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Different biological strategies for the bioremediation of naturally polluted soils

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Abstract: Finding an appropriate method with the highest rate of polycyclic aromatic hydrocarbon (PAH) removal from naturally polluted soils is an important research issue. A pot factorial experiment (using contaminated soil samples from the Isfahan Refinery, Iran) was conducted in a 90-day period to compare the following bioremediation strategies: (1) natural attenuation (NA): the inherent ability of soil for bioremediation; (2) bioaugmentation (BA): inoculating soil with PAH degrading microbes *Marinobacter hydrocarbonoclasticus*; (3) biostimulation (BS): using N, P and K nutrients for the stimulation of bioremediating soil bacteria to achieve the C:N:P ratio of 100:10:1, and (4) bioaugmentation + biostimulation (BS + BA). Treatments NA (22.8%) and BA + BS (63.9%) resulted in the least and the highest rate of PAH removal from the soil. The 2–4 ring compounds had a significantly ($P \leq 0.05$) higher rate of degradation than the 5–6 ring compounds. The highest rates were resulted by fluorene (76.41%) and acenaphthylene (72.28%) using the BA + BS treatment. However, the lowest degradation rates were resulted by indeno (1,2,3-cd) pyrene (10.05%), benzo [b] fluoranthene (10.17%), benzo (g, h, i) perylene (12.53%), and benzo [k] fluoranthene (13.67%), using NA treatment. The BA + BS treatments are the most effective method for the bioremediation of PAH polluted soils.

Keywords: oil pollutant; contamination; soil microorganism; bacterial population

Polycyclic aromatic hydrocarbons (PAHs) are the most important oil pollutants, classified by the US Environmental Protection Agency as a priority pollutant (EPA 2015) due to their undesirable effects on the environment and humans (Table 1). So far, several methods have been used to eliminate PAHs from the soil, including physical, chemical, and biological ones (Zhang et al. 2017).

Natural attenuation, which is the ability of indigenous soil microorganisms to bioremediate polluted soils with PAHs, has been repeatedly referred to as the initial mechanism of reducing soil PAHs (Jiang et al. 2018). Biostimulation, as another important bioremediation strategy, is to stimulate the degrading capacity of the indigenous bacteria by adding nutrients, which prevent metabolic limitations (Naeem and Qazi 2020).

Bioaugmentation increases the bioremediation capacity of the contaminated soil by a strain or a consortium of microorganisms with the PAH degrading potential (Mirzakhani and Nejad 2016). For example, in bioremediation with *Pseudomonas aeruginosa*, the production of siderophore and biosurfactant increases the bioavailability and solubility of organic compounds and reduces soil PAH pollutants (Das and Mukherjee 2007). Research has indicated the positive effects of bioaugmentation and biostimulation in bioremediation of the PAH contaminated soils (Suja et al. 2014).

Previous research has analysed the single effects of the above-mentioned techniques on the bioremediation of PAH-contaminated soils (Yu et al. 2019). Accordingly, we hypothesised the combined use of such techniques is more effective, which to our knowledge has not been investigated previously. The objective was to investigate such techniques in the bioremediation of PAH contaminated soils collected from the Isfahan Refinery, Iran.

MATERIAL AND METHODS

The PAH contaminated soil samples were randomly collected from Isfahan Oil Refinery, Iran, contami-

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Table 1. The examined polycyclic aromatic hydrocarbon (PAH) compounds

Number	Compound	Symbol	Number of rings
1	naphthalene	NAPH	2
2	acenaphthene	ACE	3
3	acenaphthylene	ACEN	3
4	fluorene	FLU	3
5	phenanthrene	PHE	3
6	anthracene	ANT	3
7	fluoranthene	FLUO	4
8	pyrene	PYR	4
9	benzo{a}anthracene	BaA	4
10	chrysene	CHR	4
11	benzo{b}fluoranthene	BbF	5
12	benzo{k}fluoranthene	BkF	5
13	benzo{a}pyrene	BaP	5
14	dibenzo{a,h}anthracene	DahA	5
15	benzo{g,h,i}perlene	BghiP	6
16	indeno{1,2,3,cd}pyrene	IPY	6

nated with oil hydrocarbons for a long time (Figure 1). The compressed soil samples were air-dried and passed through a 2-mm sieve, and their physical and chemical properties were determined using the standard methods (Table 2) (Miransari et al. 2008).

Marinobacter hydrocarbonoclasticus inoculum, used for the bioaugmentation treatment, was purchased from the National Center for Biological and Genetic Resources of Iran. The bacteria were first cultured on a marine agar culture medium for fresh culture and were then transferred to the culture medium: broth brain heart infusion (BHI). They were incubated for 24 h in 30 °C to reach 1 McFarland density (3 × 10^8 CFU (colony forming unit)/mL). According to the 60% WHC (water holding capacity) in physiological serum, the bacterial inoculum was prepared and used for soil inoculation (Figure 1) (Huguenot et al. 2015).

Pot experiment. The PAH contaminated soil was air-dried, passed through a 2-mm mesh, and treated with the following treatments: (1) natural attenuation (NA); (2) bioaugmentation (BA); (3) biostimulation (BS) (treating the soil with N, P and K using K_2HPO_4, NH_4NO_3

Figure 1. Different stages of the experiment including the sampling site, the production of *Marinobacter hydrocarbonoclasticus* and the experimental treatments

Table 2. Soil physicochemical properties

Texture	pH	Electric conductivity (dS/m)	Lime	Total N	Available P	Na⁺	K⁺	Organic carbon (%)
			(%)		(mg/kg)			
Sandy clay loam	7.85	6.26	28.5	0.03	17.6	256	640	2.73

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Table 3. Effect of different bioremediation treatments on soil polycyclic aromatic hydrocarbon (PAH) (mg/kg dry soil) after 90 days

Treatment	Initial PAH (mg/kg)	Final PAH (mg/kg)	Remaining PAH (%)
NA	264.38 ± 11.31	205.13 ± 7.70[a]	77.62 ± 0.42[a]
BA	264.38 ± 11.31	164.09 ± 7.63[b]	62.05 ± 0.56[b]
BS	264.38 ± 11.31	128.87 ± 6.68[c]	48.18 ± 0.96[c]
BA + BS	264.38 ± 11.31	95.41 ± 5.16[d]	36.07 ± 0.52[d]

All the values are the mean of three replicates presented with their standard error values. NA – natural attenuation; BA – bioaugmentation; BS – biostimulation

and $(NH4)_2SO_4$ at the final C:N:P ratio of 100:10:1), and (4) bioaugmentation + biostimulation (BS + BA).

Plastic pots without lids were filled with 500 g of soil and stored at room temperature for 90 days. The moisture content of the pots during this period was adjusted to 40% of the WHC by weighing the pots. The pot soil was shaken twice a week for the circulation of air containing the oxygen. The bacterial inoculation was performed every 15 days with the aim of maintaining the number of microorganisms constant during the test period (Huguenot et al. 2015). The soil samples were collected at the end of the test and stored at 4 °C for the subsequent analyses.

Measurement of soil PAH. All PAHs were measured at the beginning and end of the test. Extraction was carried out by the Soxhlet method using an equal ratio of N-hexane and D-chloromethanes (Method 8100 of the American Environmental Protection Agency) (Samimi et al. 2009). Using gas chromatography (GC) (Agilent Technologies/Model N-6890 with the HP-5 column, California, USA) and ISO 18287 (2006) of the American Environmental Protection Agency (EFSA), the density of 16 soil PAH compositions was determined (ISO 18287, 2006).

Statistical analysis. This experiment was a completely randomised block design with the four bioremediation treatments in three replicates. Data were analysed with SPSS (New York, USA), and the mean values were compared using *LSD* (least significant difference) at a 5% level.

RESULTS

All treatments reduced soil PAH levels (Table 3, Figure 2). Moreover, BS (51.82%) and BA + BS treatment (63.93%) resulted in significantly ($P \leq 0.05$) higher PAH removal compared with BA (37.95%) and NA treatments (22.8%) (Table 3). The highest rate of degradation was related to the FLU (76.41%), and ACEN (72.28%) compounds using the BA + BS treatment, and the least was related to IPY (10.05%), Bbf (10.17%), Bkf (13.67%), and BghiP (12.53%), by the NA treatment (Figure 2). BA + BS treatment was the most effective method for the degradation of the

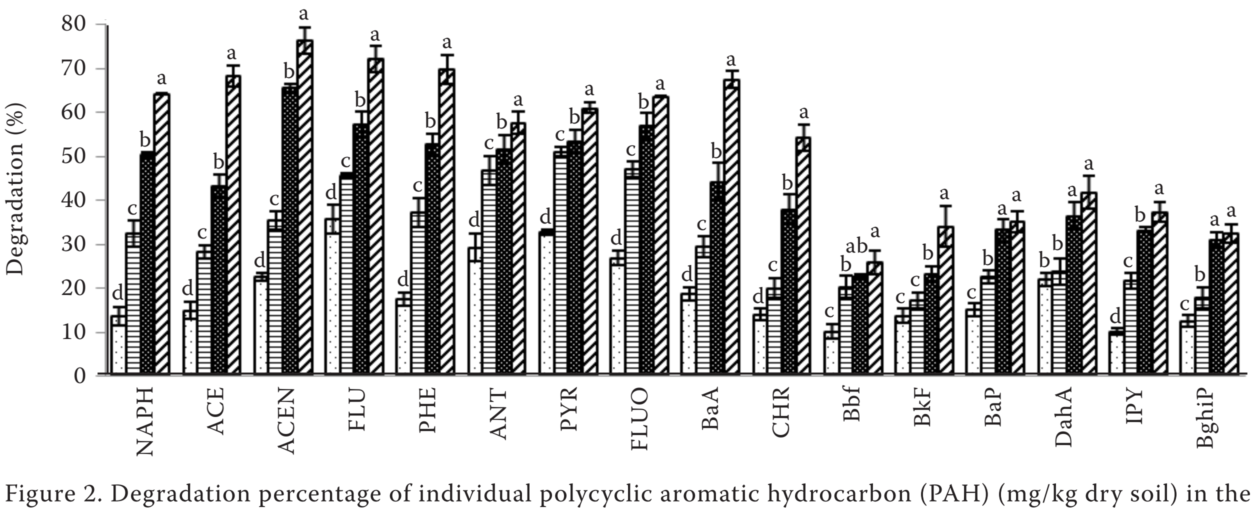

Figure 2. Degradation percentage of individual polycyclic aromatic hydrocarbon (PAH) (mg/kg dry soil) in the aged polluted soil under bioremediation treatments after 90 days. The data represent means of three replicates. Error bars show the standard error. NA – natural attenuation; BA – bioaugmentation; BS – biostimulation

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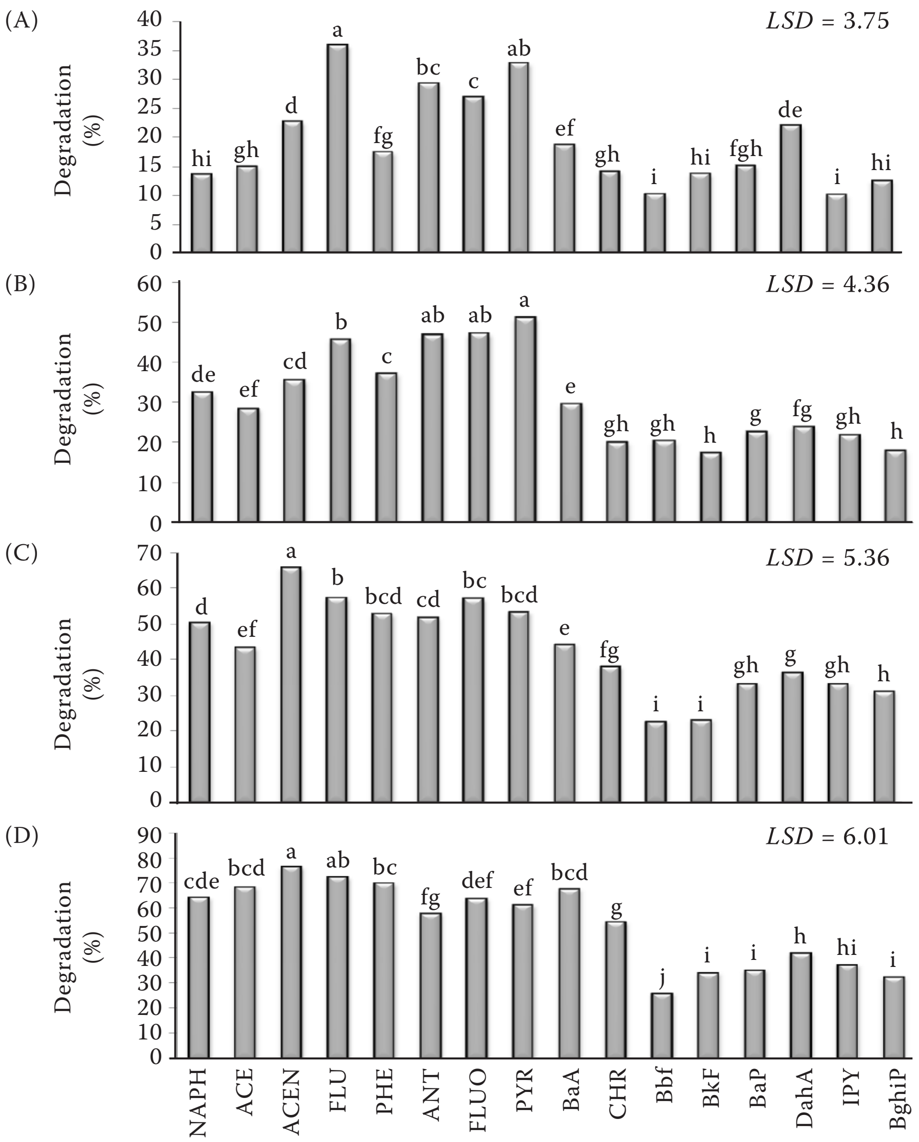

Figure 3. (A) The effect of natural attenuation (NA); (B) bioaugmentation (BA); (C) biostimulation (BS), and (D) bioaugmentation + biostimulation (BA + BS) on the degradation percentage of individual polycyclic aromatic hydrocarbon (PAH) (mg/kg dry soil) in aged polluted soil. Data are the average of three replicates

16 contaminants, resulting in the highest degradation rate for BghiP, BaP, and DahA (Figure 2).

After 90 days of using NA treatment, soil PAH reduced from the initial amount of 264.38 to the final amount of 205.13 (mg/kg dry soil) (Table 3). Using the NA treatment, the highest rate of degradation was related to FLU and PYR at 35.83%, and 32.76%, respectively, and the least was related to IPY (10.05%), Bbf (10.17%), BghiP (12.53%), and Bkf (13.67%) (Figure 3A). The NA treatment resulted in a degradation rate of 22.29, 19.38 and 13.91% for the 2–3, 4, and 5–6-ring PAH, respectively (Table 4).

The maximum rate of PAH degradation (the 2–3-ring compounds) by the BA treatment was equal to 37.66% (Table 4), which was 15.37% (significantly $P \leq 0.05$) higher than that of NA. The highest degradation was related to PYR (51.08%) and the least to BkF (17.20%), BghiP (17.77%), CHR (19.97%), BbF (20.22%), and IPY (21.71%) (Figure 3B). The corresponding values for the 4- and 5–6-ring compounds were equal to 13.56% and 6.64%, respectively (Table 4). Degradation rate of the PAHs with 5–6 rings were according to the following: DahA (23.84%), BaP (22.55%), IPY (21.74%), BbF (20.22%), Bghip (17.77%), and BkF (17.20%) (Figure 3B).

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Table 4. Polycyclic aromatic hydrocarbon (PAH) compounds degradation (%) affected by different bioremediation treatments

Number of rings	NA	BA	BS	BA + BS
2–3	22.29[a]	37.66[a]	53.57[a]	68.15[a]
4	19.38[b]	32.94[b]	48.18[b]	63.61[b]
5–6	13.91[c]	20.55[c]	30.00[c]	34.46[c]

The values are the means of three replicates. NA – natural attenuation; BA – bioaugmentation; BS – biostimulation

The BS treatment resulted in a 53.57% reduction of PAH, significantly ($P \leq 0.05$) higher than that of NA (22.29%) and BA (37.66%) treatments (Table 4). Compared with the other PAH compounds, the BS treatment, as previously mentioned, resulted in the highest reduction in the 2–3-ring compounds followed by the 4-ring (48.18%) and 5–6-ring compounds (30.00%) (Table 4). The highest rate of PAH degradation in this treatment was related to ACEN (65.73%) and the least to BkF (23.22%) and BbF (22.72%) (Figure 3C).

The BA + BS treatment was the most effective treatment, significantly ($P \leq 0.05$) decreasing PAH concentration from the initial value of 264.38 mg/kg to the final value of 95.41 mg/kg dry soil (Table 3). The least degradation rate (34.46%) by the BA + BS treatment, which was related to the 5–6-ring structures, was 20.55% (significantly) ($P \leq 0.05$) higher than that of the NA treatment (Table 4). The ACEN (25.88%) and BbF (76.24%) compounds had the least and highest rates of degradation, respectively (Figure 3D).

DISCUSSION

Different strategies were compared for the bioremediation of naturally polluted soils. BA + BS was shown to be the most effective bioremediation strategy. Bioaugmentation, which used *M. hydrocarbonoclasticus*, was an effective strategy; however, when combined with biostimulation (the use of nutrients), its effectiveness significantly increased. Various experiments have shown that the most effective treatments for the degradation of soil PAH are BA and BS (Maletic et al. 2020). Depending on the quality of the inoculation liquid and its nutrients contents, the efficiency of bioaugmentation and biostimulation may be different (Suja et al. 2014, Wu et al. 2020). This may be because the two treatments can synergically affect each other, and hence their potential bioremediation increases. Agnello et al. (2016) found that the combined use of phytoremediation and bioaugmentation was more effective on the bioremediation of PAH-contaminated soils compared with their single-use.

The results indicated the high ability of *M. hydrocarbonoclasticus* for the biodegradation of soil PAH. Naeim et al. (2020) found that among different bacterial strains, *M. hydrocarbonoclasticus* had the highest rate of PAH degradation, which was due to the activity of laccase enzyme, and the production of biosurfactant, biofilm and exopolysaccharide (Isfahani et al. 2018). The bacteria use pollutants as a source of carbon to produce biosurfactants, which entraps and solubilises the pollutant.

The least effective bioremediation strategy was NA (the use of indigenous soil microbes). These results are consistent with the findings of other researchers (Guarino et al. 2017). The indigenous microorganisms are the main cause of degradation by the natural attenuation process, although there are other factors such as evaporation and oxidation, which also result in the remediation of soil (Agnello et al. 2016).

There were significant differences in the biodegradation of different PAH compounds. The increased number of rings decreased the efficiency of the methods for the bioremediation of the polluted soils. The chemical structure is among the most important factors determining the rate of PAH degradation by the selected bioremediation method. The reason for this trend is the less ability of bacteria to decompose compounds with high molecular weight (Aziz et al. 2018). In the PAH compounds with the higher number of rings, due to the less availability of the replaced allylic acid, by its absorption on soil matrix, the bioavailability and degradation rate of PAH compounds with high molecular weight decreases (Lu et al. 2019).

Successful oil bioremediation requires a bacterial population with a high-degradation potential to withstand environmental stresses (Ghosal et al. 2016). In PAH compounds with a higher number of rings and higher molecular weight, the ability of bacteria

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to decompose the compounds decreases, which is mainly due to the presence of a stable molecular structure (Aziz et al. 2018).

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