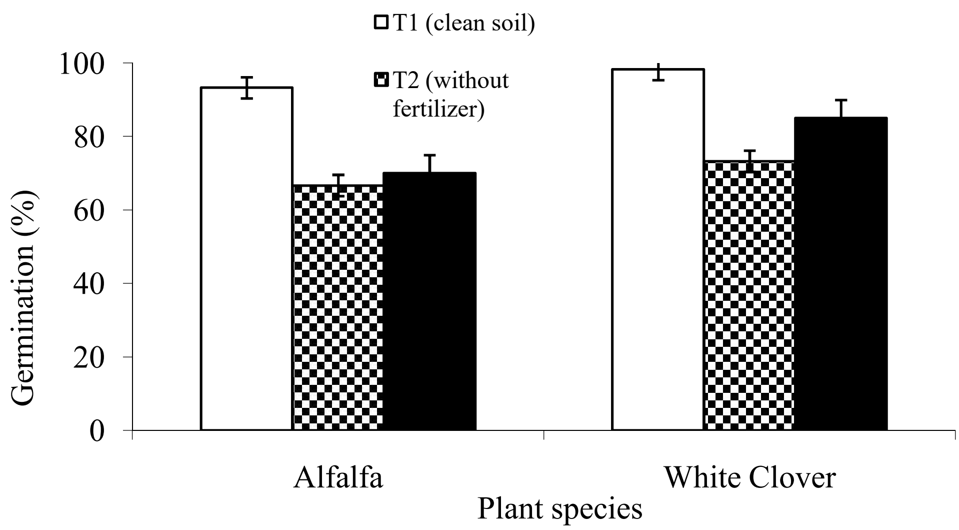


Figure 1: Germination of plant species in different treatments. Error bars represent standard deviation (n=3)

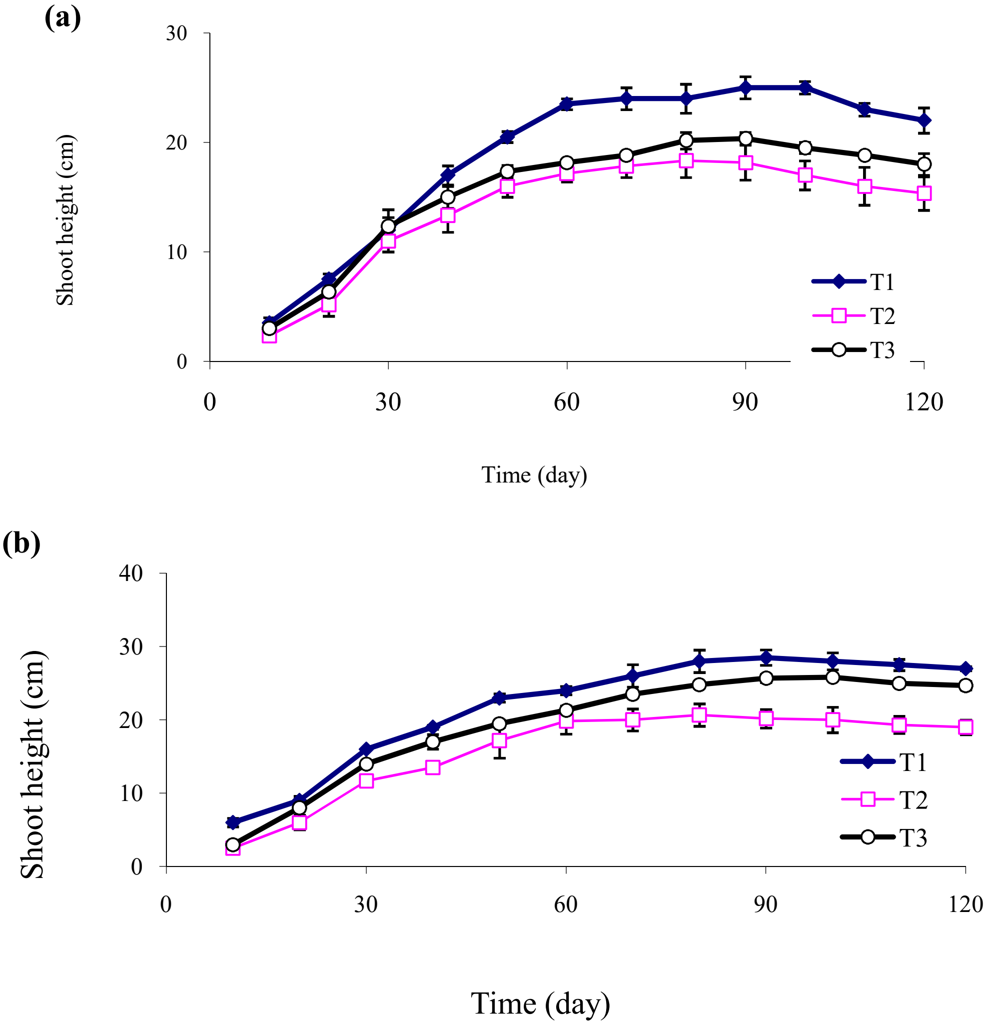
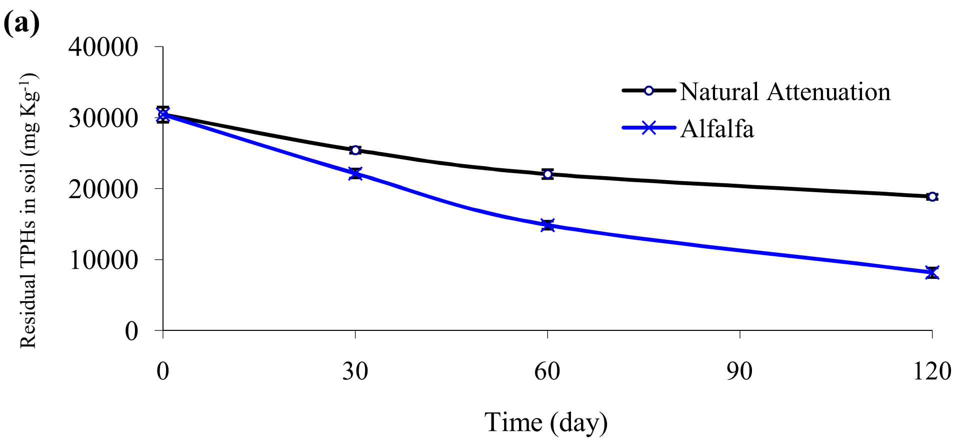


Figure 2: Shoot height monitoring during phytoremediation a) Alfalfa b) White Clover. Error bars represent standard deviation (n=3)



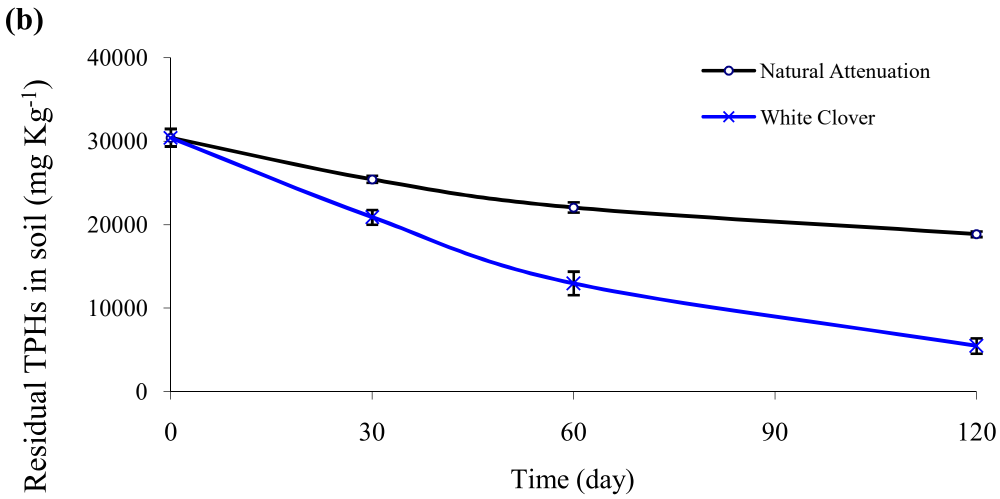


Figure 3: Residual amounts of total petroleum hydrocarbons (TPHs) in the rhizosphere of a) alfalfa and b) white clover in treatment 2 (without fertilizer) in comparison with natural attenuation treatment. Error bars represent standard deviation (n=3)

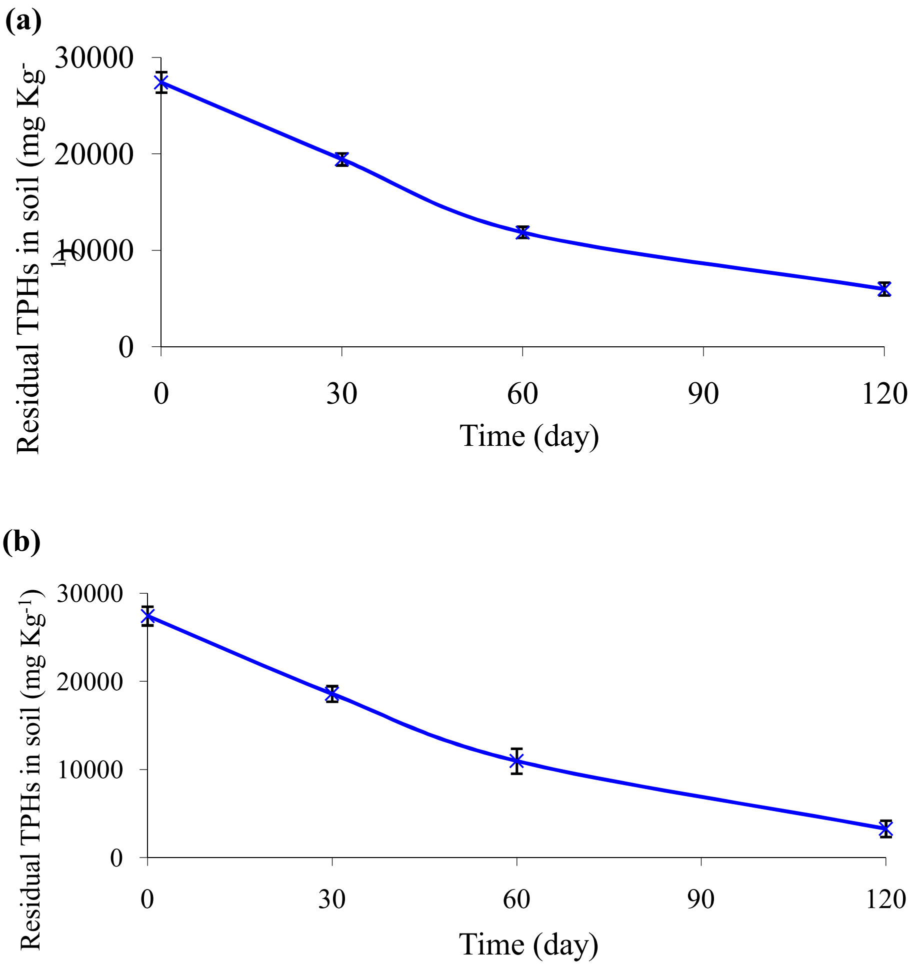


Figure 4: Residual amounts of total petroleum hydrocarbons (TPHs) in the rhizosphere of a) alfalfa and b) white clover in treatment 3 (with fertilizer). Error bars represent standard deviation (n=3)

CONCLUSIONS

Obtained results demonstrate that white clover and alfalfa, which are well-known and easy to access plant species in many countries such as Iran, are promising species for phytoremediation of aged, petroleum hydrocarbon-contaminated soils. However, petroleum hydrocarbon contamination depressed growth of the studied plants to some extent. Root biomass production of white clover was more than alfalfa in contaminated soil and caused highest hydrocarbon dissipation among the studied treatments suggesting that greater root biomass is likely to be associated with higher microbial population and activity in the rhizosphere and hence, higher phytoremediation efficiency. Petroleum hydrocarbon dissipation in unfertilized contaminated soil in presence of alfalfa and white clover were 73.16 and 82.03 percent, respectively. Peat fertilizer showed positive impact on plant growth in most cases, but it couldn't enhance phytoremediation efficiency significantly. Based on the obtained results, plant-aid remediation with white clover and alfalfa plant species can be a promising approach to manage aged petroleum hydrocarbon-contaminated sites. The investigation of microbial population in the rhizosphere as well as studying the influence of other organic and inorganic fertilizers on hydrocarbon removal through biostimulation would furthermore increase insight into the plant-based remediation of hydrocarbon contaminated soils.

ACKNOWLEDGEMENTS

Special thanks to the University of Tehran, Graduate Faculty of Environment for their support.

REFERENCES

1. Peng, S., Zhou, Q., Cai, Z., Zhang, Z. (2009). Phytoremediation of petroleum contaminated soils by *Mirabilis Jalapa L.* in a greenhouse plot experiment. J. Hazard. Mater., 168: 1490-1496.
2. Bramley-Alves, J., Wasley, J., King, C.K., Powell, S., Robinson, S.A. (2014). Phytoremediation of hydrocarbon contaminants in subantarctic soils: An effective management option. J. Environ. Manage., 142: 60-69.
3. Feng, L., Zhang, L., Feng, L. (2014). Dissipation of polycyclic aromatic hydrocarbons in soil amended with sewage sludge compost. Int. Biodeterior. Biodegrad., 95: 200-207.
4. Moubasher, H.A., Hegazy, A.K., Mohamed, N.H., Moustafa, Y.M., Kabiel, H.F., Hamad, A.A. (2015). Phytoremediation of soils polluted with crude petroleum oil using *Bassia scoparia* and its associated rhizosphere microorganisms. Int. Biodeterior. Biodeg., 98: 113-120.
5. Singh, O.V., Jain, R.K. (2003). Phytoremediation of toxic aromatic pollutants from soil. Appl. Microb. Biotechnol., 63: 128-135.
6. Perrichon, P., Akcha, F., Menach, K.L., Goubeau, M., Budzinski, H., Cousin, X., Bustamante, P. (2015). Parental trophic exposure to three aromatic fractions of polycyclic aromatic hydrocarbons in the zebrafish: consequences for the offspring. Sci. Total Environ., 524: 52-62.
7. Euliss, K., Ho, C., Schwab, A.P., Rock, S., Banks, M.K. (2008). Greenhouse and field assessment of phytoremediation for petroleum contaminants in a riparian zone. Bioresour. Technol., 99: 1961-1971.
8. Gerhardt, K.E., Huang, X., Glick, B.R., Greenberg, B.M. (2009). Phytoremediation and rhizoremediation of organic soil contaminants: Potential and challenges. Plant Sci., 176: 20-30.
9. Joner, J.E., Leyval, C., Colpaert, V.J. (2006). Ectomycorrhizas impede phytoremediation of polycyclic aromatic hydrocarbons (PAHs) both within and beyond the rhizosphere. Environ. Pollut., 142: 34-38.
10. Agamuthu, P., Abioye, O.P., Abdul Aziz, A. (2010). Phytoremediation of soil contaminated with used lubricating oil using *Jatropha curcas*. J. Hazard. Mater., 179: 891-894.
11. Merkl, N., Schultze-Kraft, R., Infante, C. (2004). Phytoremediation in the tropics-the effect of crude oil on the growth of tropical plants. Biorem. J., 8: 177-184.
12. Huang, X.D., El-Alawi, Y., Penrose, D.M. (2004). A multi-process phytoremediation system for removal of polycyclic aromatic hydrocarbons from contaminated soils. Environ. Pollut., 130: 465-476.
13. Chaineau, C.H., Morel, J.L., Oudot, J. (1997). Phytotoxicity and plant uptake of fuel oil hydrocarbons. J. Environ. Qual., 26: 1478-1483.
14. Adam, G., Duncan, H.J. (2002). Influence of diesel fuel on seed germination. Environ. Pollut., 120: 363-370.
15. Hutchinson, S.L., Banks, M.K., Schwab, A.P. (2001). Phytoremediation of aged petroleum sludge: Effect of inorganic fertilizer. J. Environ. Qual., 30: 395-403.
16. Pilon-Smits, E. (2005). Phytoremediation. Annu. Rev. Plant Biol., 56: 15-39.
17. Chang, Y.S., Chang, Y.J., Lin, C.T., Lee, M.C., Wu, C.W., Lai, Y.H. (2013). Nitrogen fertilization promotes the phytoremediation of cadmium in *Pentas Lanceolata*. Int. Biodeter. Biodeg., 85: 709-714.
18. Tahooni, S. (2000). Principles of foundation engineering. Pars Aain Press, Tehran pp.72.
19. MacKinnon, G., Duncan, H.J. (2013). Phytotoxicity of branched cyclohexanes found in the volatile fraction of diesel fuel on germination of selected grass species. Chemosphere, 90(3): 952-957.
20. Al-Ghazawi, Z., Saadoun, I., Al-Shak'Ah, A. (2005). Selection of bacteria and plant seeds for potential use in the remediation of diesel contaminated soils. J. Basic Microb., 45: 251-256.
21. Carmen, M.M., Camejo, D., Fernández-Garcia, N. (2009). Effect of oil refinery sludges on the growth and antioxidant system of alfalfa plants. J. Hazard. Mater., 171: 879-885.

22. Adam, G., Duncan, H.J. (1999). Effect of diesel fuel on growth of selected plant species. Environ. Geochem. Hlth., 21(4): 353-357.
23. Salanitro, J.P., Dorn, P.B., Huesemann, M.H. (1997). Crude oil hydrocarbon bioremediation and soil ecotoxicity assessment. Environ. Sci. Technol., 31: 1769-1776.
24. Gallegos-Martinez, M.G., Gomez-Trujillo, A.G., Gonzales, L.G. (2000). Diagnostic and resulting approached to restore petroleum-contaminated soil in a Mexican tropical swamp. Water Sci. Technol., 42: 377-384.
25. Jagtap, S.S., Woo, S.M., Kim, T.S., Dhiman, S.S., Kim, D., Lee, J.K. (2014). Phytoremediation of diesel-contaminated soil and saccharification of the resulting biomass. Fuel, 116: 292-298.
26. Banks, M.K., Schwab, P., Liu, B. (2003). Effect of plants on the degradation and toxicity of petroleum contaminants in soil: A field assessment. Adv. Biochem. Eng. Biotech., 78: 75-96.
27. Sheng-You, X.U., Ying-Xu, C., Kuang-Fei, L. (2009). Removal of Pyrene from Contaminated Soils by White Clover. Pedosphere, 19(2): 265-272.
28. Teng, Y., Shen, Y., Luo, Y. (2011). Influence of Rhizobium meliloti on phytoremediation of polycyclic aromatic hydrocarbons by alfalfa in an aged contaminated soil. J. Hazard. Mater., 186: 1271-1276.
29. Merkl, N., Schultze-Kraft, R., Infante, C. (2005). Assessment of tropical grasses and legumes for phytoremediation of petroleum-contaminated soils. Water Air Soil Pollut., 165: 195-209.
30. Escalante-Espinosa, M.E., Gallegos-Martınez, E., Favela, T., Gutierrez-Rojas, M. (2005). Improvement of the hydrocarbon phytoremediation rate by *Cyperus laxus* Lam. inoculated with a microbial consortium in a model system. Chemosphere, 59: 405-413.