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Research article

Expression of Bacterial *nir* and *nar* genes during phytoremediation of soils oil polluted

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ABSTRACT
Due to oil industry development, petroleum pollution is considered as one of the most important environmental pollutants. Iran is the world's fifth oil producer and due to remediation of petroleum hydrophobic is important. Since the hydrophobic nature of petroleum hydrocarbons and aging phenomenon, soil remediation of petroleum hydrocarbons was complicated than aqueous environment. Hence, the aim of this study was investigating the role of plant to remove of petroleum contaminants in soil. According this, petroleum contaminated soil were planted by vetiver. After plant growth, the mobility and degradation of aged-petroleum hydrocarbons were investigated in soil. The results showed that TPH degradation was increased under vetiver cultivation. The expression of *nir* and *nar* gene as the genes in nitrogen cycle genes were increased in the presence of plant roots.

Keywords:*nir* and *nar* gene, petroleum pollution,phytoremediation

INTRODUCTION

Soil pollution by crude oil and its derivatives is considered among the most dangerous types of environmental pollution. Due to the increasing growth of the oil industry and side industries which has resulted generation, transmission, and storage of these materials, categorizes various petroleum hydrocarbons in the first row of environmental pollutants in Iran. Widespread soil pollution by oil hydrocarbonate materials is visible around oil installations and locally in the transfer routes of these substances. According to the negative and dangerous effects of oil pollution in the environment, clearing these pollutantsalways is one of the raised concerns in the field of environment. All petroleum hydrocarbons mainly have been made of hydrogen and carbon ratio of two hydrogen atoms to a carbon atom. So they called hydrocarbons (Okoh, 2006).Crude oil contains a wide range of hydrocarbons such as hydrocarbons with one to one hundred carbon atoms and oil composition differences between different regions originate from differences in the type and molecular weight components. In addition to the hydrocarbon compounds in crude oil, also non-hydrocarbon organic compounds are found in samples of crude oil which have a high boiling point (Speight, 1991).Crude oil and oil pollutants can effect on biological properties of soil directly to the cause of oil Toxicity and indirectly due to physical and chemical changes imposed by the viscous fluid. Oil pollution stress can be leaded to water absorption and gas exchange efficiency (Baker, 1970). The first effects that influence the spread of oil or natural gas on soil are replacing soil air by the aforementioned gases and occurrence of an anaerobic environment. The effects will ultimately lead to the destruction of microorganisms and vegetation. According to collected reports by Robertson *et al*. (2007), after adding petroleum hydrocarbons (PHCs) to soil microbial density is decreased immediately. Hydrophobic property ofoil pollutants effects on

diffusion of water and solutes and oxygen in soil. The impact leads to many of the effects of oil pollution on plants and soil organisms. Soil fertility capacity is the highest level of microbial activity that will depend on soil conditions. These conditions include microbial population size, oxygen, water temperature and nutrient content in the soil. This capacity may be increased due to relatively large amounts of carbon from the PHCs. Strong growth PHC oxidizing micro organisms in response to increasing carbon is associated with the consumption of soil nutrients, lead to reduce nutrient elements, especially nitrogen and phosphorus for plants (Van Hamme, 2003). Scott-Denton *et al*. (2005) were reported that 4.0 percent of the oil concentration in soil leads to unmoved Nitrogen. In addition to, Nitrogen loss can result from nitrification prevention in the soils. There aren`t notrificator bacteria in new and short-term contaminated soils. With the passage of time and ageingof the pollution, nitrification process is started again and movable or mineral nitrogen content of soil increases (Scott-Denton *et al*. 2005). In addition to reducing the efficiency of water and salts in the soil contaminated with oil due to its hydrophobic effect, oil pollution effects on nitrogen uptake negatively. High consumption of nitrogen in the soil by microbes leads to incidence of the element competition between plants and microbes that effect on oil degradation and plant growth. Inorganic nitrogen is unmoved (organic) while oil pollution prevents nitrification (Kucharski*et al*., 2010).It is concluded from sources and reports that oil pollution reduces soil microbial diversity (Robertson *et al*. 2007). Another important factor in soil is soil enzyme activity that there are reports of changes in enzyme activity in the presence of oil pollution. For example, according to report of Achuba*et al*. (2008) pollutants such as motor oil lead Reduction of catalase enzyme activity and increased activity of soil dehydrogenase. Phytoremediation is use of resistant plants for remediation and cleanup of contaminated soils in different combinations. This method is also known as green remediation. In this technique the plants is used for remediation of soil, sediments and even polluted ground and surface water. Most studies have been done so far phytoremediation of heavy metals and there is very little research in the field of organic pollutants. Using plants to clean up soil from pollutants such as herbicides, Pesticides and Aromatic Hydrocarbons compounds has provided new features in order to better management of effluents and sewage. Phytoremediation depends on the nature and effects of the intensification of mutual zone of plants, microorganisms and the environment. Phytoremediation is defined in 5 main cause including Phytostabilization, Phytoextraction, Rhizofiltration, Phytotransformation and Rhizosphere Bioremediation. Due to the nature of petroleum hydrocarbons, plant deformations and biodegradation mechanisms of roots regain will be more important in clearing these compounds in the environment. Plant deformation includes Absorbing organic pollutants and polluters of soil, groundwater and transformation of plant (Haichar et *al*., 2008). This process depends on the direct absorption of pollutants from water and soil metabolites accumulate in the tissues of plants. Biodegradation in Rhizosphere (Rhizosphere Bioremediation, Rhizodegradation) also known as induction plants or cooperation plants in phytoremediation (Paul *et al*., 1996). Appropriate plant selection can improve the efficiency of phytoremediation operations. Plants growing in contaminated soils, are less affected by toxic pollutants are healthier and more resistant than other plants and they will be grow more by creating healthy root system (Tsao, 2003).Vetiver (*Vetiverzizanioides* L. Nash) is one of the plants for phytoremediation of polluted soils highly regarded today. Vetiver system is based on the use of Vetiver grass. At first, the system was developed by the World Bank to protect the soil and water in India in the mid-1980s. Vetiver due to the extraordinary characteristics now was used as a bio-engineering technique for stabilizing slopes, sewage disposal, phytoremediation of contaminated lands and waters and other environmental protection goals (Günther et al., 1996). Soil biological activities are sensitive to environmental stresses and any change in environmental conditions may lead to a shift in

species composition of soil micro flora and change the speed of the metabolic activities. In fact, soil biological activities can indirectly be reflecting soil conditions, especially of soil pollutants and even reflects of the soil clean up progress. The soil biological activities includes Carbon availability index (CAI), Soil respiration, Microbial biomass C (MBC), and metabolic quotient (qCO2), Lipase activity and Functional expression of the nitrogen cycle genes (Margesin et al., 2005). Soil nitrogen cycle genes are considered as functional genes, because they play active and crucial role in the cycling of major elements (N). Functional genes are useful indicators for evaluating performance under different conditions in soil nutrient cycling. Among these conditions can be said to soil pollution with petroleum hydrocarbons and organic materials. Nitrogen is a limiting factor decomposition of petroleum hydrocarbons. On the other hand functional genes of nitrogen cycle are sensitive to environmental conditions, in particular soil contaminants. The status investigating of genes expression in the soil is a good indicator to evaluate soil phytoremediation (Zhang et al., 2013). In general, it is concluded that Soil Microbiology indexes are helpful tools and low-cost for evaluation of bioremediation to assess progress and the biological variability of soil contaminated with petroleum hydrocarbons and the process of bioremediation. Since this study is important that environmental cleanup processes by using biological methods will be faced with cost and time. If executed performance of system is not high, it will be not economically effective. For this purpose it is necessary system or model is Optimized and adjusted. One of the optimization factors is Bioremediation process and cognitive behavioral adjustment nutrients. Nitrogen is the most important element at the forefront of attention. This research pays the expression of key genes in the nitrogen cycle in the soil. It is expected that plant roots as a driving factor in the soil increase the expression of such genes as a key factor in phytoremediation. The relative position of nitrogen behavior in contaminated soils can be realizedby studying the key genes in the nitrogen cycle and comparing it with data from chemical analysis of soil. In this study, *nir*and*nar* genes expressionand comparing with roots in soil cultivation and change activities is evaluated.

MATERIAL AND METHOD

Soil sampling and preparation of test material

Soil and sludge which have been collected from the following sites of Oil Pipeline and Telecommunication Company were transferred to laboratory. Soil pollution includes weathered heavy crude oil and sludge of oil products tanks. The Soils have been collected from pollution leakage from pipes and dredgers cases and have been accumulated safety capsules to prevent environmental pollution.

Evaluation of soil chemical characteristics before phytoremediation

100 grams samples were prepared from two under investigation soil types. The concentration of inorganic nitrogen (Kjeldahl), iron and manganese (atomic absorption method) were measured. After measuring the organic carbon content (oven dried digestion method) C / N ratio was determined. Moreover, some soil chemical properties were measured including electrical conductivity (EC) and pH. The Soxhlet method (Protocol No. 5520 EPA) was used for TPH measuring.

Biological and molecular study of soil before phytoremediation

Before phytoremediation soil conditions were studied biologically. For this purpose, DNA was extracted from soil. Two probes of index genes in the analysis of petroleum hydrocarbons were used for investigating the presence of microorganisms degrading Petroleum hydrocarbons in soil. Candidate Genes included Alkane mono-oxygenase gene (*alk*B) and catechol 2-3 oxygenase gene (*xyl*E). These genes are present in many microorganisms degrading Petroleum hydrocarbons. So the presence of this genes in DNA extracted from soil reflects the presence of these bacteria in soil (Jussila*et al*., 2006).

Planting

Plan for phytoremediation of contaminated soil was a randomized complete block design of two types. After preparing the potting soils, plant cultivation was carried out. Two species of

herbaceous such as Pampas Grass (*CortaderiaSelloana*) and Vetiver (*Vetiveriazizonioides*) and two species of tree Willow (*Salix babylonica*) and Poplar (*Populusnigra*) were selected for planting. Finally, Vetiver was established and continuation of the experiment was performed with Vetiver Grass.

Measurement of soil biological indicators

Rhizosphere soil sampling was performed in ninth months (T1), twelfth (T2) and the fifteenth (T3) phytoremediation. Polluted soil testing was conducted using a factorial randomized complete block design. Independent effects of this experiment such as plant effect, Time to reduce concentrations of TPH and changes in soil biological indicators were studied as dependent variables.

a. Study of gene expression patterns (steady state level)

To investigate the expression pattern of nitrogen cycle genes in soil contaminated with petroleum hydrocarbons in response to the Phytoremediation (At the transcriptional level), RT-PCR was used. In this method, the total soil RNA was extracted using soil RNA extraction kit (Nor gene, Canada).Fragments of cDNA were synthesized from the mRNA by using Reverse transcriptase enzyme (Roche) and random primers small (Random Hexamer (dN6). The level of gene transcription was searched and amplified among mRNA sets by using gene-specific primers (Table 1). Strong PCR product bands in electrophoresis e reflect the level of transcription.

Table 1.The used primers in the PCR reaction

Gene noun	Primers	Size (bp)
16S rDNA	27F/1492R	1500
16S rDNA	GC clamp-341F/534R (DGGE)	250
narG	F: 5-TCGCCSATYCCGGCSATGTC-3 R: 5-GAGTTGTACCAGTCRGCSGAYTCSG-3	869
nirK	F: 5-GGMATGGTKCCSTGGCA-3 5R: 5-GCCTCGATCAGRTTRTGG-3	731
nirS	F: 5-CCTAYTGGCCRCART-3 R: 5-CGTTGAACTTRCCGGT-3	202

b. The amount of residual TPH

The Soxhlet method (Protocol No. 5520 EPA) was used to determine the amount of residual TPH in soil of pots.

STATISTICAL ANALYSIS

The data from each experiment, at first residual values were calculated by Minitab Software (version 16). Their normality was evaluated by Ryan-Joiner test at 5%. Levene test at 5% of the software was used To investigate the homogeneity of variance treatments. The results were analyzed by SAS 9 software and analysis table of variance (ANOVA) was prepared to study the effects. Means were compared by Fisher's LSD was at 5%. Finally averages of data with standard deviation (SD) were reported.

RESULTS

Chemical properties of experimental soil

Two soil samples were tested in this study. Some chemical properties of the soil type are shown in table 2. Since the C / N ratio was less than 30, extra nitrogen was not added to the soil to plant (nonstop N).

Table2.Some characteristics of two soil samples used in the experiment

Measured indexes	SoilM	SoilH
Total N (Kjeldahl%)	0.8	0.8
Organic C %	3.33	5.98
C/N	4.16	7.47
Fe (mg kg^{-1} soil)	24335	96162
Mg (mg kg^{-1} soil)	902	6307
pH (in 1:2.5 extract)	7.22	7.5

EC (dSm^{-1}; in 1:2.5 extract)	0.05	1.01
TPH (mg kg^{-1} soil)	3500	700
*alk*B[1]	+	+
*xyl*E[1]	+	+
Soil Texture	Silty clay loam	Silty clay loam
[1]Petroleum hydrocarbons degrading genes		

Soil microbiological studies

a. Functional genes expression of nitrogen cycle

The concentration of RNA extracted from soil samples and concentration of synthesized cDNA from the RNA are shown in table 3 and 4 respectively.Although Nor gene kit has been used for RNA extraction, 230/260 ratio of RNA samples was less than 1. It represents impurities of humic acids. However, there were no problems in the construction of cDNA from the extracted DNA.The RNA was used at a concentration equal to cDNA synthesis in the reverse transcriptase reaction.Finally same concentration of cDNA was used in the final PCR reaction.Expression of housekeeping (16S) gene in cDNA synthesis after the concentration of the cDNA is shown in figure1. Expression of *nir*and*nar*genes was increased under cultivation of Vetiver increased in both soil (figures 2 to 4).

Table 3. Extracted RNA from soil

Samples	RNA (ng/µl)	RNA (Mean)	230/260	260/280
M1	24.0	31.5	0.81	1.28
M2	32.0		0.71	1.33
M3	37.6		0.64	1.2
CM1	15.7	17.6	0.745	1.34
CM2	20.2		0.86	1.08
CM3	17.0		0.73	1.2
H1	39.6	39.8	0.96	1.76
H2	34.8		0.83	1.56
H3	45.0		0.95	1.30
CH1	20.0	16.8	0.80	1.24
CH2	12.9		0.83	1.35
CH3	17.6		0.84	1.29

Table 4.cDNA concentration in micrograms of RNA

Samples	cDNA concentration(ng/µl)
H1	377
H2	356.6
CH1	357.2
CH2	342.2
M1	351.5
M2	346.6
CM1	351.5
CM2	349.3

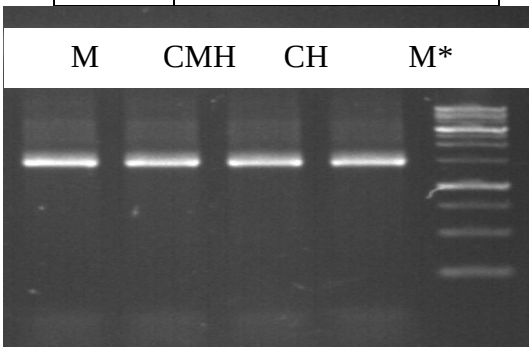

Figure1. Electrophoresis of RT-PCR reaction products Housekeeping bacterial ribosome gene (bp 1500), M* 1kb Ladder.

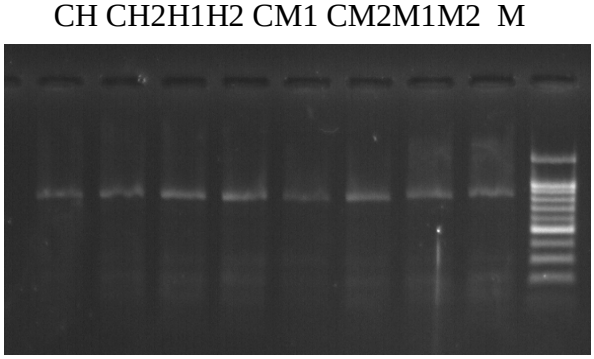

Figure 2.Electrophoresis *nir*S the bacterial ribosome gene RT-PCR reaction products (1500 bp), M 100 bp Ladder.

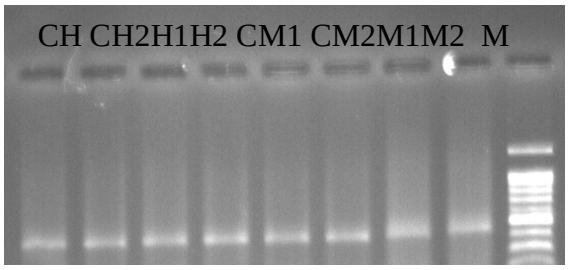

Figure 3.Electrophoresis *nir*K the bacterial ribosome gene RT-PCR reaction products (1500 bp), M 100 bp Ladder.

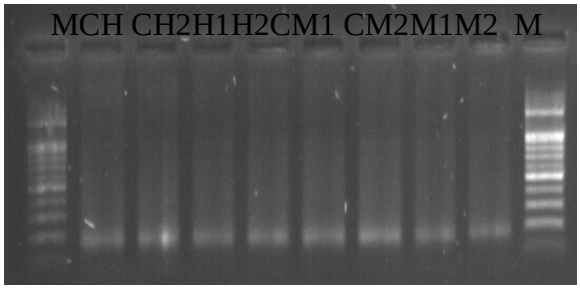

Figure 4.Electrophoresis *nar*G the bacterial ribosome gene RT-PCR reaction products (1500 bp), M 100 bp Ladder.

b. Residual TPH

Phytoremediation interaction effect over time on the amount of residual TPH in both soil were significant.The amount of TPH in soil M at each measurement showed significant reduction in both soil and soil residual TPH decreased over time was observed. The amount of TPH in soil M at each measurement showed significant reduction but reduction was significant in soil CM just in time T3.The amount of residual TPH in soil M was lower in all three time soil CM (Diagram 1).

Also amount of remaining TPH in soil H decreased over time.This difference was significant over time. While the unplanted control samples (CH) reduce the amount of residual TPH but There was no significant change since T2.The amount of residual TPH in soil H was less than CH in all the times (Diagram 1).

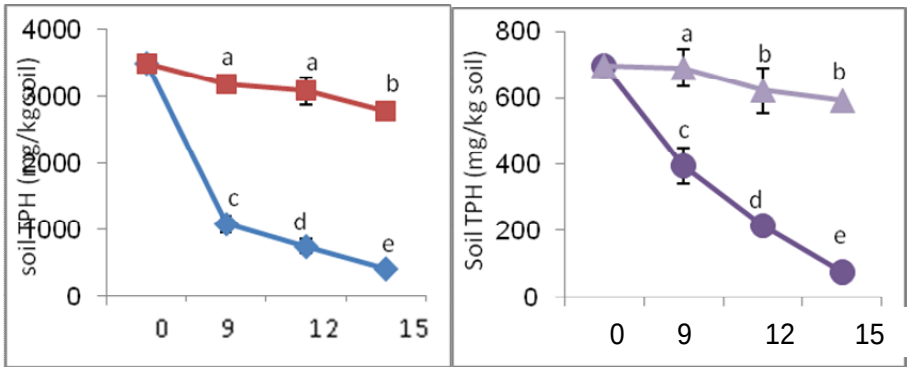

Diagram 1.Phytoremediation treatment effect over time on the soil TPH, ▲CH ● H♦ CM ■ M.

DISCUSSION

Gene expression of nitrogen cycle

Nitrogen cycle is one of the key functions of soil in bioremediation of petroleum hydrocarbons. Usually nitrogen limitation is considered as a key factor influencing Bioremediation is an organic compounds. Optimum carbon to nitrogen ratio (C / N) is recommended for 10: 1 decomposition (Yergeau*et al.*, 2012). There are few studies on the abundance and activity of nitrogen cycle genes in oil contaminated soil and especially during the phytoremediation. Based on the results obtained in research by Bin *et al*. (2014), frequency of key bacteria of nitrogen cycle is sharply reduced on the effect of petroleum hydrocarbons pollution and they were also banned from activities. Bioremediation of polluted soil in this case, frequency of bacteria and expression of *nar*G gene significantly increased. The results showed that Genes of nitrogen cycle are a good indicator to assess improvement, quality and soil health, especially in oil-contaminated soils and Evaluation of phytoremediation progress. In this study,*nir*K and *nir*S genes expression increased under Vetiver cultivation in both soil increased. Additionally, increase of *nar*G gene expression was observed to a certain extent by Vetiver cultivation.

Residual TPH in soil

Pena-Castro *et al*. (2006) Showed that Phytoremediation is useful, especially with using of family Poasea grass in removing of oil pollutants. Such that, soil toxicity decrease at the end of phytoremediation. In addition, the enrichment of bacteria in the soil adjacent to the roots of grasses that is responsible for the analysis of mass Pollution, both in number and in selecting decomposers genotypes of root can stimulate the degradation of pollutants. In the present study in both Vetiver cultivated soils, residual TPH in soil is reduced over time. When the decomposition was more in the presence of plants, it seems that plant roots in the soil could provide the conditions that lead to stimulate the decomposition of these compounds.Liste*et al*. (2006) showed that plant roots release aromatic acids and surfactant phospholipids whichis more significant than amount of the microbial production and they are able to increase their mobility and bioavailability of organic pollutants. Phytoremediation is perfect tool to remove organic compounds such as PAH See sequester resistant. In accordance with these findings, the results of numerous studies show an increase in pollutants decomposition of hydrocarbons in the presence of plants.Eulis*set al*. (2008) reported that TPH level of deposits decreased 70% after one year of planting grass. Meanwhile, the cultivation of plants such as poplar and willow only 20% was observed. The results of studies, such as Li and Banks (1993), Schwab and Banks (1994) and Qiu*et al*. (2008) showed that decomposition of condition of the lesion is oil in the plant faster. Based on the results, similar by Hui et al. (2007), Vetiver cultivation in oil contaminated soil increases the soil TPH decomposition. The Vetiver roots in of contaminated soil hydrocarbons concentration in solution increased soil water. This phenomenon can be attributed to the role of root exudates. Its concentration in soil water will be decreased over time by decomposition of TPH in Soil.

CONCLUSION

One of the indicators which represent improvement in the rhizosphere microbial activity is increasing of genes expression in nitrogen cycle as one of the key functions of soil. Increased expression of these genes represents the positive role of plant in the recirculation elements in soil and it indicates that this cycle activates is essential element in the soil. Finally, Vetiver cultivation in the oil contaminated soil causes increasing soil TPH decomposition. Regarding the role of roots in improving the bioavailability of petroleum hydrocarbons, also carbon availability index increased in presence of plant roots. High growth of this plant can cause a large amount of photo assimilate in soil. They are suitable substrates for microbial activity. In addition to it increases bioavailability of petroleum compounds, volume of water, the mobility of pollutants and microorganisms in the soil root zone and quickly decomposes pollutants. In general, the use of phytoremediation by a suitable plant with the root mass is an

appropriate method for remediation of contaminated soil.

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