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Potential of phytoremediation for the removal of petroleum hydrocarbons in contaminated soils associated with *Rhodococcus erythropolis*

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Degradation of petroleum hydrocarbons of the contaminated soils from the region of Isfahan refinery in colonized and un-colonized soils with the *Cynodon dactylon* and *Poa pratensis* plant species associated with *Rhodococcus erythropolis* Bacteria was investigated during 90 days in experiment greenhouse. It was in a completely randomized design with 12 treatments in 3 replications. On 90th day, the rate of decline of petroleum hydrocarbons in soil was measured by the EPA41 8 / 1 method. Statistical analysis was carried out using SPSS software as a regular and factorial means compared with LSD tests at P <0.01 for the effect of the bacteria, species and the interaction effect of them. The highest average rate of reduction was observed for the effects of species of *Cynodon dactylon* as well as the effects were caused by the bacteria in the ideal level. However the higher impact obtained by bacteria inoculation. The *Cynodon dactylon* treatments inoculated with bacteria showed the higher impact for removal petroleum hydrocarbons in the contaminated soils. Although both plants and their associated microorganisms presented a potential for removal of petroleum hydrocarbons in contaminated soils, results highlighted that potential may be distinct among plant species, which should be accounted for when designing cleanup strategies.

Keywords: Contaminated soils, *Cynodon dactylon*, petroleum hydrocarbons, *Poa pratensis*, Region of Isfahan Refinery, *Rhodococcus erythropolis.*

INTRODUCTION

Organic compounds, released in the environment by various human activities, are posing serious threat to the environment due to their toxicity, hydrophobic nature and persistence in the environment for a longer period of time. The presence of organic compounds in soil, such as hydrocarbons, polyaromatic hydrocarbons, polychlorinated biphenyls, phenols, chlorophenols, toluene, trinitrotoluene, benzene, herbicides and pesticides, inhibits growth and metabolic activities of soil-associated microbes, even at very low concentrations [1-4]. Furthermore, organic compounds can enter the food chain, and due to their toxic nature they can cause mutagenicity and carcinogenicity in animals and humans [5-7]. Therefore, the removal of these organic compounds from soil and water is one of the main issues in the field of environmental sciences and engineering [8-11].

Recently, the focus of the studies is to investigate the pollutants removal in soil using the organic and inorganic methods [12].

Phytoremediation is a promising technology for the removal of hydrocarbons from polluted soil and it depends on synergistic relationships between plants and their associated rhizosphere microbial communities [13]. The root system of the plant is one of the most important factors. Plants can indirectly influence degradation by altering the physical and chemical conditions of the soil [14]. Plant roots exude organic and inorganic substances to their neither exterior during normal metabolism. Root exudates act as substrates for soil microorganisms, thereby enhancing the degradation of toxic organic chemicals [15-16]. showed that some tropical grasses and legumes are resistant to petroleum pollution, and root surface was increased in the graminoids *Brachiaria brizantha, Cyperus aggregatus*, and *Eleusine indica* in petroleum-polluted soils. In addition, bioremediation is using plants and microorganisms to remove or detoxify environmental contaminants. Bioremediation has been intensively studied over the past two decades, driven by the need for a low-cost, in situ alternative to more expensive engineering-based remediation technologies [17-19]. Under petroleum-polluted conditions, plants or plant-associated microflora can convert hydrocarbons (HCs) to non-toxic forms. Bioremediation has been applied to remove crude oil [20-21], motor oil [22-23] from soil.

The remediation processes include treating the petroleum pollutants with hydrocarbon-degrading microorganisms [24]. such as *Rhodococcus*

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erythropolis. *R. erythropolis* cells possess various kinds of enzymes that allow them to biodegrade different pollutants [25]. mainly utilizing different types of monooxygenases and dioxygenses to degrade pollutants and also facilitate cell growth [26]. The biodegradability of *R. erythropolis* is related somewhat to the tolerance to the toxic substrates and solvents. It was reported that *R. erythropolis* tolerates both water-miscible, such as ethanol and butanol up to 50% (v/v), and water-immiscible solvents such as dodecane and toluene (5%, v/v). Tolerance mechanisms to the hydrocarbons involve the adaption of the cell surface to the environment [27]; (e.g. more mycolic acids or fatty acids in the cell surface) and thus to enhance the direct contact between cells and oil pollutants for a better biodegradation rate [28].

Iran is the world's fourth largest producer of crude oil and oil pollution is therefore widespread in this region during production and transport activities. Biological methods such as enhanced microbial degradation and phytoremediation are promising green and cost effective tools for large scale remediation [29-31]. However, the time span for biological methods is often long and the techniques are less efficient on highly polluted sites and for remediation of heavier oil products [32-34]. Therefore, finding new approaches to enhance efficiency of bioremediation is desired. The aim of this study is to determine the most efficient short term strategy for phytoremediation of severely oil contaminated soils from Isfahan refinery region in Iran. So that, (i) examined the potential of the phytoremediation the *Cynodon dactylon* and *Poa pratensis* (ii) The assessment of the bioremediation efficiency was based on *Rhodococcus erythropolis* inoculation on the *Cynodon dactylon* and *Poa pratensis* (iii) evaluated the effect of species interactions and bacteria in removal of petroleum hydrocarbons.

MATERIALS AND METHODS

Sampling

Samples were randomly collected from locations in several soil samples in the area of the depot of sulfur in Isfahan Refinery region. The non-contaminated soil was randomly sampled from the closest locate to the soil contaminated with petroleum hydrocarbons.

Sample processing

Each soil sample was crushed, thoroughly mixed then sieved through a 2 mm pore size sieve (RETSCH, Germany) to get rid of large debris. The sieved soil was then mixed with 50% garden soil and soil analysis was conducted to determine the physical and chemical characteristics of the soil (Table 1).

Table 1. Chemical and physical characteristics of the soil

Name	Unit	measure
EC	ds/m	11.5
pH		7.9
Lime	%	18.5
Gypsum	%	0.25
Total nitrogen	%	0.061
Organic carbon	%	0.56
Available phosphorus	mg/kg	17
Available Potassium	mg/kg	230
Clay	%	22
Sand	%	37
Silt	%	41
Soil texture	Loamy	-
Petroleum hydrocarbons in non- contaminated soil	g/kg	0.2
Petroleum hydrocarbons in contaminated soil	g/kg	4

The electrical conductivity (EC) measured of the saturated extract. Acidity (pH) was determined in saturation mud. The gypsum measured by EDTA, soil organic carbon by Nelson method and total nitrogen in soil by Kejeldal, restorable phosphorus by Olsen method were measured. Hydrometer method was used to determine soil texture. As well as minimum and maximum levels of petroleum hydrocarbons in contaminated soil and normal soil (non-contaminated control soil) was selected and the concentration of petroleum hydrocarbons was measured in both cases by EPA418 /1.

Soil sterilization

To remove the microorganisms of soil, 1.5 kg of soil contaminated with petroleum hydrocarbons was transferred to the 15 bags of polypropylene moisture resistant and autoclave at 121 degree and was maintained for 20 minutes under these conditions.

Preparation of the used materials

Rhodococcus erythropolis strain PTCC 1767 was purchased from the collection of fungus and bacteria Regional Center of Industrial Research Organization of Iran. Material culture medium, the antibiotic kanamycin, N-hexane, naphthalene, anthracene and phenantrains 1 and 10 with 99% purity was purchased from Sigma-Aldrich Company.

Preparation of the stocks

Stoke antibiotic kanamycin was prepared at a concentration of 150 ppm in the water solvent, and the stocks of naphthalene, anthracene and phenanthrene 1 and 10 were each prepared separately by the solvent n-Hexane at a concentration of 10 ppm.

Preparation of BHI culture medium

Preparation of BHI culture medium (Table 2) was performed to the volume 1 liter with combinations of enzymatic digestion of animal tissues, extract of calf brain without water, glucose, sodium chloride, and disodium hydrogen phosphate in grams per liter. Then it was adjusted to pH 7 and at 110 °C for 20 minutes was autoclaved, and after the cooling the stock kanamycin the concentration of 150 ppm at a rate of 10 cc by the syringe filter 0.22 µm, filtered and added to culture medium.

Preparation of BH specific solid culture medium

BH specific solid culture medium (Table 3) was prepared to the volume of 1 liter with the compounds of magnesium sulfate, calcium chloride, monopotassium phosphate, diammonium phosphate, potassium nitrate, ferric chloride and glucose in grams per liter. The pH was adjusted to 7, agar added and the medium was autoclaved at 110 °C for 20 minutes. After cooling the stock kanamycin with the 150 ppm concentration, 10 cc of it was filtered by the syringe filter 0.22 µm, and added to the culture medium. Before closing of agar, 20 cc of the culture medium was poured into petri dish.

Preparation of NA general solid culture medium

General solid culture medium NA was prepared to the volume of 1 liter of peptone, meat extract expressed in grams per liter. The pH was adjusted to 7, agar (gr/l) added to the culture medium and the medium was autoclaved at 110 °C for 20 minutes. After cooling the stock kanamycin with the 150 ppm concentration, 10 cc of it was filtered by the syringe filter 0.22 µm, and added to the culture medium. Before closing of agar, 20 cc of the culture medium was poured into petri dish (Table 4).

Preparation of MSM liquid culture medium

Liquid culture medium MSM (Table 5) was prepared to the volume of 1 liter with compounds of ammonium nitrate, potassium dehydrogenate phosphate, magnesium chloride, calcium chloride and zinc chloride in grams per liter. The pH was adjusted to 7, and the medium was autoclaved at 110 °C for 20 minutes. After cooling the stock kanamycin with the 150 ppm concentration, 10 cc of it was filtered by the syringe filter 0.22 µm, and added to the culture medium. Before closing of agar, 20 cc of the culture medium was poured into petri dish. After cooling the stocks kanamycin the concentration 150 ppm the amount 10cc by the syringe filter 0.22 µm filtered. Moreover 20 cc from each of naphthalene stoke, anthracene and phenanthrene 1 and 10 with 100 ppm concentration were filtered by the syringe filter 0.44 µm and as the carbon source added to the culture medium.

Table 2. Preparation of BHI culture medium

Animal tissue of enzymatic digested	Extract of calf brain without water	Glucose	Disodium hydrogen phosphate	Kanamycin
(gr/L)				(cc)
10	12.5	4	2.5	10

Table 3. Preparation of BH solid culture medium

magnesium sulfate	calcium chloride	monopotassium phosphate	diammonium phosphate	potassium nitrate	ferric chloride	glucose	agar	Kanamycin
(gr/l)								(cc)
0.2	0.02	0.1	1	1	0.5	2	15	10

Table 4. Preparation of NA solid culture medium

Peptone	Meat extracts	Agar	Kanamycin
gr/l			(cc)
5	3	15	10

Table 5. Preparation of MSM liquid culture medium

Ammonium nitrate	Potassium dihydrogen phosphate	dipotassium hydrogen phosphate	$MgCl_2.6H_2O$	$CaCl_2.6H2O$	$MnCl_2.6H_2O$	$ZnCl_2.6H_2O$	Kanamycin	Naphthalene	Anthracene	phenanthrene
(gr/l)							(cc)			
1	2	7	2	1	0.1	0.1	10	20	20	20

Morphology of the Rhodococcus erythropolis bacteria

Linear *Rhodococcus erythropolis* bacteria culture was carried out in tow medium of specific solid culture of Hess Bushnell and general nutrient agar. Petri dish was transferred into the incubator at 30 °C for 48 hours, and then gram staining was carried out on colonies of *Rhodococcus erythropolis* bacteria. The purple color of colonies of Rhodococcus erythropolis bacteria under a light microscope was observed.

Transfer of Rhodococcus erythropolis bacteria to the suspension of inoculation

After activating bacteria in liquid culture medium BHI, 1 cc of deposits with bacterial cells was prepared by centrifugation of 3000 rpm for 20 minutes, and was transferred to the liquid culture medium of MSM to the volume of 1 liter and then was placed in an incubator shaker with 30 °C temperature and 180 rpm for 12 hours.

Inoculation of bacteria to the contaminated soil

1cc of liquid culture medium MSM with bacterial colonies in the centrifuges 3000 rpm for 20 minutes was taken and the centrifugation was transferred to the 12 petri dish containing 10 grams of contaminated soil sterile.

Preparation of seeds

First the impurities were isolated from the seeds of the plant species of Bermuda grass (*Cynodon dactylon*) and Kentucky bluegrass (*Poa pratensis*) to sterilize the seeds. In the second stage the seeds were washed 5 times with tap water, the seeds were soaked in 75% ethanol solution for 30 seconds. In the fourth stage were washed three times with distilled water for 10 minutes. In the fifth stage the seeds were soaked for 15 minutes with 25% sodium hypochlorite. In the final stage (stage six) the seeds were washed 3 times with distilled water each time for 10 minutes. The amount of 0.1 gr seed annual meadow grass species and 0.1 gr of seed of Bermuda grass species was added to the pots.

The seeds were added to the 36 pots with 1.5 kg of soil contaminated with petroleum hydrocarbons. The contaminated soil with inoculation suspension of Bactria was mixed with soil of 12 pots; 0.1 grams the seed of the each species was added to these pots.

Implementing the plan

The experiment was carried out as factorial and ordinary in a completely randomized design with 12 treatments and 3 replications (Table 6). Two species Bermuda grass (*Cynodon dactylon*) and annual meadow grass (*Poa pratensis*), the bacterium *Rhodococcus erythropolis* on 2 levels the bacterial inoculation and without inoculation, ordinary soil and soil contaminated with petroleum hydrocarbons completely (100% contaminated) made the experimental treatments. The treatments were carried out in the two levels of sterile and non-sterile for 90 days in research greenhouse of Islamic Azad University Isfahan (Khorasgan). Rate reduction of petroleum hydrocarbons were measured at 90 days.

Measuring the concentrations of petroleum hydrocarbons

To measure the concentrations of petroleum hydrocarbons, sampling was done of the soil contaminated with petroleum hydrocarbons and uncontaminated soil, each in three replications of the treatments. Concentration of petroleum hydrocarbons was measured according to the Global Environment Facility 418.1 and IR spectrophotometer Jackv model 450. Concentrations of petroleum hydrocarbons were also measured in ordinary soil.

Extractable petroleum hydrocarbons

Petroleum hydrocarbons were extracted based on the method by ultrasonic EPA 35 / 50C and the solvent for 3 times. 1 gram of contaminated soil and ordinary soil was solved in 10 cc of fluorine

carbon chloride solvent by sonic ultrasonic tour, soil particles became micron droplets then at 3000 rpm were centrifuged for 2 minutes to obtain a homogeneous extract. Cleaning and separation of polar compounds by combining 1.0 grams of silica gel was carried out in a homogeneous extract by Vertex. The extract obtained by Whatman filter paper was smooth. Dilution of the extract was carried out by the solvent fluorocarbon 1, 1, and 3 according to the concentration of each sample if necessary. The extract was filtered by the syringe filter 0.22 µm.

The data were analyzed by SPSS software and data means were compared using the least significant difference at 1%, and charts' drawing was done by SPSS software.

RESULTS

The effect of treatments on the mean concentration of petroleum hydrocarbons of contaminated soils

Analysis of variance of the concentration of petroleum hydrocarbons in contaminated soils indicated the significant effect (p= 0.01) of *Cynodon dactylon* species on the average concentration of petroleum hydrocarbon of the soil contaminated treatments in day 90 (Table 7).

Comparing the means with LSD post hoc tests at P <0.01 indicated a statistically significant effect in the reduction of the petroleum hydrocarbons by *Cynodon dactylon* plant species compared with *Poa pratensis*. The maximum reduction in petroleum hydrocarbons (mean 65.1 gr/kg) related to *Cynodon dactylon* species (Fig. 1).

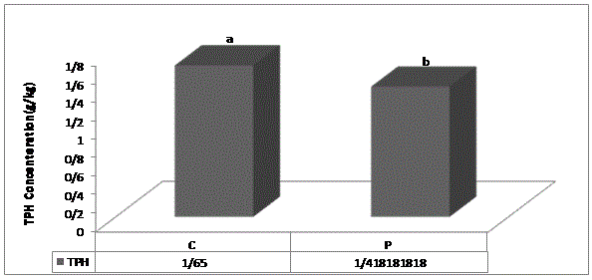

Fig. 1. The effect of the species of *Cynodon dactylon* and *Poa pratensis* on the average concentration of petroleum.

Table 6. The experimental treatments

Row	Treatments	Symptom
1	Soils contaminated sterilized uncultivated	T1
2	Non-sterilized soils contaminated without cultivation	T2
3	Sterilized soils contaminated inoculated with bacteria *Rhodococcus erythropolis* + *Poa pratensis*	S+P+B
4	Sterilized soils contaminated without inoculation of bacteria *Rhodococcus erythropolis* + *Poa pratensis*	S+P+NB
5	Non-Sterilized soils contaminated inoculated with bacteria + *Poa pratensis*	NS+P+B
6	Non-Sterilized soils contaminated without inoculation of bacteria *Rhodococcus erythropolis* + *Poa pratensis*	NS+P+NB
7	Sterilized soils contaminated inoculated with bacteria *Rhodococcus erythropolis* + *Cynodon dactylon*	S+C+B
8	Sterilized soils contaminated without inoculation of bacteria *Rhodococcus erythropolis* + *Cynodon dactylon*	S+C+NB
9	Non-Sterilized Soils contaminated inoculated with bacteria *Rhodococcus erythropolis* and *Cynodon dactylon*	NS+C+B
10	Non-Sterilized soils contaminated without inoculation of bacteria *Rhodococcus erythropolis* + *Cynodon dactylon*	NS+C+NB
11	Ordinary soil + *Cynodon dactylon*	C1
12	Ordinary soil + *Cynodon dactylon*	C2

Table 7. Analysis of variance of the average concentration of petroleum hydrocarbon soil contaminated treatments in day 90.

Sources of variation Petroleum hydrocarbons (gr/kg)	Df	Mean square
Species	1	**0.29
Error	16	0.1
CV%		6.73

ns, * and ** symbolize not significant and significant at the 5% and 1% levels of probability, respectively.

Hydrocarbons of contaminated soils

Means comparison of concentration by LSD post hoc tests at P <0.01 showed that the average decrease of hydrocarbon petroleum is in the ideal level by the treatments of inoculated with bacteria and without inoculated bacteria. So that the concentration was reduced in the inoculated with bacteria treatment with mean of 1.57 gr/kg and the mean of reduction in without inoculation of bacteria treatment was 1.5 gr/kg (Fig. 2).

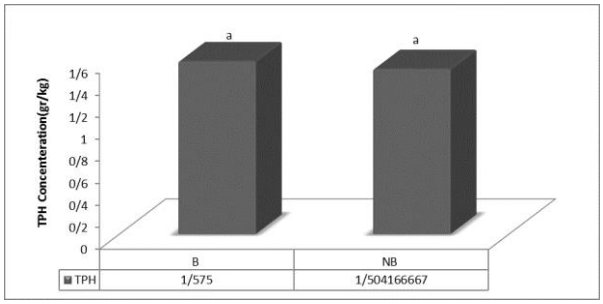

Fig. 2. The effect of inoculation and without inoculation of bacteria treatments on the average concentration of petroleum.

The average concentration of petroleum hydrocarbons were compared by the LSD post hoc tests at P <0.01 of the simultaneous effect the species of *Cynodon dactylon* and *Poa pratensis* with soil. Significant differences were observed between the soil treatment and *Cynodon dactylon* specie with *Poa pratensis* specie and soil. The specie of *Cynodon dactylon* reduced concentrations of petroleum hydrocarbons in ideal level, so that the average reduction of 1.56 gr/kg related to the treated sterile soil with *Cynodon dactylon*, and the mean of petroleum hydrocarbons reduction was 65.1 gr/kg in non-sterile conditions (Fig. 3).

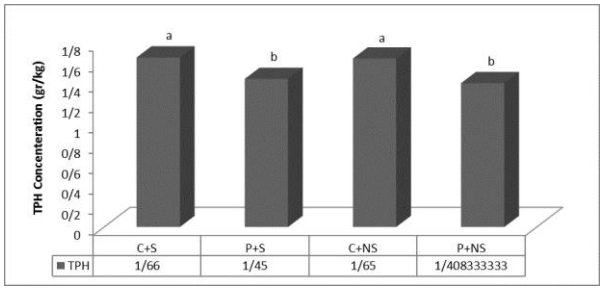

Fig. 3. The effect of treatments with *Cynodon dactylon* and sterile soil, *Poa pratensis* and sterile soil, *Cynodon dactylon* and non-sterile soil and *Poa pratensis* with non-sterile soil on the average concentration of petroleum.

In order to evaluate the simultaneous effect of species and bacteria, the average concentrations of petroleum hydrocarbons were compared based on the LSD post hoc tests test at P <0.01. The test showed no significant difference in the simultaneous effect of species and bacteria.

However 1.7 gr/kg mean rate of decline obtained in the treatment of *Rhodococcus erythropoli*s inoculated with the *Cynodon dactylon*, the rate mean of decline was 1.6 gr/kg in the treatment without inoculating *Rhodococcus erythropolis* with the *Cynodon dactylon*.

DISCUSSION

Phytoremediation has now emerged as a promising strategy for removal of a variety of soil contaminants [35-37]. The effect of planting plants on contaminated soil for refined petroleum hydrocarbons revealed the highest mean rate of decline is related to the *Cynodon dactylon* specie. This can be caused by the more stimulate of the roots of *Cynodon dactylon* to grow compared with *Poa pratensis* specie (Fig. 1). The efficiency of phytoremediation relies on the establishment of active plants with sufficient biomass growth, active root proliferation and/or root activities that can support a flourishing microbial consortium assisting phytoremediation in the rhizosphere [38-41]. It is reported that planting plants in areas contaminated with petroleum hydrocarbons may increase the rate of production of metabolites of the enzyme degrading effect. Therefore they require more source of carbon for bacteria to increases the decomposing process and reduce the average concentration of petroleum hydrocarbons. The diversity of root morphology amongst plant species may explain some of this variability. For example, species from *Poaceae* are often chosen due to their high rooting density which is capable of supporting large microbial communities [42-45].

Bioremediation of petroleum-contaminated soil is mainly based on biodegradation in the rhizosphere [46-47]. Bioremediation uses microbes or other biological systems to degrade environmental pollutants [48-54]. In this study, performance *Rhodococcus erythropolis* bacteria in the soil contaminated with petroleum hydrocarbons revealed the ideal level of the average rate of decline of petroleum hydrocarbons in both inoculation and without inoculation bacteria. However, the highest rate of decline obtained of the inoculated treatment with *Rhodococcus erythropolis. Rhodococcus erythropolis* inoculation in the soil may be modified the providing of nutrients for plants. Rhizoremediation, which can be used at the final stages of polluted soil re-cultivation, is based on the use of microorganisms

to utilize environmental pollutants as carbon and energy source and leads to the destruction of organic pollutants by microorganisms associated with plant roots [55-56].

Phytoremediation associated with microbial bioremediation led to a more successful remediation method of contaminants, particularly organic compounds [57]. .The mechanism of combination plants and bacteria in contaminated soil for refined petroleum hydrocarbons (Fig.3) showed that the rate of decline of Petroleum hydrocarbons is in the ideal level for the interaction of bacteria and specie and it obtained of *Cynodon dactylon* specie for inoculation and without inoculation bacteria. The highest effect was related to the inoculated treatments. The action of good root-colonizing and pollutant-degrading bacterial strains can result in efficient cleaning of polluted soils. It has been reported that root systems of some plants such as tall fescue, sorghum, maize, alfalfa, ryegrass, Bermuda grass, and rice contain highly potent hydrocarbon-degrading microorganisms. [58]. it is stated that perennial plant species (*Lolium perenne)* with degrading bacteria *Rhodococcus erythropolis* to the removal of soil contaminated with petroleum hydrocarbons in soil is one of the most effective methods (Fig. 4).

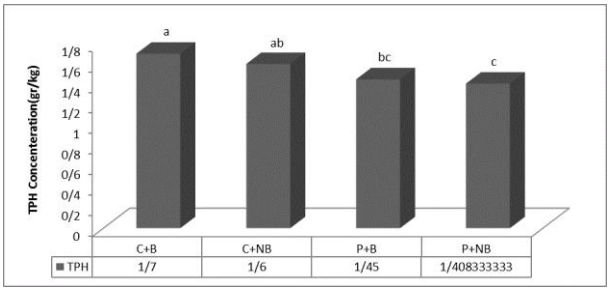

Fig. 4. The effect of treatments with *Cynodon dactylon* and inoculated Bacteria, *Cynodon dactylon* and without inoculated Bacteria, *Poa pratensis* and inoculated Bacteria and *Poa pratensis* without inoculated Bacteria on the average concentration of petroleum hydrocarbons

CONCLUSION

Degradation of hydrocarbons in 100% soils during the study was negligible, regardless of the applied treatments. Low percentages of hydrocarbon degradation, suggesting that a longer time period may be required. Phytoremediation proved to be an effective strategy for hydrocarbon removal, and colonized soils showed high rates of degradation in most experiments. So that the presence of plants clearly enhanced hydrocarbon degradation compared to un-colonized soils, significant hydrocarbon degradation was found in *Cynodon dactylon* specie and also significant *Cynodon dactylon* potential for hydrocarbons.

Phytoremediation seems to depend on root system development. Overall results point that *Cynodon dactylon* and *Poa pratensis* and their associated microorganisms present a potential for phytoremediation of petroleum hydrocarbons in contaminated soils, but the response to bioremediation treatments varied with plant species. In general the information obtained in this study may be useful for the decision on which phytoremediation strategies to apply to a specific contaminated soil. One of the most important mechanisms for remediation of hydrocarbons contaminated soil seems to be based on the synergy of plant roots of *Cynodon dactylon* and microorganisms. Therefore, due to the hard seed germination of this species in contaminated soils needs to be the seeds germinated in the out of soils contaminated and then transferred to contaminated soil. This makes the plant to seamlessly cover the entire surface the contaminated soil and creates a uniform root mass.

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