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Biotreatment of Aliphatic and Aromatic Fractions of Crude Oil-contaminated Water by Oil-degrading Bacterial Consortium

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ABSTRACT

Contamination of water by oil or its by-products can damage the environment. Some microorganisms use oil as the sole carbon source and degrade it to simple and non-toxic products. Bioremediation is an economic and efficient method to decrease oil contamination. In this report, bioremediation of aliphatic and aromatic fractions of crude oil in oil-contaminated water obtained from a water well near a Tehran refinery by a bacterial consortium was studied. The reduction of total crude oil, aliphatic and aromatic reduction was 28, 60 and 35% respectively after 96 h. Most aliphatic reduction occurred between C_{14} and C_{24}. The reduction of phenanthrene, anthracene, fluoranthene, pyrene and chrysene was studied. The highest reduction was observed in fluoranthene (43%) and phenanthrene (35%). Our results show that the bacterial consortium had a high potential to remove oil from contaminated water in a short period of time.

Keywords: biodegradation, contaminated water, microorganism, petroleum, pollution

INTRODUCTION

In oil-producing countries, in areas of oil extraction and oil processing, crude oil is one of the main soil and water contaminants. The leakage of crude oil from pipelines and refineries damages the environment. Various bacteria and fungi are able to use crude oil fractions as a sole carbon source and change them to non-toxic compounds such as CO_2 (Leahy and Colwell 1990; Cerniglia 1992; Hamamura *et al.* 2006). The aliphatic and some aromatic fractions in crude oil are the most biodegradable components, and resins and asphalts are believed to be resistant to biodegradation (Atlas 1981; Leahy and Colwell 1990). Some reports have shown that aliphatic and aromatic fractions of crude oil are efficiently biodegraded by oil-degrading bacteria in soil (Minai-Tehrani and Herfatmanesh 2007). In aerobic conditions oxygen plays an important role in the biodegradation of oil and its components (Odu 1978).

Water contamination by oil is harmful to humans. It can damage drinking water and also penetrate surface and subsurface water and damage cultivation areas and plants. Li *et al*. (2005) showed that about 32% of chemical oxygen demand (COD) was reduced in biodegradation of organic compounds in oil-contaminated waste water by bacterial treatment after 4 days. Some factors such as nutrients play an important role in bacterial growth and biodegradation of oil-contaminated water (Atlas 1992). The bacterial population can also enhance the efficiency of biodegradation (Obahiagbon and Akhabue 2009). The saturated fraction of crude oil-contaminated water was the main biodegradable part and the asphaltene fraction was hardly biodegraded (Thouand *et al.* 1999).

In this report the effect of oil-degrading bacteria isolated from contaminated soil near a refinery in Tehran on bioremediation of aliphatic and aromatic fractions of crude oil in liquid medium was studied. This study can be useful to clean up oil-contaminated water and promote a healthier environment.

MATERIALS AND METHODS

Topographic position of contaminated site

There is an old oil refinery in the south of Tehran and around the refinery there are many villages and cultivation areas. The drinking water and water for cultivation are provided from well water. In the last decade, some samplings from the wells' water demonstrated a high contamination of water by oil (Mesdaghinia *et al.* 2005; Safavi *et al.* 2005). This contamination affects agricultural products as well as the health of the population. In recent years, the Environmental Protection Agency of Iran has pushed the refining industry to find and fix leakages sites and clean surface and underground waters. Predominantly, their priority is to eliminate pollution from contaminated waters by biodegradation.

For this study oil-contaminated water was obtained from a well that was chosen randomly in the Tehran refinery area. The sample was collected in a sterilized flask and transferred to the laboratory at 4°C for further study.

Culture medium preparation

Analysis of the elements in the water sample (**Table 1**) shows that the inorganic elements were not available in sufficient quantities

Table 1 Characteristics of oil-contaminated water used for microcosm study.

EC mV	25.5
DO mg/L	6.2
Chloride ppm	280
Alkanity ppm	215
Hardness ppm	510
pH	7.3
TPH[a] %	2.3
Aromatics %	0.6
Aliphatics %	1.5
Asphaltine %	0.11

[a] TPH = total petroleum hydrocarbons

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for acceptable growth of microorganisms, hence some inorganic nutrients such as 1 g KH_2PO_4, 1 g Na_2HPO_4 and 0.25 g NH_4NO_3 were added to 1 L of water and sterilized to induce a suitable medium for their growth.

All chemicals were of reagent grade and obtained from Merck Co.

Isolation of bacterial consortium

The oil-degrading bacteria were isolated from the soil near the site where water was sampled according to the following procedure. 1 g of oil-contaminated soil was added to mineral salt liquid medium containing 2.5 g/L KH_2PO_4, 2.5 g/L Na_2HPO_4, 1 g/L NH_4NO_3, 0.2 g/L $MgSO_4$ and 0.01 g/L $CaCO_3$; pH was adjusted to 7 and 2% (v/v) sterilized crude oil was added to this medium as the only carbon source. The medium was cultured for a week in a reciprocal shaker, 150 rpm at 30°C. After 1 week, 2 ml of cultured medium suspension was transferred to new medium under the same conditions. This transfer was repeated twice and the bacteria in the last transfer were used for inoculating the water microcosm. To ensure the presence of oil-degrading bacteria, the growing bacteria in the last medium culture were also transferred to a plate with solid medium, containing agar-agar enriched with 0.5% hexadecane as the carbon source and incubated for 72 h at 30°C. The growing colonies were considered to be oil-degrading bacteria.

Oil extraction and analysis

About 2 ml of cell culture (with 0.25 McFarland turbidity or about 7.5 $\times 10^7$ CFU/ml) of the last liquid medium were transferred to contaminated water obtained from the contaminated site. The dispersion of oil was observed in the medium which suggested the ability of bacteria to degrade oil. The bacteria were cultured for 96 h in a reciprocal shaker, 150 rpm at 30°C. After 96 h, the medium was centrifuged (5000 × *g* for 30 min) to precipitate the cells and the supernatant was separated for oil analysis. The supernatant was mixed with an equal volume of chloroform and transferred to a separating funnel. The suspension was shaken firmly to dissolve the oil fraction of supernatant in the solvent phase. The solvent phase was separated and evaporated in a vacuum chamber for 24 h. The residue was weighed to determine the amount of total crude oil. The total aliphatic and aromatic fractions of oil were extracted and analyzed according to Minoui and Minai-Tehrani (2009). In this method the residue was dissolved in *n*-hexane and filtered. 5 ml of filtered solution was loaded to a 1 × 25 cm glass column filled with silica gel (20 cm) and sodium sulfate (5 cm as a moisture capturing material). The column was pre-washed with *n*-hexane and 30 ml of *n*-hexane was used as the mobile phase to release the aliphatic fraction. The fraction was collected and the solvent was evaporated. The residue was weighed to determine the amount of total aliphatic fractions. The residue was dissolved in 150 µl *n*-hexane, and 1 µl was injected into a gas chromatograph (Hewlett-Packard GC 5890) column equipped with an FID detector and a fused silica capillary column. The carrier gas was H_2 with a flow rate of 60 cm/sec and the injection temperature was 300°C and that of the detector 330°C. The total peak areas of the hydrocarbons were taken as a quantitative amount of their concentrations and compared with time zero (T_0).

To release aromatic fractions from the column, 30 ml of *n*-hexane/dichloromethane (1: 1, v/v) was used as the mobile phase. The aromatic fractions were collected and the solvent was evaporated. The residue was weighed to determine the amount of total aromatic fractions. The residue was dissolved in 5 ml acetonitrile and 20 µl was injected into a HPLC (Shimadzu LC 10A) column equipped with a UV detector at 254 nm with water/acetonitrile (1: 2, v/v) as the mobile phase and a flow rate of 1 ml/min. Some polycyclic aromatic hydrocarbons (PAHs) such as phenanthrene, anthracene, fluoranthene, pyrene and chrysene were prepared as standards and injected into the HPLC column. Their peaks were used to localize them in the main chromatogram.

The total peak area of each compound in the chromatogram was used to determine the reduction of PAHs and compare them with T_0.

The above procedures were also done for the extraction of total aliphatic, aromatic and crude oil in the control sample containing the oil-contaminated water with inorganic nutrients but no bacteria. The control sample was also incubated in a reciprocal shaker for 96 h at 30°C. Each sample was prepared in triplicate.

Statistical analysis

Results were expressed as mean ± standard deviation (±SD) and the analysis of variance and statistical significant difference ($P < 0.05$) was performed by one-way ANOVA with mean separation using Tukey's test. The statistical results were analyzed by Graphpad Prism 5 program.

RESULTS

Oil reduction

The reduction of total aliphatic, aromatic and crude oil after 96 h of incubation and its comparison with the control are shown in **Fig. 1**. For all cases, the difference between the treated samples (with bacteria) and the control (without bacteria) was significant. The total aliphatic fractions were decreased with a higher efficiency than the total aromatic fractions. The reduction of total crude oil was 30% in treated samples but only 7% in the control. The reduction of total aliphatic and aromatic fractions in the treated samples was 60 and 35%, respectively, in comparison with the control (20 and 10% reduction, respectively)

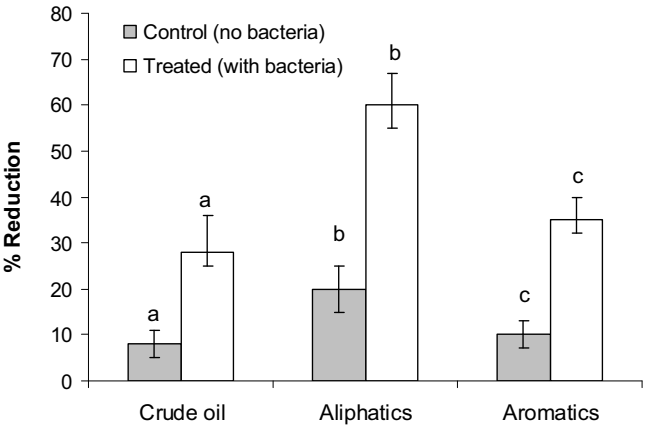

Fig. 1 Reduction of total crude oil, aliphatic and aromatic fractions in the control and treated samples. In all cases the difference of reduction between the control and treated samples was significant. The letters indicate significant difference within groups. Average values given ± standard deviation ($n = 3$ for each group, significant levels were determined by one-way ANOVA followed by Tukey's test).

Aliphatic reduction

The GC analyses of aliphatic fractions are shown in **Fig.2**. **Fig. 2A** shows the aliphatic fractions at T_0. In the control sample the aliphatic fractions decreased after 96 h of incubation in comparison with T_0 (**Fig. 2B**) while the aliphatic reduction was higher in the treated sample after 96 h (**Fig. 2C**). The aliphatic fractions were efficiently decreased in the treated sample in comparison with the control. Most aliphatic reduction occurred between C_{14} and C_{24}. The highest aliphatic reduction was observed in C_{17} (70%) and C_{16} (67%), while in the control, the reduction was 40 and 30%, respectively.

The reduction in the ratios of normal C_{17} and C_{18} alkanes to their branched isomers, pristane and phytane, showed that these ratios were significantly reduced in the treated sample while their reduction in the control sample was not significant after 96 h of incubation (**Fig. 3**).

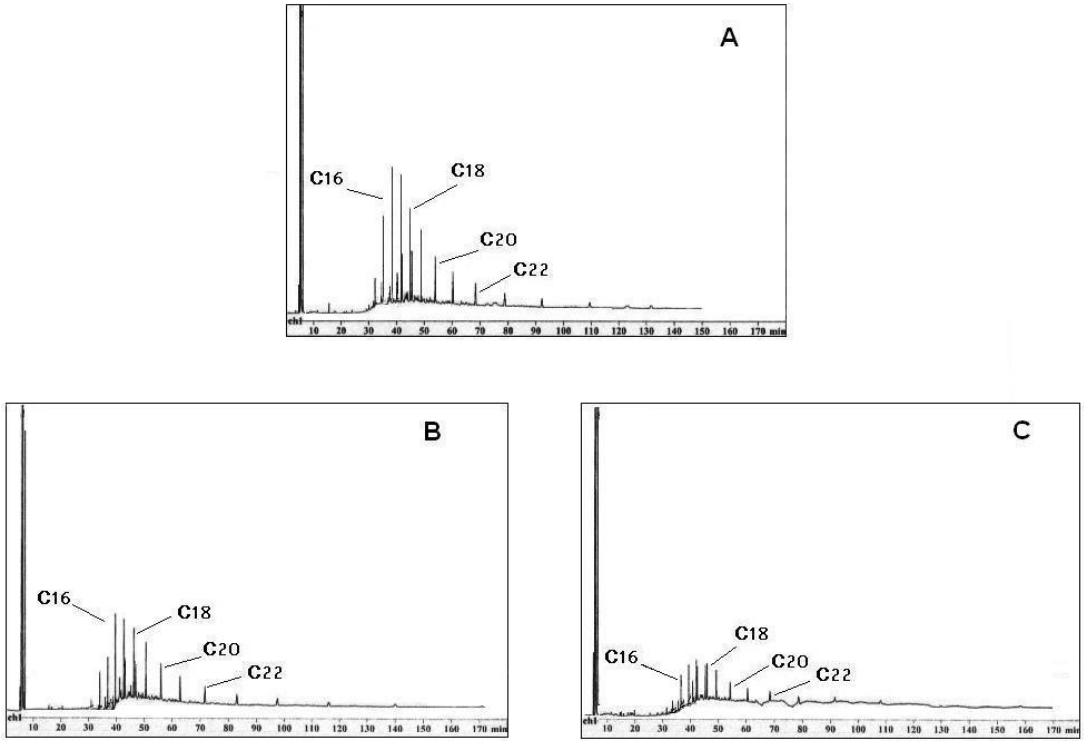

Fig. 2 Comparison of G.C chromatographs in the control and treated samples for detection of aliphatic fractions. (**A**) The aliphatic fractions at T_0, (**B**) in the control and (**C**) treated sample after 96 h. The numbers show the amount of carbons in fractions.

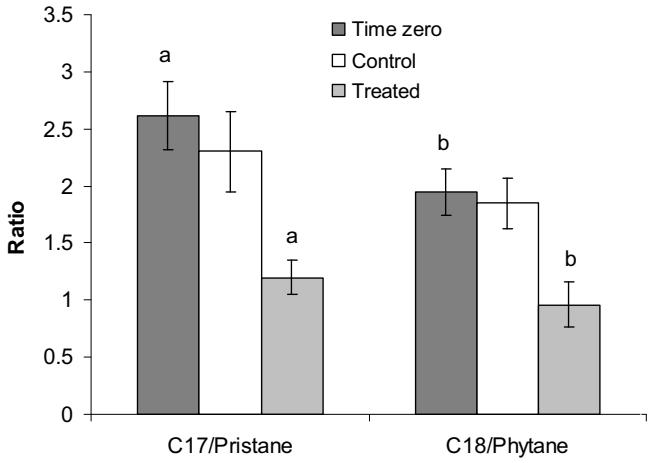

Fig. 3 The ratios of normal C_{17}/pristane and C_{18}/phytane. There was a significant difference between the treated samples and control and also samples at T_0 (see the letters), while no significant difference was observed between the control and the samples at T_0 (n = 3 for each group, significant levels were determined by one-way ANOVA followed by Tukey's test).

Aromatic reduction

HPLC analyses were done to determine the reduction of aromatic fractions in both control and treated samples. The reduction of some PAHs such as phenanthrene, anthracene, fluoranthene, pyrene and chrysene was analyzed. **Fig. 4A** shows the amount of aromatic fractions at T_0. The comparison of the peaks in the control (**Fig. 4B**) and treated sample (**Fig. 4C**) after 96 h of incubation showed that the reduction of PAHs in the treated sample was higher than in the control. The highest reduction was observed for fluoranthene (43%) and phenanthrene (35%), while the reduction in the control was 23 and 7%, respectively (**Fig. 5**).

DISCUSSION

One of the most important risks of water contamination is oil leakage, which affects drinking or cultivation water. It can affect the health of humans, plants or other organisms. The bioremediation of oil-contaminated water has always been an important issue. Some bacteria have a high potential for biodegradation of crude oil (Belhaj *et al.* 1992; Marge-sin *et al.* 2003; Johnson and Hyman 2006). This report focuses on bioremediation of aliphatic and aromatic fractions of crude oil by oil-degrading bacteria. Our results show that the bacterial consortium isolated from soil had a high potential to biodegrade crude oil (30% of total petroleum hydrocarbons (TPH) was biodegraded in 4 days). Some reports indicated that the reduction of different crude oils in seawater was between 26-50% after 42 days of treatment (Atlas 1975) and a microbial consortium has reduced total hydrocarbon content of crude oil-contaminated water for about 99% after 9 weeks treatment (Ibhadode *et al.* 2009). Aromatic and aliphatic fractions of crude oil are the most biodegradable parts of the oil, and microorganisms prefer to use the aliphatic fractions (Leahy and Colwell 1990; Prince 1993). According to **Figs. 2** and **4**, the isolated bacteria degraded the aliphatic fractions with higher efficiency than the aromatic fractions. This suggests that the bacteria preferred to use the aliphatic fractions as the primary carbon source. Biodegradation of diesel oil in liquid culture, which is mainly composed of aliphatic fractions, was about 90% after 13 days of incubation (Márquez-Rocha *et al.* 2001).

Biodegradation and volatilization are two main factors for reduction of aliphatic fractions in soil and water (Nicodem *et al.* 1997). The reduction ratios of normal C_{17} and C_{18} alkanes to their branched isomers, pristane and phytane, are usually used as indices of biodegradation and for monitoring the biological effect of microorganisms on the aliphatic fractions of oil (Atlas 1981; Seklemova *et al.* 2001). In the present work, the reduction of these ratios was highest in the treated sample, more than the control, suggesting that biodegradation plays the main role in treated samples in reducing the aliphatic fractions, while in the control sample, volatilization is primarily responsible for the reduction of aliphatic fractions. Most oil-degrading bacteria such as

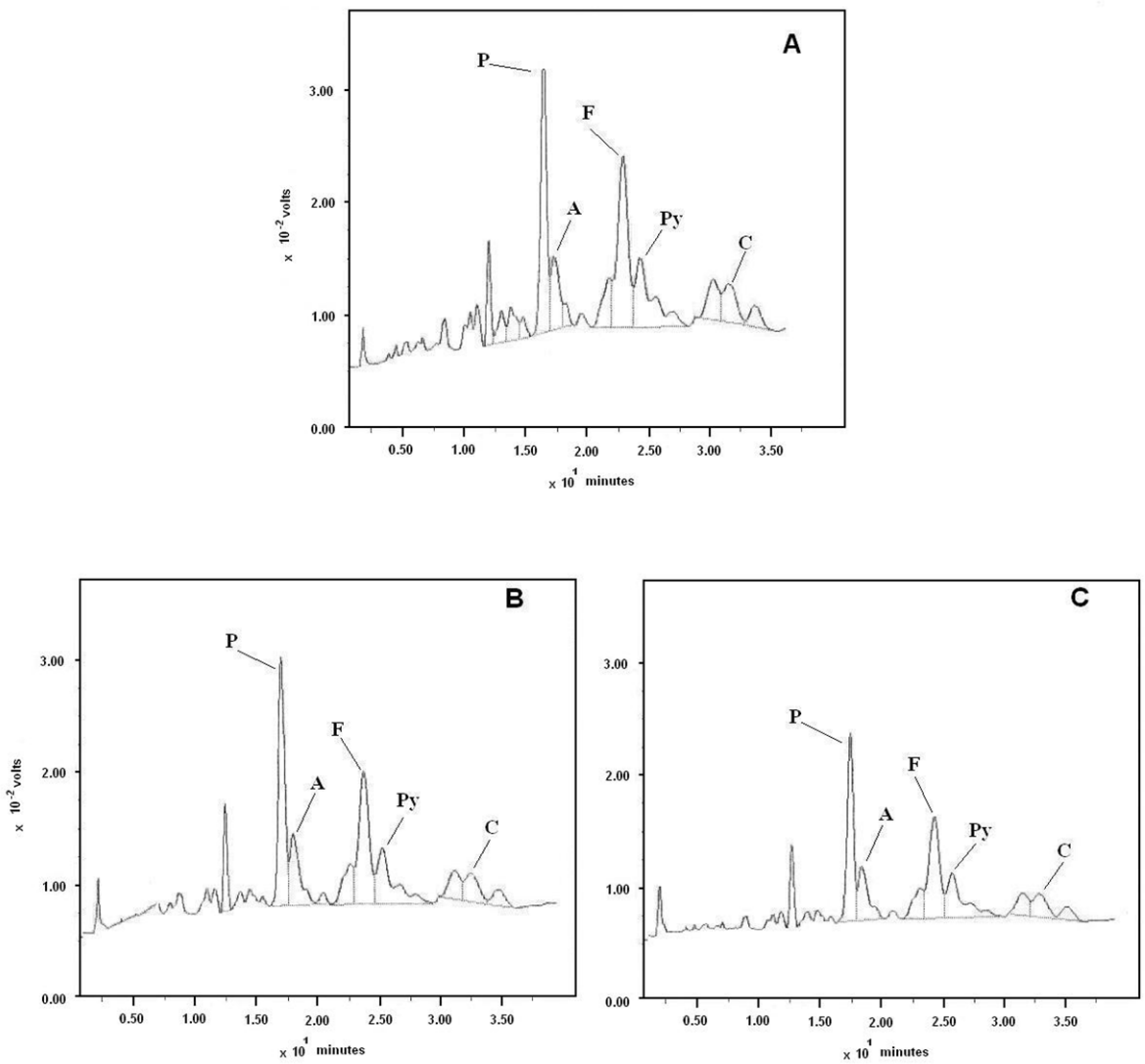

Fig. 4 HPLC chromatograms for determination of the aromatic fractions. (**A**) T_0, (**B**) in the control and (**C**) treated sample after 96 h. P = phenanthrene, A = anthracene, F = fluoranthene, Py = pyrene, C= chrysene.

Pseudomonas aeruginosa strains preferentially degrade middle to moderately long-chain alkanes (Ko and Lebeault 1999; Abalos *et al.* 2004).

Among the aromatic fractions biodegraded by the bacterial consortium, phenanthrene and anthracene were used more efficiently than fluoranthene, pyrene and chrysene. **Fig. 5** shows that, compared with phenanthrene and anthracene, the reduction of fluoranthene, pyrene and chrysene was high in the control sample. This suggests that these PAHs were decreased in the medium by both volatilization and biodegradation, while in phenanthrene and anthracene, biodegradation was the main factor for their reduction. In other words, these two PAHs were biodegraded more efficiently than the others PAHs. This suggestion does not imply that the rate of volatilization of fluoranthene, pyrene and chrysene was higher than that of phenanthrene and anthracene, but rather, in our short experiment, the presence of aliphatic fractions and simple PAHs (like phenanthrene and anthracene) in the culture medium forced the bacteria to use these materials, and probably there was insufficient time to use the heaviest aliphatics and more complex PAHs. Previous reports have also indicated that low molecular weight PAHs with less than three rings disappeared rapidly from the contaminated water, while the PAHs with more than four rings remained for longer (Lee *et al.* 1978; Yamada *et al.* 2003).

In conclusion, our results showed that in the soil near the Tehran oil refinery there are potent oil-degrading bacteria which can biodegrade the oil from contaminated water with high efficiency in a short period of time. The addition of some inorganic nutrients to the contaminated water can produce a suitable medium for biodegradation (some of

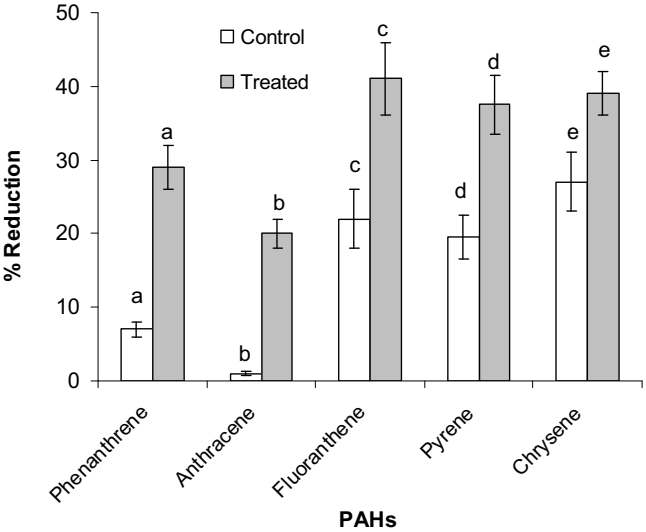

Fig. 5 Reduction of aromatic fractions in the control and the treated samples. In all the PAHs the difference of reduction between the control and treated samples was significant. The letters indicate significant difference in each PAH. Average values given ± standard deviation (n = 3 for each PAH, significant levels were determined by one-way ANOVA followed by Tukey's test).

these inorganic nutrients may penetrate the water from inorganic manure used in the cultivation lands). Nutrients play an important role in bacterial growth and oil bioremediation (Rodríguez-Blanco *et al.* 2010). Our findings also showed that the aliphatic fractions of crude oil were used up with a higher rate than the aromatic fractions and therefore, biodegradation can be used as an efficient and inexpensive method for removal of oil from the water around the oil refinery zone.

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