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Phytoremediation of an aged petroleum contaminated soil using endophyte infected and non-infected grasses

Mohsen Soleimani [a,*,1], Majid Afyuni [b,2], Mohammad A. Hajabbasi [b,2], Farshid Nourbakhsh [b,2], Mohammad R. Sabzalian [c], Jan H. Christensen [d,3]

[a] Department of Soil Science, Guilan University, Rasht 1814, Iran
[b] Department of Soil Science, Isfahan University of Technology, Isfahan 84156-83111, Iran
[c] Department of Agronomy and Plant Breeding, Isfahan University of Technology, Isfahan 84156-83111, Iran
[d] Department of Basic Sciences and Environment, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

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ABSTRACT

Phytoremediation is a promising technique for cleaning petroleum contaminated soils. In this study, the effects of two grass species (*Festuca arundinacea* Schreb. and *Festuca pratensis* Huds.), infected (E^+) and non-infected (E^-) by endophytic fungi (*Neotyphodium coenophialum* and *Neotyphodium uncinatum*, respectively) on the degradation of petroleum hydrocarbons in an aged petroleum contaminated soil was investigated. Plants were grown in the soil for 7 months and unplanted soil considered as control. At the end of the experiment, total and oil-degrading bacteria, dehydrogenase activity, water-soluble phenols, total petroleum hydrocarbons (TPH) and polycyclic aromatic hydrocarbons (PAHs) contents were measured in the soil. The results demonstrated that E^+ plants contained more root and shoot biomass than E^- plants and created higher levels of water-soluble phenols and dehydrogenase activity in the soil, while there was no significant difference in bacterial counts of planted soils. Planting stimulated total and oil-degrading bacterial numbers, dehydrogenase activity and the soil content of water-soluble phenols. Regardless of endophyte infection, PAH and TPH removal in the rhizosphere of plants were 80–84 and 64–72% respectively, whereas the removals in controls were 56 and 31%, respectively. It was revealed that TPHs in retention time range of *n*-alkanes with C_{10}–C_{25} chain lengths and TPH were more degraded in the rhizosphere of E^+ plants compared to E^- ones. Thus, grasses infected with endophytic fungi could be more efficient for removal of TPH from oil-contaminated soils.

1. Introduction

The presence of crude oil and refined petroleum products in the soil can lead to toxic effects on plants and soil microorganisms and act as a source of groundwater contamination (Scott, 2003). Petroleum hydrocarbon contamination of soil occurs through extraction, accidents, transportation, leakage from tanks, pipeline ruptures, consumption and refining (Scott, 2003). Most of the crude oil reservoirs and oil refineries in Iran are located in areas with agricultural activities and urban zones. Tehran refinery is located south of Tehran surrounded by crop fields and is one of the oldest in Iran. Due to oil pollution in some parts of this region, ecosystems are subjected to serious challenges (Besalatpour et al., 2008; Shirdam et al., 2009). Consequently, the remediation of soil impacted by oil production and transport is not only of importance considering environmental problems but also for the preservation of agricultural productivity. Chemical and physical methods applied for remediation of petroleum contaminated soils such as thermal treatment, soil washing, solidification and stabilization are expensive, disruptive to the environment and also involve high energy consumption (Kaimi et al., 2007). Therefore, natural remediation techniques have been developed to provide more environmentally friendly and cost-effective cleanup of sites impacted by petroleum spills (Alkorta and Garbisu, 2001).

Phytoremediation is an emerging green technology that uses plants to remediate soil, sediment, surface and ground water contaminated with toxic metals, organics and radionuclides (Alkorta and Garbisu, 2001; Gerhardt et al., 2009). This technique has been shown to be effective for petroleum contaminated soils in several laboratory and field studies (Newman and Reynolds, 2004; Euliss et al., 2008; Gerhardt et al., 2009; Phillips et al., 2009).

Biodegradation of polycyclic aromatic hydrocarbons (PAHs) which are associated with oil contamination and are recalcitrant to microbial degradation, can be promoted by rhizosphere effects

* Corresponding author. Tel.: +98 9131071865.
E-mail address: soleimani57@yahoo.com (M. Soleimani).
[1] Tel.: +98 9131071865.
[2] Tel.: +98 3113913470.
[3] Tel.: +45 35332456; fax: +45 35332398.

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of plants (Huang et al., 2004a; Cheema et al., 2009; Haritash and Kaushik, 2009). Although plants with highly branched fine fibrous root systems which have higher total rhizosphere volume have been reported to enhance biodegradation of organic contaminants more than plants with taproot systems (Aprill and Sims, 1990), a fine fibrous root system is not critical for phytoremediation (Banks et al., 2000).

The plant roots seem to provide an ideal environment for degradation of organic compounds as a result of several mechanisms. Plant root system allows rapid movement of water and gases through the soil due to the improvement of soil structure. It also provides a biologically active soil region (i.e. the rhizosphere), which encourages microbial activity and enhances contaminant bioavailability (Newman and Reynolds, 2004; Gerhardt et al., 2009; Wenzel, 2009). Hence, the use of plants and their associated microorganisms such as endophytes is a promising green technology for remediation of contaminated soils (Weyens et al., 2009).

Endophytes are a group of bacteria or fungi which live asymptomatically within a plant and may increase host plant tolerance to biotic and abiotic stresses (Malinowski and Belesky, 2000; Soleimani et al., 2010). During phytoremediation of organic contaminants, plants can further benefit from endophytes possessing appropriate degradation pathways and metabolic capabilities, leading to more efficient contaminant degradation and reduction of both phytotoxicity and evapotranspiration of volatile contaminants (Weyens et al., 2009). Engineered endophytes have also been shown to enhance phytoremediation of organic pollutants (Barak et al., 2004; Newman and Reynolds, 2005; Doty, 2008).

Although, phytoremediation of organic contaminated soils using endophytic bacteria have been the subject of several studies (Barak et al., 2004; Khan, 2007; Phillips et al., 2008), there is little information about the effect of infected plants with endophytic fungi on petroleum contaminated soils. Therefore we hypothesized that endophyte infected plants may enhance phytoremediation efficiency of petroleum contaminated soils in comparison to non-infected plants. The objective of this study was therefore to investigate the effect of two grasses (*Festuca arundinacea* Schreb. and *Festuca pratensis* Huds.) infected (E^+) and non-infected (E^-) with *Neotyphodium* endophytes on the remediation of an aged petroleum contaminated soil. The grasses were selected because they grow relatively fast, endophytic fungi can exist in their tissues and they are natural rangeland plants of Iran. Furthermore, *F. arundinacea* has been used in several phytoremediation studies on oil-contaminated soils without *Neotyphodium* endophyte consideration (Banks et al., 2000; Cheema et al., 2009).

2. Material and methods

2.1. Soil

The aged oil-contaminated soil used for this study was obtained from the oily waste landfills around the Tehran Oil Refinery, Iran. The composite soil samples were collected from 0 to 20 cm depth and include five sub-samples, which were thoroughly homogenized by mixing the soil. The homogenized soil was passed through a 2 mm sieve and selected physical and chemical properties were measured: Soil pH was 7.3 ± 0.1 as determined in a 1:2.5 suspension of soil in 0.01 M $CaCl_2$. Soil calcium carbonate and organic carbon contents were 18 ± 3 and 13 ± 3% respectively. Cation exchange capacity of the soil was 10 ± 1 cmol(+) kg^{-1} and total nitrogen and phosphorous contents of the soil were 1.3 ± 0.4% and 29 ± 8 mg kg^{-1} respectively. The soil contained 29 ± 3% clay, 34 ± 3% silt and 37 ± 5% sand. Electrical conductivity of the soil was 5.9 ± 0.6 dS m^{-1}. The amount of total petroleum hydrocarbons (TPH) and PAHs in the soil were 4700 ± 2100 and 8.5 ± 0.9 mg kg^{-1} respectively.

2.2. Plant materials and experimental conditions

Tall fescue (*F. arundinacea* Schreb.) and meadow fescue (*F. pratensis* Huds.) were used as plant materials. Seeds of two plant species were originally collected from natural rangelands of Iran. These two species were chosen because of high infection rates (almost 100%) of their seeds with *Neotyphodium coenophialum* and *Neotyphodium uncinatum* which was confirmed by using direct staining method (Saha et al., 1988). Fifty seeds from each plant population naturally infected by endophytes were sown in plastic pots and grown in a greenhouse. After 6 months, one single plant was selected from each population based on the fungus viability using microscopic detection and its hyphal density in leaf sheath tissue (Saha et al., 1988). Then all plants were clonally propagated by separation of secondary tillers from the main tillers. After 4 months, propagated plants were transferred to the field. Plants were allowed to grow in the field for 3 months and then half of the plants in each species were treated with a mixture of propiconazole and terbuconazole fungicides to eliminate endophytes and to prepare E^- plants (Soleimani et al., 2010) according to a modified approach of Hill and Brown (2000). After heading and seed maturity of the field clonally propagated plants in spring and early summer, E^+ and E^- seeds were harvested and kept in refrigerator (4 °C).

The seeds of E^+ and E^- plants were grown in the sand culture under controlled conditions (temperature 27 ± 3 °C, relative humidity 45 ± 8% and 12 ± 0.5 h daylight). Plants were microscopically examined for the presence of endophytes before and after the experiment (Saha et al., 1988). Ten 30-d old seedlings of each plant (E^+ and E^-) were grown in the plastic pots which contained 1 kg oil-contaminated soil for 7 months in the greenhouse (temperature 29 ± 4 °C, relative humidity 41 ± 9% and 13 ± 0.7 h daylight). Control pots were also considered with no planting. All the pots including planted and unplanted (control) pots were watered based on 75% of soil field capacity that showed no water leaching out of the pots. At the end of the experiment, plant samples were collected and washed with deionized water before being separated into shoots and roots. Then all samples were oven-dried (65 °C) for 48 h to reach a constant weight and dry weight of shoots and roots were determined. Soil was sampled at the start and end of the experiment. Soil samples in planted pots were collected from the rhizosphere area. All the samples were kept in 4 °C until analysis.

Beside the above experiment, germination of the plants was monitored daily during 4 weeks. Thirty seeds of each plant (E^+ and E^-) were grown in the contaminated soil. The experimental condition was the same as mentioned above. The number of germinated seeds in each pot was recorded and expressed as a percentage of the number of planted seeds.

2.3. Microbial plate counts

Aerobic heterotrophic bacterial cells and petroleum degrading aerobic bacteria were enumerated in triplicate using the drop plate method over a range of serial dilutions (10^{-1}–10^{-8}) on Nutrient agar (Fluka Biochemika, Spain) as a general media for growth of bacteria, and oil agar, respectively (Kirk et al., 2005). Composition of oil agar media (g L^{-1}) was 990 mL sterile solution of agar (15), KH_2PO_4 (1), K_2HPO_4 (1), NH_4NO_3 (1), $MgSO_4{\cdot}7H_2O$ (0.2), $FeCl_3$ (0.05) and $CaCl_2{\cdot}H_2O$ (0.02) adjusted to pH 7.0 and 10.0 mL of filtered sterilized oil (fresh crude oil from Tehran oil refinery, Iran). Plates were incubated at 30 °C for 48 h in the dark prior to counting the numbers of colony forming units (CFU).

2.4. Dehydrogenase activity

Measurement of dehydrogenase activity by indigenous microorganisms in soil has the potential to serve as a useful indicator of the microbial activity to determine the relative effectiveness of the plant rhizosphere in remediation of oil-contaminated soils and sediments (Balba et al., 1998; Mathew and Obbard, 2001). "Soil dehydrogenase activity was measured by the reduction of 2,3,5-triphenyl tetrazolium chloride (TTC) to 1,3,5-triphenyl formazan (TPF). Briefly, 5 g soil sample was incubated for 24 h at 37 °C in 5 mL of TTC solution (5 g L^{-1} in 0.2 M Tris–HCl buffer, pH 7.4). Two drops of concentrated H_2SO_4 were immediately added after incubation to stop the reaction. The sample was then blended with 5 mL of toluene to extract TPF and shaken for 30 min at 250 rpm (25 °C), followed by centrifugation at 5000 rpm for 5 min, and absorbance in the extract was measured at 492 nm. Finally, soil dehydrogenase activity was calculated as μg TPF g^{-1} dry soil d^{-1}" (Cheema et al., 2009).

2.5. Water-soluble phenols

Water-soluble phenols (or the total reducing capacity) were measured colorimetrically using the Folin–Ciocalteau reagent, which is a mixture of phosphomolybdate and phosphotungstate (Cheema et al., 2009). "Soils were extracted with 25 mL distilled water for 4 h with shaking, followed by centrifugation at 3000 rpm for 15 min. A 20 mL aliquot of extract or standard was placed in a test tube, and then 3 mL of Na_2CO_3 solution was added followed by 1 mL of Folin–Ciocalteau reagent. The solution was mixed well and allowed to stand for 1 h at room temperature. Absorbance was measured at 750 nm. Vanillic acid was used as the standard and the amount of phenolic compounds was expressed as vanillic acid equivalents" (Lee et al., 2008; Cheema et al., 2009).

2.6. Reagents and chemicals for TPH and PAH analyses

Dichloromethane (Rathburn) and *n*-pentane hexane (Rathburn) of HPLC grade were used in the extraction and cleanup. All glassware was rinsed thoroughly in ethanol after washing to remove organic residues. Two hundred gram of anhydrous sodium sulphate (pro analysis, Merck) was rinsed three times with 200 mL dichloromethane and left to dry in a fume hood over night before being dried in an oven at 85 °C for 20 h. Silica gel 60 (0.063–0.200 mm, Merck) was rinsed sequentially with acetone (Baker), *n*-pentane and dichloromethane. When dry, the silica gel was activated at 180 °C for 20 h.

Mixtures of 19 individual PAHs: naphthalene, dibenzothiophene, anthracene, phenanthrene, fluoranthene, chrysene, pyrene, benzo(a)anthracene, benz(a)pyrene, perylene, benzo(g,h,i) perylene, benzo(k)fluoranthene, dibenzo(a,h)anthracene, fluorene, benzo(e)pyrene, acenaphthene, acenaphthylene, benzo(b) fluoranthene and indeno(1,2,3-c,d)pyrene, including the 16 PAHs suggested by the United States Environmental Protection Agency were used as quantification standards. A surrogate standard solution was prepared, which consisted of 20 μg mL^{-1} of each of the following in isooctane (CIL, Cambridge, UK): naphthalene-d_8, acenaphthene-d_{10}, fluorene-d_{10}, dibenzothiophene-d_8, phenanthrene-d_{10}, pyrene-d_{10}, chrysene-d_{12}, benzo(k)fluoranthene-d_{12}, benzo(g,h,i)perylene-d_{12}. An injection spike contained 20 μg mL^{-1} of acenaphthylene-d_8, anthracene-d_{10}, fluoranthene-d_{10}, benzo(a) anthracene-d_{12} and benzo(a)pyren-d_{12} in isooctane.

2.7. Extraction and quantification of TPH and PAHs

Integrated extraction and cleanup was performed by pressurized liquid extraction (PLE) with a Dionex ASE 200 accelerated solvent extractor. Briefly, 5 g of soil was ground in a mortar with about 5 g of deatomous earth. A 33 mL extraction cell was packed with two cellulose filters and 5 g of activated silica gel (for cleanup). The homogenized and dried sample was then transferred quantitatively to the extraction cell, and 200 μL of the surrogate standard solution was added directly on top of the sample and left for 20 min to make sure of full percolation through the sample. The remaining cell volume was filled with Ottawa sand (20–30 mm mesh) from AppliChem (Darmstads, Germany) as an inert matrix. Ottawa sand was precleaned by heating at 450 °C over night.

The PLE program was as follows: A mixture of *n*-pentane and dichloromethane (9:1 v/v) was used as solvent at a pressure of 1.5 kPa and a temperature of 100 °C; the oven heat up time was 7 min and the program had two extraction cycles with 5 min static time and a flush volume of 70%. The soil samples were extracted twice and the extracts combined and preconcentrated to <2 mL under a gentle stream of nitrogen and transferred quantitatively to a 5 mL volumetric flask. After that, 200 μL of the injection spike solution was added and Isooctane added to the mark.

TPH concentrations were measured using an Agilent 6890 gas chromatograph equipped with a flame ionization detector. Hydrogen was used as the carrier gas at a flow rate of 3.0 mL min^{-1}, and 1 μL aliquots were injected in splitless mode. The injector temperature was 325 °C and the oven temperature program was 45 °C (held for 2.5 min) then increased to 320 °C with 20 °C min^{-1} (held for 10 min) leading to a total analysis time of 26.5 min. A seven-point external calibration curve based on dilutions between 0.3 and 10 mg mL^{-1} weathered crude oil (25% by evaporation, Tehran oil refinery) was used for the quantification of TPH as well as three boiling point fractions of TPH: C_{10}–C_{25}, C_{25}–C_{35} and >C_{35}.

The concentrations of the 19 target PAHs were analyzed using a gas chromatograph (Agilent 6890N) interfaced to an HP-5975B quadrupole mass spectrometer operating in electron ionization mode (Agilent 5975B). The gas chromatographs were equipped with a 40 m ZB-5 capillary column with special dimensions (0.18 mm id × 0.25 μm film thickness). Helium was used as the carrier gas, and the gas flow was 0.8 mL min^{-1}. Aliquots of 1 μL were injected in splitless mode. Injector, ion source, and quadrupole temperatures were 325, 230, and 150 °C, respectively. The oven program was: 35 °C (held for 3 min), increased to 100 °C (25 °C min^{-1}), then to 247 °C (5 °C $min^{-1)}$ and to 320 °C with the rate of 3 °C min^{-1} (held for 10.67 min) leading to a total analysis time of 70 min. Selected ion monitoring was used to analyze 21 *m*/*z* values in the range of *m*/*z* 128–288, divided into eight groups with 2–4 ions in each. Two six-point internal calibration curves (high and low concentrations) were used for quantification of the 19 PAHs. Concentrations were corrected for recovery.

2.8. Statistical analysis

A randomized complete design in a factorial scheme was implemented with two plants, two levels of endophytes (E^+, E^-) and three replications. Analysis of variance procedure (one way ANOVA) for all treatments was conducted using the SAS program (Release 9.1). The difference between specific pairs of mean was identified using Tukey test (P <0.05).

3. Results and discussion

3.1. Seed germination and plant growth in the contaminated soil

The infection percentage of inoculated plants with endophytic fungi was 100% while, no infection was detected in E^- plants. Results showed that regardless of endophyte infection, oil

contamination of the soil had a negative effect on seed germination of *F. arundinacea* and *F. pratensis*. Seed germination for all plants (E^+, E^-) was about 60% after 4 weeks, while a 13-d delay of seed germination in contaminated soil was observed for both grasses regardless of endophyte infection (Fig. 1). The plant seeds usually start to germinate at 4–6 d after sowing in non-contaminated soils (Besalatpour et al., 2008). Huang et al. (2004b) showed that existence of 2 g kg^{-1} creosote in soil could decrease germination of tall fescue (<50%) and it was delayed for 19 d. Besalatpour et al. (2008) reported that petroleum hydrocarbons in the soil decreased seed germination of tall fescue more than 50%. Petroleum hydrocarbons may decrease soil and nutrient availability to the seeds for germination and also have inhibitory effect on germination by physically impeding water and oxygen transfer between the seed and the surrounding soil environment (Adam and Duncan, 2002).

Endophyte infected plants showed higher root and shoot biomass than non-infected plants after 7 months growth in contaminated soil, but there was no significant difference in root and shoot biomass between the two plant species (Fig. 2). Total biomass of endophyte infected plants including root and shoot was 1.5 times higher than of non-infected plants. Endophyte infection could increase plant growth of grasses such as tall fescue (*F. arundinacea*) and perennial ryegrass (*Lolium perenne*) and it may be due to physiological response of the grasses from an increase in endogenous levels of plant hormones, which may be an additional effect of the fungal infection (Kuldau and Bacon, 2008). Although, the plants were grown in highly oil-contaminated soil, E^+ plants still produced more biomass than E^- plants. The results demonstrate that endophyte infection can increase plants ability to grow in oil-contaminated soils. Endophyte infected plants have shown to be more tolerant towards biotic and abiotic stresses than do non-infected plants (Malinowski and Belesky, 2000; Soleimani et al., 2010). However, to our knowledge this is the first report showing endophyte infected plants produce more biomass than non-infected ones in soils contaminated with petroleum hydrocarbons. It may be due to the effects of rhizosphere on the bioavailability and phytotoxicity of pollutants and release of secondary metabolites such as phenolic-like compounds into the rhizosphere which can act as plant defense metabolites to biotic and abiotic stresses (Malinowski and Belesky, 2000; Schulz et al., 2002; Wenzel, 2009; Soleimani et al., 2010).

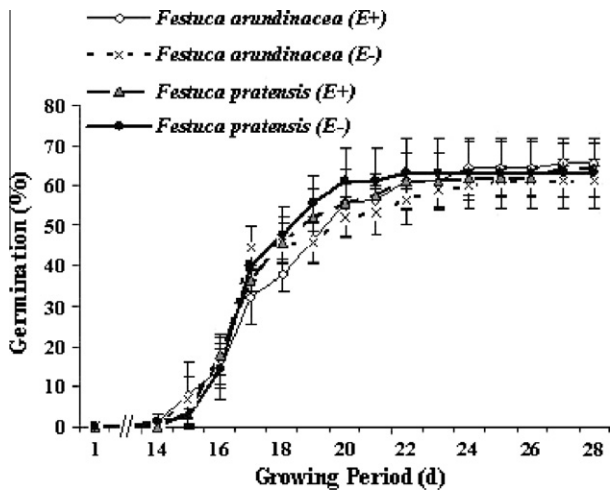

Fig. 1. Seed Germination rate of *F. arundinacea* and *F. pratensis*, infected (E^+) and non-infected (E^-) with endophytic fungi in petroleum contaminated soil during 4 weeks. Error bars are standard deviations.

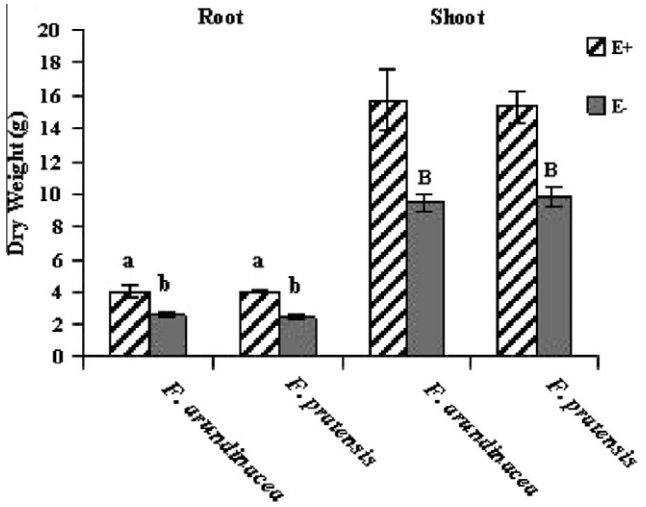

Fig. 2. Biomass of root and shoot of *F. arundinacea* and *F. pratensis*, infected (E^+) and non-infected (E^-) with endophytic fungi after 7 months growth in petroleum contaminated soil. Different letters (capital letters for shoot and small letters for root) represent significant differences with Tukey test ($P < 0.05$). Error bars are standard deviations.

3.2. Microbial activity and water-soluble phenols in rhizosphere of plants

At the end of the experiment, total and oil-degrading bacterial counts in planted soils were 16–20 and 6–7 times higher than those found in control treatments with no plants (Table 1). However, significant differences were neither found between the two grass species nor between E^- and E^+ treatments. The growth of microorganisms was stimulated by presence of the plant roots and the presence of endophytes did not adversely affect bacterial populations in the rhizosphere. Plants can generally promote soil microbial activity through the release of organic compounds from the root system, e.g. amino acids, organic acids, sugars, enzymes and carbohydrates, which provide carbon source and energy for microbial growth (Van Hecke et al., 2005). Despite of stimulation of microbial community growth due to release of carbohydrates and phenolic-like compounds from the roots of E^+ plants (Van Hecke et al., 2005), some of the rhizodeposits which are mostly found in the E^+ plants can act as microbial growth inhibitors (Firáková et al., 2007). Hence, there was no significant difference of bacterial population between E^+ and E^- plants.

Significant difference ($P < 0.05$) of dehydrogenase activity in planted and control treatments (Table 1) also revealed that microbial abundance and activity were increased by the presence of plant roots. There was a significant difference ($P < 0.05$) between dehydrogenase activity in the rhizosphere of E^+ and E^- plants, whereas it was insignificant for the two plant species (Table 1). The measurement of dehydrogenase activity provides an overall indication of activity of various microbial communities including bacteria, fungi and algae (Sandrin et al., 2009), which may have a role in degradation of organic pollutants in the soil. Oil degradation was more related to the dehydrogenase activity than the number of oil-degrading bacteria. This was in accordance with the results of Bento et al. (2003) for diesel oil. These results indicate that dehydrogenase activity is a suitable method for measurement of soil microbial activity in the presence of E^+ and E^- plants.

Plant growth increased dehydrogenase activity in the soil up to 12–23 times. Endophyte infection of plants increased this parameter up to 1.7 times, which may have been caused by an increase in root exudates such as water-soluble phenols (Table 1). Endophytic fungi in grasses such as tall fescue could enhance release of phenolic-like

Table 1
Water-soluble phenols content, dehydrogenase activity, total and oil-degrading bacterial counts in the soil planted with *F. arundinacea* and *F. pratensis*, infected (E^+) and non-infected (E^-) with endophytic fungi and control after 210 d (Control $_{T210}$) growth in petroleum contaminated soil. Data are mean ± standard deviation.

	Control $_{T210}$	*F. arundinacea*		*F. pratensis*	
		E^+	E^-	E^+	E^-
Water-soluble phenols (μg vanillic acid g^{-1} dry soil)	4.3 ± 0.2[cA]	8.2 ± 0.1[a]	6.7 ± 0.2[b]	8.1 ± 0.2[a]	7.0 ± 0.1[b]
Dehydrogenase activity (μg TPF g^{-1} dry soil)	19 ± 4[c]	394 ± 38[a]	234 ± 24[b]	427 ± 17[a]	242 ± 21[b]
Total bacterial count (CFU[B] × 10^8 g^{-1} dry soil)	0.6 ± 0.1[b]	11 ± 1[a]	10 ± 2[a]	12 ± 2[a]	11 ± 2[a]
Oil-degrading bacterial count (CFU × 10^4 g^{-1} dry soil)	2.3 ± 0.5[b]	15 ± 3[a]	16 ± 3[a]	14 ± 3[a]	14 ± 3[a]

[A] Different letters in each row represent significant differences with Tukey test ($P < 0.05$).
[B] Colony forming unit.

compounds and other secondary metabolites into the rhizosphere which can act as plant defense metabolites to biotic and abiotic stresses (Malinowski and Belesky, 2000; Schulz et al., 2002; Soleimani et al., 2010). It has been reported that production of phenolic compounds through the shikimate and acetate pathways tended to be enhanced by environmental stresses like the presence of PAHs in the soil (Siqueira et al., 1991; Lee et al., 2008; Cheema et al., 2009). The changes in rhizodeposition (e.g. organic carbons and carbohydrates) associated with endophytic fungi could enhance microbial activity and mineralization processes in the soil (Van Hecke et al., 2005) and resulted in more dehydrogenase activity in E^+ plants in comparison to E^- plants. There was also a considerable amount of water-soluble phenols in the control treatment without plants (Table 1). This could be the result of aromatic ring cleavage of PAHs while, in planted soil, phenolic compounds derive from two sources; degradative intermediates of PAHs and plant root exudates (Lee et al., 2008; Cheema et al., 2009).

3.3. PAH degradation in soil

Among the 19 PAHs, naphthalene, acenaphthene, acenaphthylene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, perylene and indeno(1,2,3-c,d)pyrene were not detected in the tested soils. PAHs with 2 and 3 aromatic rings (viz. flourene, dibenzothiophene, phenanthrene and anthracene) and also a 5-ring PAH (dibenzo(a,h)anthracene) were found in low concentrations in the soil (<100 μg kg^{-1}). The soil contents of PAHs with 4–6 aromatic rings were up to 0.2–3.1 mg kg^{-1} (Table 2). The main reasons for low concentrations of PAHs with 2 and 3 aromatic rings are most likely evaporation, photooxidation and biological degradation during a long time in the aged tested soil (Huang et al., 2004a). Removal efficiency of PAHs was enhanced in planted soils in comparison to unplanted soils (Table 2) which revealed the enhancing effect of plants in degradation of these compounds. There was no significant difference between PAH removal in the rhizosphere neither between the two plant species nor between E^+ and E^- plants. It might be due to low initial concentrations of PAHs in the soil. Due to degradation of most of the PAHs in the rhizosphere of E^+ and E^- plants after 7 months, decreasing the time scale would reveal the effect of endophytic fungi on degradation of these compounds. The lowest degree of degradation was observed for benzo(g,h,i)perylen (a 6-ring PAH). Since high-molecular-weight PAHs do not serve as a carbon and energy source for microbial populations during degradation, benzo(g,h,i)perylene could be removed in the presence of plant root exudates through the co-metabolism mechanism (Haritash and Kaushik, 2009).

Regardless of endophyte infection and type of plant species, the presence of plants in the soil enhanced total PAH (∑PAHs) degradation as well as degradation of 2–5 ring PAHs (Table 2). The loss of PAHs from soil and sediment could be due to biotransformation, biodegradation, plant uptake, or abiotic dissipation, including leaching and volatilization. Abiotic losses by leaching were insignificant because the water content of the soil was maintained at about 75% of the field capacity, thus no leachate was produced during the experiment. The losses of high-molecular-weight PAHs via volatilization from soil were also unlikely to occur due to their low vapor pressure. The loss of PAHs from soil by plant uptake/accumulation can be assumed to be negligible, due to no detection of these compounds in most plant materials (and/or detection of

Table 2
Degradation of PAHs and TPH in the soil planted with *F. arundinacea* and *F. pratensis*, infected (E^+) and non-infected (E^-) with endophytic fungi and control after 210 d (control$_{T210}$). Data are mean ± standard deviation.

Compound (total rings number)	Control$_{T0}$ (mg kg^{-1})		*F. arundinacea*		*F. pratensis*	
		Control $_{T210}$	E^+	E^-	E^+	E^-
		% Remediation				
Fluorene (3)	0.012 ± 0.001	30 ± 1[bA]	100[a]	100[a]	100[a]	100[a]
Dibenzothiophene (3)	0.061 ± 0.008	13 ± 7[b]	100[a]	100[a]	100[a]	100[a]
Phenanthrene (3)	0.032 ± 0.009	35 ± 5[b]	100[a]	100[a]	100[a]	100[a]
Anthracene (3)	0.071 ± 0.009	37 ± 3[b]	65 ± 1[a]	63 ± 2[a]	66 ± 2[a]	58 ± 8[a]
Fluoranthene (4)	0.230 ± 0.096	22 ± 5[b]	80 ± 2[a]	81 ± 2[a]	84 ± 0.1[a]	83 ± 6[a]
Pyrene (4)	3.095 ± 0.446	73 ± 20[b]	93 ± 3[a]	94 ± 2[a]	97 ± 0.3[a]	96 ± 4[a]
Chrysene (4)	2.716 ± 0.522	78 ± 5[b]	91 ± 3[a]	92 ± 3[a]	96 ± 0.4[a]	93 ± 8[a]
Benzo(e)pyrene (5)	0.764 ± 0.029	25 ± 10[b]	73 ± 2[a]	73 ± 2[a]	81 ± 1[a]	74 ± 13[a]
Dibenzo(a,h)anthracene (5)	0.033 ± 0.006	6 ± 2[b]	100[a]	100[a]	100[a]	100[a]
Benzo(g,h,i)perylene (6)	1.527 ± 0.014	10 ± 0.2[b]	36 ± 3[a]	35 ± 1[a]	36 ± 2[a]	32 ± 3[a]
∑PAHs	8.542 ± 0.912	56 ± 11[b]	84 ± 1[a]	81 ± 8[a]	79 ± 2[a]	80 ± 2[a]
n-alkanes [C_{10}–C_{25}]	21 300 ± 1400	37 ± 5[d]	69 ± 0.5[ab]	60 ± 2[c]	70 ± 0.5[a]	61 ± 3[bc]
n-alkanes [C_{25}–C_{35}]	24 700 ± 500	28 ± 6[b]	75 ± 2[a]	69 ± 2[a]	74 ± 1[a]	68 ± 4[a]
n-alkanes [>C_{35}]	1500 ± 300	1.3 ± 0.0[b]	4.7 ± 0.2[a]	3.7 ± 0.6[a]	4.6 ± 0.5[a]	3.2 ± 0.9[a]
TPH	47 400 ± 2100	31 ± 5[c]	72 ± 1[a]	64 ± 2[b]	72 ± 0.1[a]	64 ± 0.3[b]

[A] Different letters in each row represent significant differences with Tukey test ($P < 0.05$).

some of the 3 ring PAHs in very low concentrations in some of the plant roots in comparison to those lost from the soil) (contents in plants not shown). Furthermore, most PAHs cannot move appreciably into plants from the soil due to a log $K_{OW} > 4$, i.e. equilibrium constant that provide an indication of constituent sorption onto soil, and remediation depends almost totally on degradation in the rhizosphere (Alkorta and Garbisu, 2001; Newman and Reynolds, 2004). Therefore, enhancement of PAH removal might be caused by an increase in the microbial activity of oil-degrading bacteria and degradation mediated by plant-secreted enzymes in the rhizosphere compared to that in unplanted soil. The data obtained from microbial counts and dehydrogenase activity (Table 1) supported this idea.

Although, there was a significant difference in dehydrogenase activity in soils planted with E^+ compared to E^- plants, we did not see a significant difference of PAH degradation in those treatments where the compounds were mostly degraded. Also there were not significant differences in PAH degradation in the rhizosphere of *F. arundinacea* and *F. pratensis* which showed similar biomass production and also similar microbial activity in their rhizosphere. However, further investigation considering higher initial PAH concentrations and also various time scales in the presence of E^+ and E^- plants should be addressed to identify the effects of endophytic fungi on remediation of these compounds.

3.4. TPH degradation in soil

Degradation of petroleum hydrocarbons including three subsets of TPH (C_{10}–C_{25}, C_{25}–C_{35} and $>C_{35}$) and TPH increased in the presence of plant roots in the soil after 7 months. The removal of C_{10}–C_{25}, C_{25}–C_{35} and $>C_{35}$ fractions and TPH were 60–70, 68–75, 3–5 and 64–72% respectively in planted soil, and 37, 28, 1 and 31% in control treatments (Table 2). There was no significant difference between degradation of TPH and the three fractions in the rhizosphere of the two plant species while, E^+ plants in comparison to E^- plants showed more capability to enhance removal of C_{10}–C_{25} fraction and TPH in the soil (Table 2). It may be due to more root biomass and root surface area which increased secretion of microbial enhancing metabolites such as water-soluble phenols and therefore stimulated microbial activity in the soil. Oxidation of alkanes in the soil depends on enzyme classes which are mostly related to the type of oil-degrading bacteria (Van Beilen et al., 2003). More extensive degradation of alkanes with shorter chains (C_{10}–C_{25}) than alkanes with longer chains (C_{25}–C_{35} and $>C_{35}$) in the rhizosphere of E^+ plants might be due to changes in the bacterial community structure with special enzyme class(es) to oxidize these compounds (Van Beilen et al., 2003). It has been reported that endophytic fungi could enhance rhizodeposition of plants and consequently influence microbial mineralization processes in the soil considering no effect on the basic structure of the microbial community (Van Hecke et al., 2005). However, according to our knowledge there is no report claiming increase of degradation of hydrocarbons in the rhizosphere of E^+ plants. Regardless of endophyte infection TPH fractions C_{10}–C_{25} and C_{25}–C_{35} chain lengths were the preferred substrates compared to alkanes with $>C_{35}$ chain lengths. Alkanes with shorter chain length are most readily degradable and could be rather more toxic than long-chain alkanes (Balba et al., 1998). Alkanes with long chain lengths are hydrophobic solids and consequently are difficult to degrade due to their poor water solubility and bioavailability (Balba et al., 1998). However, regardless of the effect of endophyte infected plants, our finding about enhancing degradation of TPH in planted soils were in line with other reports (Merkl et al., 2005; Kaimi et al., 2007; Euliss et al., 2008).

4. Conclusions

Growth of *F. arundinacea* and *F. pratensis*, infected and non-infected with endophytic fungi, could enhance dissipation of PAHs and TPH in the aged contaminated soil with no significant differences between the two plant species. Planting increased the number of total and oil-degrading bacteria, dehydrogenase activity and content of water-soluble phenols in the soil. Endophyte infected plants showed more capability to stimulate degradation of TPHs in the C_{10}–C_{25} fraction and TPH in the rhizosphere than non-infected counterparts. This observation might be due to enhancement of microbial degradation which could be a result of more root biomass and release of more water-soluble phenols into the soil. Increasing water-soluble phenols in the presence of E^+ plants may enhance activity of microbial communities with certain enzyme class(es) to degrade C_{10}–C_{25} chain length alkanes. However, further detailed investigations should be addressed.

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