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# Bioremediation Combined with Ozonation Treatment of Polycyclic Aromatic Hydrocarbon Contaminated Soil of an Aged Oil Refinery Using Local Bacteria

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The amounts of polycyclic aromatic hydrocarbons (PAHs) were determined in a silty-clay soil sample of an aged oil refinery field in Abadan, Iran. More than 70 aerobic bacteria were isolated from the soil sample. Then 13 of them selected based on ability of growing in liquid mineral media which added anthracene as the sole carbon source. The selected bacteria were used to bioremediation of the same polycyclic aromatic hydrocarbon-contaminated soil in the present of Tween 80. The results show 78.1 % remediation after 30 days incubation at 37 ºC and 180 rpm. Two-phase ozonation and integration of ozonation and bioremediation methods also were studied achieved to 91.1 and 98.4 % in polycyclic aromatic hydrocarbons elimination, respectively. The polycyclic aromatic hydrocarbon contents of all samples were determined instrumentally by high performance liquid chromatography. It was found that the using of local bacteria resulted in a high efficiency bioremediation of soil. The results also demonstrated that removal of 3-ring polycyclic aromatic hydrocarbons is faster than 4-ring and 5-ring polycyclic aromatic hydrocarbons during bioremediation. No significant different seen in polycyclic aromatic hydrocarbon degradation between 3-ring, 4-ring and 5-ring polycyclic aromatic hydrocarbons during ozonation and combined ozonation and biological treatment. The highest polycyclic aromatic hydrocarbon removal was obtained by combination of two-phase ozonation and bioremediation.

**Key Words: Ozonation, Bioremediation, Polycyclic aromatic hydrocarbon, Tween 80.**

## INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are one of the most hazardous material groups reported by US Environmental Protection Agency (EPA) since 1970. They are toxic, carcinogenic and mutagenic. Because of these dangerous effects of polycyclic aromatic hydrocarbons, it is necessary to degradation them from environment. Polycyclic aromatic hydrocarbons often found in soil of old gasworks, oil refineries and forests. Various studies have reported high concentration of polycyclic aromatic hydrocarbons in soil of oil refineries and old gasworks sites[1-4]. Polycyclic aromatic hydrocarbons adsorb strongly into soil particles because they are hydrophobic and neutral in charge[5,6].

The persistence of polycyclic aromatic hydrocarbons in the soil of many industrial sites since above 70 years coupled with hydrophobicity, give them a high potential for bioaccumulation[7]. The USEPA accepts several processes for polycyclic aromatic hydrocarbons remediation including ozonation and bioremediation. The ability of using bioremediation technologies to remediation polycyclic aromatic hydrocarbon-contaminated soil is attended in recent years[8-13].

Polycyclic aromatic hydrocarbons may undergo adsorption, volatilization, photo-oxidation, chemical oxidation and bioaccumulation. However the major polycyclic aromatic hydrocarbon decomposition processes are ozonation and biological degradation[14-18]. The polycyclic aromatic hydrocarbon-degrading microorganisms could be algae, bacteria and fungi[19]. Some bacteria capable of degrading polycyclic aromatic hydrocarbons are discovered. Some of the more efficient bacteria belong to the genera *Bacillus*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus* and *Sphingomonas*[20-22].

However some disadvantages inhibit wide application of bioremediation included: (i) It is difficult to make sufficient microbial population in contaminated sites to receive a satisfied rate for *in situ*. Bioremediation[23]. (ii) *Ex situ* treatment methods although bring an acceptable rate for biodegrading of polycyclic aromatic hydrocarbon-contaminated soil *via* optimize the growth conditions, but are more expensive[24,25]. (iii) The slow release of the polycyclic aromatic hydrocarbons from soil particles to the aqueous phase is known as a limiting-rate factor for bioremediation processes[11,26].

Some strategies are used in recent years to increase efficiency of bioremediation technologies included: (i) Slurry

phase treatment to help to contaminants adsorbed on soil particles to transfer to water phase. (ii) Use of surfactants and vegetable oils to maximize the contact between soil and water to increase the bioavailability of contaminants[12,27]. (iii) Use of higher plants as an alternative to stimulate the microbial activities and enhance the remediation activities[25]. (iv) Integrated treatment by the combination of ozonation and bioremediation is based on the capability of ozone to transformation polycyclic aromatic hydrocarbons, regarding them into more biodegradable intermediates and oxidize the soil organic material releasing adsorbed contaminants so they are more biologically available[17,28,29].

In order to achieve an efficient biodegrading process it is necessary to find a new bacterium or a group of bacteria involved in complete degradation path way so that potentially toxic metabolites do not accumulate[30].

In case of old polluted sites, it seems isolation of the required bacteria from contaminated soil of the polluted site, can cause to a more effective bioremediation process in the same site soil, because of adaptation of inoculated bacteria to soil and climate conditions.

However the efficiency of the ozonation and bioremediation processes depended strongly on the soil matrix and humidity of the soil samples[28].

The integrated ozonation-bioremediation process has been investigated by a number of authors[17,28,29]. Using ozonation in combination with bioremediation is based on capability of ozone to increase the water solubility and bioavailability of polycyclic aromatic hydrocarbons *via* oxidizing polycyclic aromatic hydrocarbons and the other organic materials[17,28,31].

The objectives of the research are: (i) Determine major of the 16 USEPA-listed polycyclic aromatic hydrocarbons from soil sample of an aged oil refinery located in Abadan (west-south of Iran). (ii) Isolation of a polycyclic aromatic hydrocarbon-degrading bacteria groups from contaminated soil and evaluation ability of the isolated bacteria to losses of the polycyclic aromatic hydrocarbons from contaminated soil during bioremediation. (iii) Evaluation of polycyclic aromatic hydrocarbons degradation during two-phase ozonation. (iv) Integrated treatment of the contaminated soil samples using combination of ozonation and bioremediation. The results obtained were compared with previous methods.

## EXPERIMENTAL

This work was needed for polycyclic aromatic hydrocarbon-contaminated soil sample of an old industrial site, taken from the site of Abadan oil refinery (about 100 years old). Polycyclic aromatic hydrocarbons contaminating pollutants has been released in soil without any control for near one century and hence total organic carbon (OC) of the soil was high relatively.

The soil samples were selected from 5 areas on contaminated site from depth of 0, 30, 60 and 90 cm. Stones and woods were removed. The soil was then milled, mixed and screened through 1 mm mesh screen and stored at 4 ºC until used.

The soil type was silty clay loam (SCL). Selected physical and chemical properties of the soil sample are shown in Table-1. Total organic carbon content of soil sample was determined gravimetrically by drying in an oven at 105 ºC for 12 h followed by ignition in a 550 ºC electric furnace for another 12 h. The initial concentration of 15 polycyclic aromatic hydrocarbons in soil sample are given in Table-2. The concentration of anthracene increased to 200 ppm artificially in spiked soil sample.

TABLE-1
SELECTED PHYSICAL AND CHEMICAL PROPERTIES OF THE SOIL SAMPLE

| | |
|---|---|
| Organic carbon (%) | 21 |
| Clay (%) | 39 |
| Silt (%) | 45 |
| Sand (%) | 16 |
| pH | 7.72 |
| Ec (ds $m^{-1}$) | 35.7 |
| Saturated (%) | 30.88 |
| ρs (g $cm^3$) | 2.65 |
| ρd (g $cm^3$) | 1.83 |

TABLE-2
INITIAL CONCENTRATION OF 16 USEPA REPORTED POLYCYCLIC AROMATIC HYDROCARBONS IN SOIL SAMPLE OF ABADAN OIL REFINERY

| Polycyclic aromatic hydrocarbon | (mg $Kg^{-1}$) |
|---|---|
| Naphthalene | 9.40 |
| Acenaphtene | 11.00 |
| Fluorene | ND |
| Phenanthrene | 97.00 |
| Anthracene* | 2.40 |
| Flt. | 0.50 |
| Pyrene | 6.80 |
| B. (a) A. | 0.61 |
| Chry. | 4.50 |
| B. (b) F. | 0.96 |
| B. (k) F. | 0.18 |
| B. (α) P. | 0.16 |
| D. (ah) A. | 0.14 |
| B. (ghi) P. | 0.40 |
| Indeno (1,2,3-cd) Py. | 0.13 |

Anthracene, naphthalene, phenanthrene and pyrene were obtained from Merck chemical Co. (Germany) with purities higher than 96 %. Anhydrous sodium sulphate was heated in an electric furnace at 400 ºC for 3 h and then cooled to room temperature in a vacuum flask before using as drying agent. Fine $HgCl_2$ used as a microbiological inhibitor in ratio 1:100. The mineral media (MM) used for culture of the polycyclic aromatic hydrocarbon-degrading bacterium was composed of: NaCl 0.5 g, $Na_2HPO_4$ 3.4 g, $KH_2PO_4$ 1.5 g, $NH_4Cl$ 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.25 g and $CaCl_2$ 7.4 mg. Mineral media also contained trace elements consist of: $CuSO_4$ 0.2 mg, KI 0.5 mg, $MnSO_4{\cdot}H_2O$ 0.2 mg, $ZnSO_4$ 0.1 mg, $H_3BO_3$ 0.2 mg and $FeCl_3$ 0.1 mg. Solid mineral media was prepared of 15 g/L of mineral media.

The present study was used high-performance liquid chromatography instrument belongs to Tehran Petroleum Research Center, Iran. The determination of polycyclic aromatic hydrocarbon contents was carried out with waters high performance liquid chromatography (HPLC) system equipped with 410 binary pumps, 470 scanning fluorescence detector, Reodyne 7725i injection loop (20 μm) and a polycyclic aromatic hydrocarbon $C_{18}$ column (250 mm × 4.6 mm, particle size-5 μm).

The following program was developed in the laboratory for separation at the constant flow rate of 1 mL/min: (i) Isocratic elution with a 65:35 (v/v) mixture of acetonitrile and water for 14.5 min. (ii) Gradient elution by increasing acetonitrile proportion to 100 % within 5 min. (iii) Isocratic elution with pure acetonitrile for 15.5 min. The excitation and emission wavelengths of the detector were programmed *versus* time. The individual polycyclic aromatic hydrocarbon compounds were identified by comparison of retention times with the retention times of reference standard solutions.

**Preparation of spiked soil samples:** The soil samples were spiked for degrading assay as follows: the soil was dried, grinded and sieved through a 1 mm mesh and sterilized by autoclaving to eliminate microorganisms. Then 200 ppm filter sterilized anthracene was dissolved in acetone and added to 50 % of soil sample. Acetone evaporated in a fume hood and finally the soil mixed again with remainder of the sample.

**Isolation of polycyclic aromatic hydrocarbon-degrading bacteria:** For isolation of polycyclic aromatic hydrocarbon-degrading bacteria in soil of aimed site, 1 g of polycyclic aromatic hydrocarbon-contaminated soil sample added to 50 mL of BHI broth media and incubated at 37 ºC and 180 rpm for 48 h.

A serial dilution of the enrichment broth from $10^{-1}$-$10^{-10}$ prepared and then each diluted prepared added to 4 plates consist of 2 solid nutrient and 2 solid sabouraud dextrose agar media. The plates then incubated at 37 and 44 ºC for 24 h. Then in several steps bacteria purified and isolated on solid media to reach all bacteria growth in polycyclic aromatic hydrocarbon-contaminated soil sample.

All isolated bacteria individually inoculated to 250 mL flasks contained 50 mL BHI Broth Media including 30 ppm filter sterilized anthracene and incubated at 37 ºC and 180 rpm. The optical density of each samples detected at 600 nm every 24 h 3 times. Then 1 mL of each flasks added to a 250 mL Erlenmeyer flask containing 50 mL mineral media including 100 ppm filter sterilized anthracene and incubated at the same condition. The optical density 600 nm of each samples detected every 24 h.

The best growth bacteria were selected based on optical density 600 nm results. The selected bacteria inoculated to 250 mL Erlenmeyer flasks containing 50 mL mineral media including 250 ppm anthracene and this procedure was repeated by increasing anthracene concentration to 400, 600 and finally 1000 ppm in 4 steps.

The results were used to detect bacteria capable to growth in polycyclic aromatic hydrocarbon-contaminated mineral media contained anthracene as the sole carbon source.

**Polycyclic aromatic hydrocarbon-degrading assay:** A series of contaminated soil samples (50 g) spiked with 200 mg/kg anthracene in an acetone solution and sterilized to eliminate microorganisms. Tween 80 added to spiked samples in concentration of 5 g/mL. The samples were inoculated by 1 mL of pre-culture media (*ca.* $5 \times 10^7$ cell/mL) of isolated bacteria with demonstrated ability of polycyclic aromatic hydrocarbons degrading (13 bacteria). The samples then incubated at 37 ºC and 180 rpm for 30 days without pH adjustment. The water content was kept at 70 % of the water-holding capacity of the soil. A soil sample without addition of microorganisms was used as the control. Finally the polycyclic aromatic hydrocarbon content of samples were determined instrumentally.

**Ozonation method:** Ozonation of soil samples were carried out in a two-phase system including semi-continuous bubble column containing 50 g of soil. A flow of ozone gas produced by a laboratory compact ozone generator (model COG 5S belong to Arda Company, France) was transported to the soil trough a porous glass ozone dispersion disc located at the bottom of the column. The concentration of ozone in feed gas was kept at 1 mg/L and the gas flow rate at 0.4 L/min. The ozonation time was 30, 60, 90 and 120 min. The soil sample was treated without pH adjustment. The inlet and outlet concentration of ozone were measured using a UV/visible spectrophotometer (Shimadzu, Japan) at the wavelength $\lambda = 258$ nm.

## RESULTS AND DISCUSSION

A series of bacteria (13 bacteria) belong to *Pseudomonas* and *Bacillus genera* were isolated from polycyclic aromatic hydrocarbon-contaminated soil samples of oil refinery field in Abadan, based on growing in liquid mineral media added anthracene as the sole carbon source. Almost of selected bacteria belong to the genera *Bacillus*, *Pseudomonas* and *Sphingomonas*. The climate type of the investigated site was tropical and hence the growth temperature ranges of the isolated bacteria were broad.

**Ozonation:** Fig. 1 shows anthracene degradation using ozone gas after 30, 60, 90 and 120 min during two-phase ozonation. It shows maximum decreasing in anthracene content (91.1 %) after 2 h ozonation although 89 % of degradation observed after half time (1 h) ozonation.

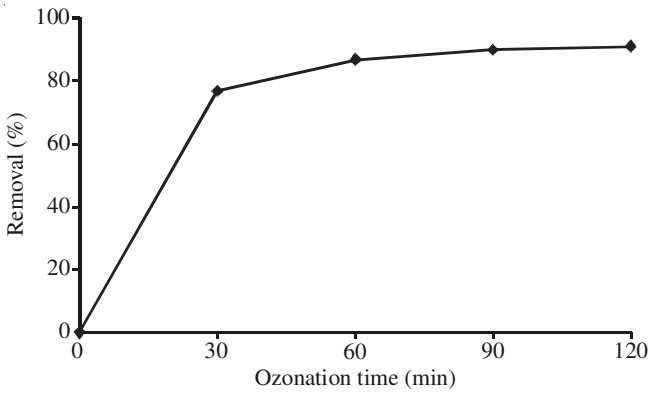



Fig. 1. Anthracene removal by two-phase ozonation of spiked soil sample of Abadan oil refinery (*versus* time)

Treatment of 14 polycyclic aromatic hydrocarbons in contaminated soil using 2 h, two-phase ozonation is shown in Fig. 2. It shows a decreasing of 66.0-99.2 % (total 91 %) in 14 polycyclic aromatic hydrocarbons content of the soil sample. The results demonstrate that two-phase ozonation is an effective method to remediation of SCL soils contaminated by polycyclic aromatic hydrocarbons. Metal oxides on surface of soil particles are known as heterogeneous catalyst to generate OH radicals *via* reaction with ozone[32].

Ozone demand of 0.96 mg/g soil was observed for the soil samples. These results are comparable to ozone break-

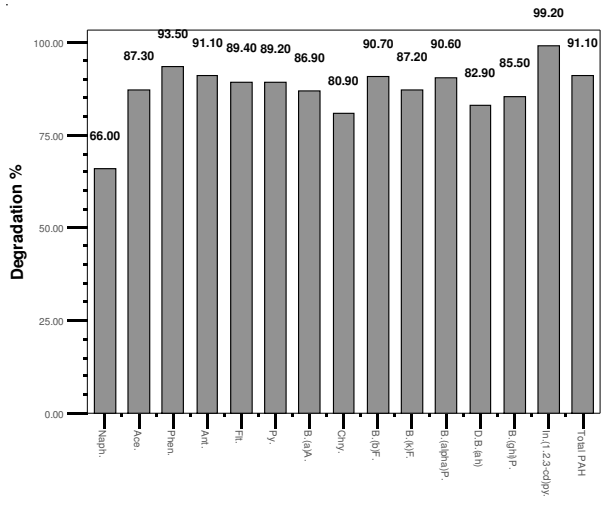



Fig. 2. Polycyclic aromatic hydrocarbons removal by 2 h two-phase ozonation of contaminated silty clay soil sample of Abadan oil refinery

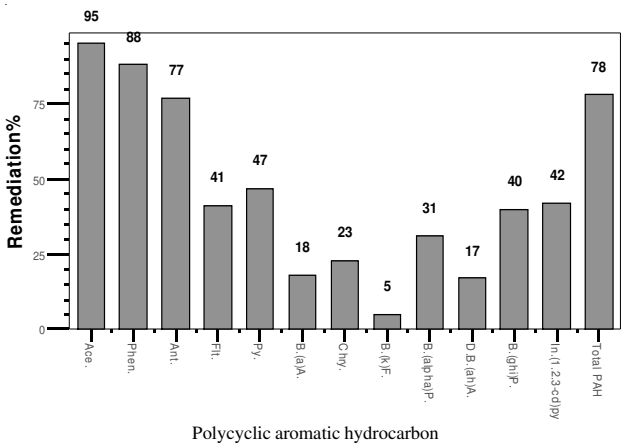
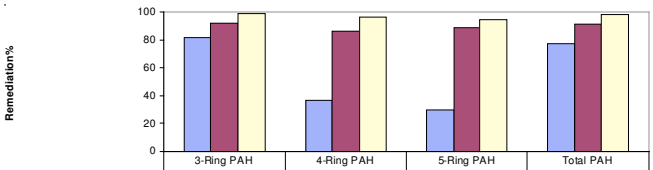



Fig. 4. Polycyclic aromatic hydrocarbon removal by 30 days bioremediation of contaminated silty-clay soil sample of Abadan oil refinery using local bacteria whit Tween 80 (5 g/kg) whitout pH adjustment

through studies conducted by Masten and Davies[33] and Pierpoint *et al.*[34]. The average applied ozone dose was 3.4 mg ozone per mg polycyclic aromatic hydrocarbons degraded in soil for two-phase ozonation.

As shown in Fig. 3, there is no significant different seen in polycyclic aromatic hydrocarbon-degrading between 3-ring, 4-ring and 5-ring polycyclic aromatic hydrocarbons during two-phase ozonation. 2 h two-phase ozonation of soil resulted in 91.7 % degradation of 3-ring polycyclic aromatic hydrocarbons, 86.1 % degradation of 4-ring polycyclic aromatic hydrocarbons and 88.6 % degradation of 5-ring polycyclic aromatic hydrocarbons. The results are not similar to those obtained by other authors, who determined different degrading values between 2-ring and 3-ring polycyclic aromatic hydrocarbons[28,30,35]. The results direct us to conclude that degradation of polycyclic aromatic hydrocarbons during two-phase ozonation is not dependent on the number of fused rings.




| | 3-Ring PAH | 4-Ring PAH | 5-Ring PAH | Total PAH |
|---|---|---|---|---|
| Bioremediation | 81.9 | 36.8 | 29.7 | 77.1 |
| 2-Phase Ozonation | 91.7 | 86.1 | 88.6 | 91.1 |
| Ozonation/Bioremediation | 98.5 | 96.5 | 94.3 | 98.36 |

Fig. 3. Removal of 2-ring, 3-ring and 5-ring polycyclic aromatic hydrocarbons in contaminated soil of Abadan oil refinery by bioremediation, two-phase ozonation and combined ozonation/ bioremediation methods

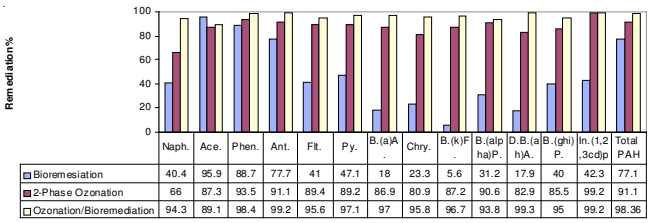
**Bioremediation:** The reduction per cent of polycyclic aromatic hydrocarbons in contaminated soil during 30 days bioremediation in laboratory condition is presented in Fig. 4. The average total polycyclic aromatic hydrocarbons concentration after 30 days bioremediation was 68.7 mg/kg, reduced from an initial concentration of 313.8 mg/kg. This is a reduction in total polycyclic aromatic hydrocarbons of 78.2 %. The good results obtained might because of the advantages of using local bacteria. Beside we inoculated all selected bacteria collectively to degradation assay because it is believed that bacteria can cooperate with each other for biodegradation of polycyclic aromatic hydrocarbons.

On the basis of previous studies, the concentration of Tween 80 was fixed at 5 g/L[13]. No pH adjustment was employed because the pH of soil was at an optimum level for the development of bacteria[36].

Although the bioremediation results are lower compared to ozonation results, hence bioremediation known as a cheap and effective method to remediation of polluted SCL soils, lonely and in coupled with other methods such as ozonation.

The slow release of the polycyclic aromatic hydrocarbons from soil particles to the aqueous phase is known as a limiting-rate factor for bioremediation processes[11,26]. Hence more soluble contaminants are available for biodegrading processes. Fig. 3 shows a significant decreasing in bioremediation of polycyclic aromatic hydrocarbons while the number of fused rings was increased. Present results show the largest remediation value for 3-ring polycyclic aromatic hydrocarbons (81.9 %) and lower values for 4-ring and 5-ring polycyclic aromatic hydrocarbons (36.8 and 29.7 %, respectively). The results are in agreement with previous authors who determined that 3-ring polycyclic aromatic hydrocarbons degraded by microorganisms faster than 4-ring and 5-ring polycyclic aromatic hydrocarbons[28].

**Combined ozonation and bioremediation:** The removal of polycyclic aromatic hydrocarbons by two-phase ozonation and biological post treatment in comparison with previous single methods is shown in Fig. 5. The results show that this method is more effective than using each of combined methods individually. The integrated ozonation and bioremediation method resulted in 98.4 % removal of total polycyclic aromatic hydrocarbons in contaminated SCL soil. The improvement of polycyclic aromatic hydrocarbon degradation results could be explained by the fact that, pre-ozonation of polycyclic aromatic hydrocarbon contaminated soil can increase the bioavailability of polycyclic aromatic hydrocarbons *via* oxidizing the soil organic material releasing adsorbed contaminants and conversion them to more water soluble metabolites[17,28,29].




| | Naph. | Ace. | Phen. | Ant. | Flt. | Py. | B.(a)A. | Chry. | B.(k)F. | B.(alpha)P. | D.B.(ah)A.. | B.(ghi)P. | In.(1,2,3cd)p | Total PAH |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bioremesiation | 40.4 | 95.9 | 88.7 | 77.7 | 41 | 47.1 | 18 | 23.3 | 5.6 | 31.2 | 17.9 | 40 | 42.3 | 77.1 |
| 2-Phase Ozonation | 66 | 87.3 | 93.5 | 91.1 | 89.4 | 89.2 | 86.9 | 80.9 | 87.2 | 90.6 | 82.9 | 85.5 | 99.2 | 91.1 |
| Ozonation/Bioremediation | 94.3 | 89.1 | 98.4 | 99.2 | 95.6 | 97.1 | 97 | 95.8 | 96.7 | 93.8 | 99.3 | 95 | 99.2 | 98.36 |

Fig. 5. Polycyclic aromatic hydrocarbon removal by 2 h pre-ozonation followed by 30 days bioremediation in comparison with individual ozonation and bioremediation methods

In the other hand, some authors determined that pre-ozonation decrease the bioremediation efficiency because it may destroy the indigenous microbial population of soil which may help to inoculated bacteria to bioremediation. They believe that ozonation may also break the same polycyclic aromatic hydrocarbon chemical bonds used by microorganisms and produce the intermediates that might be more toxic than the parent compounds[37]. However the results of the present study are not in agreement with the recent authors.

Fig. 3 shows that the number of fused ring has not significant effect on degradation results during integrated treatment of polycyclic aromatic hydrocarbon-contaminated soil by two-phase ozonation and biological treatment (98.5, 96.5 and 94.3 % degradation for, respectively 3-, 4- and 5-ring polycyclic aromatic hydrocarbons).

## Conclusion

The present study has shown that the content of polycyclic aromatic hydrocarbons in the soil of Abadan oil refinery is much higher than USEPA accepted limit values. The present investigation indicated that using of local bacteria can result in high efficiency bioremediation of polycyclic aromatic hydrocarbon-contaminated silty-clay soils (78.1 %). Two-phase ozonation was also achieved to 91.1 % in polycyclic aromatic hydrocarbons elimination. The highest polycyclic aromatic hydrocarbon removal was obtained by combination of ozonation and bioremediation methods. The results of the investigation also demonstrated that removal of 3-ring polycyclic aromatic hydrocarbons is faster than 4-ring and 5-ring polycyclic aromatic hydrocarbons during bioremediation. However no significant difference seen in polycyclic aromatic hydrocarbon degradation between 3-, 4- and 5-ring polycyclic aromatic hydrocarbons during ozonation and combined ozonation and biological treatment.

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