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Increase in Removal of Polycyclic Aromatic Hydrocarbons During Bioremediation of Crude Oil–Contaminated Sandy Soil

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Abstract

A 2^3 full factorial experimental design was adopted to estimate the effects of three variables on the biodegradation of oil during soil bioremediation: bioaugmentation seeding a mixed culture, addition of fertilizer or mineral media, and correction of initial pH of the soil to 7.0. The tests were carried out in polyvinyl chloride reactors with 5.0 kg of crude oil–contaminated soil at 14 g/kg. After screening the variables, soil bioremediation tests were conduced with varied C:N ratios, yielding an increase in biodegradation of the oil heavy fraction from 24 to 65%, consumption of total *n*-paraffins, and a remarkable decrease in the concentration of residual polycyclic aromatic hydrocarbons of the soil.

Index Entries: Soil bioremediation; crude oil; hydrocarbons; biodegradation; experimental design.

Introduction

The increase in the worldwide production of petroleum products and their transportation has increased the number of accidental releases, which contributes to environmental contamination and health problems. Petroleum is a complex mixture composed mainly of hydrocarbons, which are sometimes recognized as carcinogenis *(1,2)*. Hydrocarbons can be removed from the soil by traditional chemical and physical operations *(3)*, which are limited by cost and efficiency factors *(4)*. Environment preservation claims, regulatory system recommendations, and the increase in costs related to the environmental industry image have roused interest in cost-effective biologic alternatives for the cleanup of hydrocarbon-contaminated soil. The biologic treatment of contaminated soils can be carried out using several techniques such as land farming and composting. Among these techniques, degradation employing bioreactors has been studied

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owing to its simple process operation, control, and monitoring *(3,5)*. However, the bioremediation process is complicated by the chemical properties of the contaminant, and also by factors related to the availability of a degrading microbial consortium and establishment of adequate process conditions.

In recent years, there have been considerable research efforts to use the potential of indigenous and exogenous microorganisms to degrade hydrocarbons and to optimize the bioprocess *(6–8)*. In many cases, bioremediation tests were conducted without adequate experimental planning and supporting statistical analysis, yielding a large number of experiments and incorrect evaluation of experimental errors. Statistical tests and experimental design tools can evaluate the effect of selected process variables and their interaction on the bioremediation. The purpose of the present study was to optimize the efficiency of bioremediation treatment over a fine sandy soil from Galeão Beach, Brazil, contaminated with crude oil by using statistical experimental design tools. Considering only the year 2000, more than 2,000,000 L of crude oil was spilled on the Guanabara Bay region as a result of ruptures in oil ducts, causing the subsequent contamination of some beaches, including Galeão Beach.

Materials and Methods

Soil and Oil

The sandy soil selected was collected from Guanabara Bay, Rio de Janeiro State, Brazil, according to the procedure described by Balba et al. *(9)*. It is an inorganic, fine sandy soil presenting very low carbon and nitrogen contents, 1.6 and 0.55 ppm, respectively. The natural soil pH was 8.3, and some other properties of the soil can be found in ref. *10*. Arabian light oil was used in the experiments. The oil composition indicated the presence of 46.5% saturated compounds, 32.23% aromatic compounds, and 21.27% resins.

Experimental Design for Soil Bioremediation Tests

Soil bioremediation tests were executed in two steps. The first one refers to the screening of the process variables. The screening method chosen was a two-level factorial experiment *(11)*. Based on previous soil bioremediation investigation results *(7,8,12)*, the following variables were investigated: X_1, seeding of exogenous microorganisms; X_2, macronutrient sources; and X_3, correction of the initial pH of the soil to 7.0. In the experimental design, process variable values were normalized to –1 and +1 for each variable. The range of values of the studied variables were as follows: X_1, bioaugmentation by Nd mixed consortium seeding (+1) and not seeding of exogenous microorganisms (–1); X_2, use of mineral medium (+1) and a single amendment of commercial mineral fertilizer (–1); X_3, correction of the initial pH of the soil to 7.0 (+1) and initial soil pH of 8.3 (–1), the

natural pH of the soil. Using these values, the design matrix was constructed, and the measurements of the heavy oil fraction losses were used as the response variable. Experimental results were presented as a mean of two independent tests, and the design and statistical analysis were developed using Statistica 5.5 software.

Nd mixed culture, composed of *Pseudomonas* sp., *Serratia* sp., *Bacillus* sp., *Candida* sp., *Streptomyces* sp., and *Penicillium* sp., was previously isolated from a Brazilian refinery soil by Del'Arco and de França *(7)*. This culture was used to verify the bioaugmentation effect on our experiments. Commercial 10:10:10 NPK mineral fertilizer (Ouro Verde) in a single amendment to provide a 100:1 C:N ratio or a mineral medium with the composition described in refs. *7* and *8*, was used as the macronutrient source.

With the intent of optimizing oil biodegradation, a second batch of bioremediation tests was executed considering the results of the experimental design. The experiments were conducted employing previously selected process conditions and using 100:1, 100:2, 100:5, and 100:10 C:N ratios. All tests were carried out using polyvinyl chloride boxes (22 width × 32 length × 12 cm height). Each reactor was loaded with 5.0 kg of soil previously contaminated with 70.0 g of Arabian light crude oil (14 g/kg contamination level). The sampling process, moisture maintenance, and soil aeration were executed according to the procedures described by Del'Arco and de França *(7,8)*.

Analytical Methods

Oil extraction from the soil was performed according to Stout and Lundegard *(13)*. The heavy oil fraction from the oil extract was executed according to the procedure described by Del'Arco and de França *(7,8)*. Paraffin analyses were performed in the oil extract using a gas chromatography/mass spectrometry apparatus (HP, model HP5880 A/GC/EM 5987) equipped with a flame ionization detector and a phenylmethylpolysiloxane (5:95) column (0.25 mm × 30 m) according to the method proposed by Del'Arco and de França *(7,8)*. Quantification of polycyclic aromatic hydrocarbons (PAHs) was done using a deuterated internal standard of each selected PAH. All assays were executed in triplicate. Viable fungi and bacterial cell counts were performed at 0, 28, and 56 d by pour plate method using, respectively, bacto nutrient agar and Sabouraud agar. Petri dishes were incubated at 30 ± 1°C, and the number of bacterial and fungal colonies was counted after 48 and 120 h respectively.

Results and Discussion

Application of a 2^3 Full Factorial Design for Variable Screening

The following discussion focuses on the factorial design and the effects of three entrance variables on bioremediation of crude oil–contaminated soil.

Table 1
Full Factorial 2^3 Experimental Design Matrix[a]

Experiment	X_1	X_2	X_3	Y
1		Control		7.3 ± 0.9
2	–1	–1	+1	24.1 ± 0.7
3	–1	+1	+1	26.5 ± 0.5
4	–1	–1	–1	24.2 ± 0.5
5	+1	+1	+1	41.1 ± 1.6
6	+1	+1	–1	40.8 ± 2.0
7	–1	+1	–1	24.3 ± 0.8
8	+1	–1	–1	39.2 ± 1.1
9	+1	–1	+1	39.5 ± 0.8

[a]Results considering abiotic losses, 7.3 ± 0.9.

Table 1 summarizes the experimental results of heavy oil fraction biodegradation in conjunction with dependent variables. It can be noted that there was a considerable variation in oil degradation depending on the process conditions. In this case, the experimental data were analyzed using statistical methods appropriated to the experimental planning, and the oil biodegradation behavior was modeled according to a polynomial equation. Multiple regression analysis of the experimental data resulted in the following empirical model (Eq. 1):

$$Y = 23.98 + 17.2X_1 + 15.04X_2 + 0.26X_3 - 13.44X_1X_2 - 0.26X_1X_3 - 1.81X_2X_3 + 1.81X_1X_2X_3 \quad (1)$$

In which Y is the heavy oil fraction biodegradation loss (%); and X_1, X_2, and X_3 were previously defined.

The empirical model was validated using analysis of variance (ANOVA) (see Table 2). The correlation measures for testing the adequacy of the fit were made using the multiple correlation coefficient, R, and the determination coefficient, R^2. The fact that the value of R (0.9893) for the statistical model is close to 1 indicates a high degree of correlation between the observed and predicted values. The value of the determination coefficient, R^2 (0.9788), suggests that the model fails to explain only about 3% of the total variations. F- and p values (Table 2) indicate that the Nd mixed-culture seeding, X_1, is the most significant factor for oil biodedegradation. Nd culture was isolated from a petrochemical land farm, and subsequently maintained for 5 yr in a crude oil–enriched mineral medium, leading to the mixed culture adaptation to oil *(14)*. In addition, this culture was composed of hydrocarbon-utilizing and biosurfactant-producing microorganisms *(7,8,15)*. Microbial surfactant production may promote an increase in the bioavailability of hydrophobic compound and, consequently, its biodegradation *(15)*. The data provided in Table 2 show a slight significance associated with X_2, indicating that the biodegradation of oil was not

Table 2
ANOVA of Empirical Model

Factor	Sum of squares	Degrees of freedom	Mean square	*F* value	*p* Value
X_1	1421.42	1	1421.42	754.41	0.0001
X_2	11.900	1	11.900	6.316	0.022
X_3	3.020	1	3.020	1.598	0.223
X_1 by X_2	0.220	1	0.220	0.117	0.736
X_1 by X_3	1.000	1	1.000	0.531	0.476
X_2 by X_3	1.984	1	1.984	1.053	0.319
X_1 by X_2 by X_3	1.984	1	1.984	1.053	0.319
Error	32.030	16	1.884		
Total squares	1471.56	23			

influenced by the use of mineral medium and fertilizer as macronutrient sources. This result is not in agreement with the finding of Lin et al. *(16)*, who showed that a higher amount of hydrocarbon removal was achieved when fertilizer was added in relation to the use of a mineral medium. This difference may be justified by the soil properties, type of crude oil, and microbial population. High *p* values associated with the variable X_3 suggest that correction of the initial pH of the soil to 7.0 is not required, as opposed to Alexanders, *(15)* findings whose reports that soil presenting high pH should have its pH corrected to neutral before initiating treatment. It is important to emphasize that although the interaction factors were not significant at $p = 0.05$, the model showed significant lack of fit when they were not included, due to marginal significance.

To verify the increase in the biodegradation of some crude oil constituent hydrocarbon compounds, analyses were performed on the oil extract. *n*-C10 to *n*-C30 hydrocarbon compounds were analyzed because these are the most abundant in the employed crude oil and are less volatile than minor carbon chain hydrocarbons that tend to be eliminated by evaporation. Figure 1A presents the linear hydrocarbon biodegradation profiles after 56 d in tests 6 and 4, which presented, respectively, the most and the least heavy oil fraction biodegradation losses. It can be noted that the paraffin biodegradation was influenced by process conditions. In test 4, the smallest cumulative biodegradation losses of *n*-paraffins were achieved, and 55–60% C20–C30 *n*-alkanes was removed. However, in test 6, the biodegradation of paraffins exceeded 80%. This indicates the relation between *n*-paraffins and heavy oil fraction biodegradation. These specific hydrocarbon biodegradation results are in agreement with the claims of Baker and Herson *(3)*, who suggested that the C10–C20 linear chain hydrocarbons were preferentially biodegraded in comparison with the longest linear paraffin.

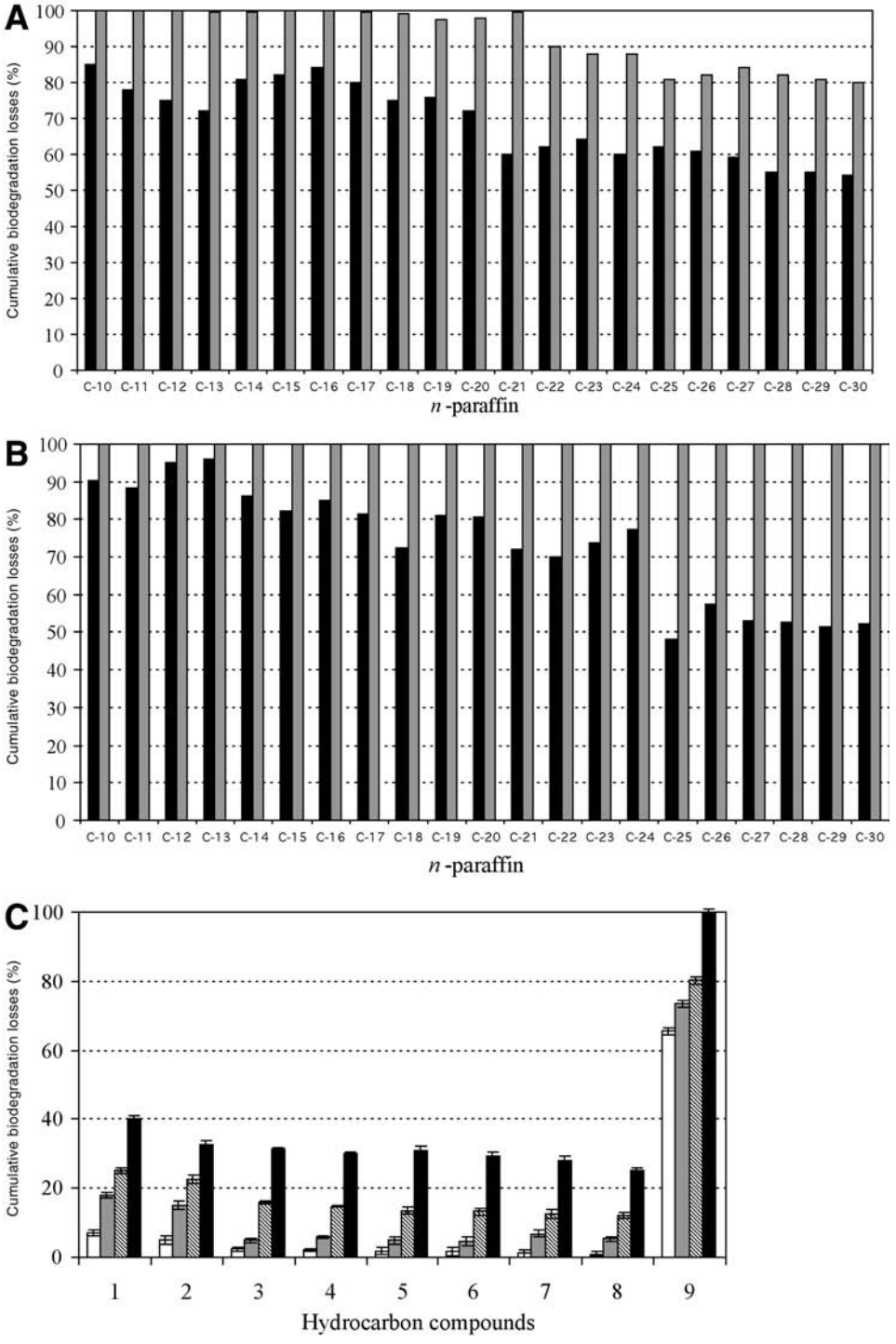
A
Cumulative biodegradation losses (%)
100
90
80
70
60
50
40
30
20
10
0
C-10 C-11 C-12 C-13 C-14 C-15 C-16 C-17 C-18 C-19 C-20 C-21 C-22 C-23 C-24 C-25 C-26 C-27 C-28 C-29 C-30
n -paraffin
B
Cumulative biodegradation losses (%)
100
90
80
70
60
50
40
30
20
10
0
C-10 C-11 C-12 C-13 C-14 C-15 C-16 C-17 C-18 C-19 C-20 C-21 C-22 C-23 C-24 C-25 C-26 C-27 C-28 C-29 C-30
n -paraffin
C
Cumulative biodegradation losses (%)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9
Hydrocarbon compounds

Table 3 presents the soil microbial concentration data. Examining the initial microbial concentration data, one can verify that all uninoculated experiments started with similar microbial concentrations. Inoculation provided an increase in soil bacterial concentration from about 10^4 to about 10^5 colony-forming units (CFU) and fungal soil concentration from about 10^3 to about 10^4 CFU. After 28 d the soil bacterial and fungal concentrations reached maximum value. After 56 d the soil fungal and bacterial concentrations decayed drastically, possibly owing to microbial selection processes and/or to remaining recalcitrant compounds, as defended by Sepic et al. *(6)*.

Optimization of C:N Ratio

With the aim of increasing oil biodegradation, we conducted bioremediation experiments considering the previous experimental design results and using different C:N ratios. When a soil is contaminated with petroleum or petroleum-derived compounds, an elevated soil carbon content is frequently observed. Nevertheless, the microbial hydrocarbon metabolism is dependent not only on carbon, but on other macronutrients, such as nitrogen, which are required in different metabolic pathways. Hence, inorganic fertilizer was used as a nitrogen source to provide 100:1, 100:2, 100:5, and 100:10 C:N ratios. After 56 process days, heavy oil fraction biodegradation losses of 49 ± 2, 52 ± 3, and 47 ± 2% were observed for the conducted tests at C:N ratios of 100:1, 100:2, and 100:5, respectively. Considering standard deviations, these values were similar. However, 64 ± 2% of biodegradation losses were observed at a 100:10 C:N ratio, indicating a nitrogen-limited process. These results clearly show that implementation of a proper amount of fertilizer during the process was essential for the greatest removal of hydrocarbons from the soil. Figure 1B presents the *n*-paraffin accumulated biodegradation losses obtained in the experiments conducted using a 100:10 C:N ratio. It can be noted that *n*-C10 to *n*-C24 were totally

Fig. 1. Hydrocarbon biodegradation. **(A)** Cumulative biodegradation losses of linear hydrocarbons in different experiments: (■)experiment 4 using indigenous microorganisms, X_1 (–1); mineral medium as macronutrient source, X_2 (–1) and natural soil pH (8.3), X_3 (–1); and (■) experiment 6 conducted using Nd mixed-culture seeding (exogenous microorganisms), X_1 (+1); inorganic fertilizer at 100:01 C:N ratio as macronutrient source, X_2 (+1); and natural soil pH (8.3), X_3 (–1). All results were collected during a 56-d process period. **(B)** Cumulative *n*-alkane biodegradation during crude oil–contaminated bioremediation tests using Nd mixed-culture seeding (exogenous microorganisms), inorganic fertilizer at 100:10 C:N ratio, and natural soil pH (8.3) after (■) 28 and (■) 56 d. **(C)** PAH and total *n*-paraffin content biodegradation during bioremediation processes using inorganic fertilizer as macronutrient source and natural soil pH (8.3) and seeding Nd mixed culture: 1 = 1-methylnaphthalene, 2 = 2-methylnaphthalene, 3 = fluorene, 4 = pyrene, 5 = chrysene, 6 = phenanthrene, 7 = benzo (*a*)anthracene, 8 = benzo (*b*)fluoretane, and 9 = *n*-paraffin total content after (□) 28 and (■) 56 d using 100:01 C:N ratio and after (■) 28 and (■) 56 d using 100:10 C:N ratio.

Table 3
Soil Microbial Concentration Data During Experimental Planning Design Experiments

	Bacteria (CFU/g of soil)			Fungi (CFU/g of soil)		
Experiment	0 d	28 d	56 d	0 d	28 d	56 d
1	<30	<30	<30	<30	<30	<30
2	$4.07 \pm 0.6 \times 10^4$	$1.08 \pm 0.1 \times 10^7$	$2.73 \pm 0.6 \times 10^5$	$1.63 \pm 0.4 \times 10^3$	$3.00 \pm 0.9 \times 10^5$	$4.13 \pm 0.8 \times 10^1$
3	$3.00 \pm 0.2 \times 10^4$	$1.30 \pm 0.1 \times 10^9$	$2.33 \pm 0.8 \times 10^5$	$4.30 \pm 0.3 \times 10^3$	$1.77 \pm 0.3 \times 10^5$	$7.10 \pm 0.9 \times 10^1$
4	$3.42 \pm 0.4 \times 10^4$	$2.28 \pm 0.4 \times 10^7$	$3.73 \pm 0.2 \times 10^5$	$2.92 \pm 0.2 \times 10^3$	$1.36 \pm 0.7 \times 10^5$	$3.16 \pm 0.9 \times 10^1$
5	$3.30 \pm 0.4 \times 10^5$	$3.87 \pm 0.2 \times 10^{10}$	$2.67 \pm 0.5 \times 10^5$	$6.57 \pm 0.2 \times 10^4$	$1.90 \pm 0.1 \times 10^7$	$3.77 \pm 0.8 \times 10^1$
6	$3.87 \pm 0.6 \times 10^5$	$1.08 \pm 0.1 \times 10^{10}$	$2.73 \pm 0.6 \times 10^5$	$1.35 \pm 0.1 \times 10^4$	$2.70 \pm 0.5 \times 10^7$	$3.17 \pm 0.6 \times 10^1$
7	$2.95 \pm 0.4 \times 10^4$	$8.40 \pm 1.0 \times 10^9$	$2.45 \pm 0.1 \times 10^5$	$2.30 \pm 0.8 \times 10^3$	$1.48 \pm 0.1 \times 10^5$	$2.53 \pm 0.6 \times 10^1$
8	$3.36 \pm 0.5 \times 10^5$	$2.55 \pm 0.8 \times 10^{10}$	$8.43 \pm 1.0 \times 10^5$	$3.75 \pm 0.2 \times 10^4$	$1.86 \pm 0.2 \times 10^7$	$2.33 \pm 0.8 \times 10^1$
9	$3.28 \pm 0.2 \times 10^5$	$2.57 \pm 0.8 \times 10^{10}$	$5.02 \pm 0.4 \times 10^5$	$2.96 \pm 0.2 \times 10^4$	$1.67 \pm 0.2 \times 10^7$	$2.33 \pm 0.7 \times 10^1$

Table 4
Soil Microbial Concentration Data During Crude Oil Bioremediation Tests Using Bioaugmentation with Nd Mixed-Culture Seeding (Exogenous Microorganisms), Initial Soil pH (8.3), and Different C:N Ratios

	Bacteria (CFU/g of soil)			Fungi (CFU/g of soil)		
C:N ratio	0 d	28 d	56 d	0 d	28 d	56 d
Control	<30	<30	<30	<30	<30	<30
100:01	$3.40 \pm 0.9 \times 10^5$	$9.53 \pm 2.0 \times 10^{10}$	$8.13 \pm 0.7 \times 10^4$	$2.40 \pm 0.2 \times 10^3$	$4.47 \pm 0.6 \times 10^4$	$1.67 \pm 0.3 \times 10^1$
100:02	$3.87 \pm 0.7 \times 10^5$	$2.21 \pm 0.1 \times 10^{10}$	$2.43 \pm 0.5 \times 10^5$	$2.70 \pm 0.5 \times 10^3$	$3.17 \pm 0.6 \times 10^4$	$2.77 \pm 0.2 \times 10^1$
100:05	$4.23 \pm 0.6 \times 10^5$	$5.60 \pm 0.5 \times 10^{10}$	$8.90 \pm 0.2 \times 10^4$	$2.93 \pm 0.2 \times 10^3$	$4.63 \pm 0.3 \times 10^4$	$3.30 \pm 0.6 \times 10^2$

biodegraded before the d 28, whereas *n*-C25 to *n*-C30 were only about 95% metabolized. By 56 d, total removal of all analyzed *n*-paraffin was observed, revealing that the major heavy oil fraction biodegradation might be related to the degradation of nonlinear hydrocarbon compounds. Figure 1C presents the biodegradation profiles of the total content of *n*-paraffin and some PAH compounds in the experiments conducted using 100:01 and 100:10 C:N ratios. It can be seen that the PAHs were less biodegradable than the *n*-paraffins, and that a significant increase in PAH degradation occurred with *n*-paraffin total consumption, suggesting preferential biodegradation of linear hydrocarbons, in agreement with the results presented by Del'Arco and de França *(7)*. Figure 1C also reveals that the PAHs with less aromatic rings, such as methylnaphthalenes and pyrene, were preferentially degraded in comparison with other PAHs, such as, benzo (*b*) fluoretane, as statedby Sepic et al. *(6),* who studied aromatic compounds from diesel biodegradation. Is important to emphasize that the proper C:N implementation promoted the increase in PAH biodegradation.

Table 4 presents the soil bacterial and fungal concentrations. Examining the initial microbial concentration data, one can verify that all the experimented conditions started with similar microbial concentrations. Despite the C:N ratio employed, an increase in the bacterial soil concentration from about 10^5 to about 10^{10} CFU and fungal soil concentration from 10^3 to about 10^4 CFU was observed during the first process days. After 56 d, microbial population decayed; however, a higher microbial concentration was observed at the 100:10 C:N ratio, in comparison with the other studied C:N ratios, which, as shown previously, also yielded the greatest heavy oil fraction removal. This can be explained by the maintenance of sufficient nitrogen available for the biodegradation of additional hydrocarbon compounds at lower C:N ratios, confirming the hypothesis of a nitrogen-limited process.

Conclusion

Experimental design tools were used to study the effects of process conditions on heavy oil fraction biodegradation losses during batch tests of crude oil soil bioremediation. The variables studied were bioagumentation seeding with an Nd mixed culture, macronutrient supply using mineral medium or inorganic fertilizer, and correction of initial pH of the soil to 7.0. A variable screening procedure showed that the oil removal depended primarily on the Nd mixed-culture seeding, whereas the use of fertilizer or mineral medium had a slight effect on oil biodegradation. It is concluded that the experimental design tool led to an increase in heavy oil fraction biodegradation losses from 24 to 40% after 56 process days. Subsequent bioremediation tests executed using bioaugmentation with Nd mixed culture, a single amendment of inorganic

fertilizer to provide a 100:10 C:N ratio, and initial *in natura* soil pH led to an increase in heavy oil fraction biodegradation losses up to 64% and total *n*-paraffin removal. Preferential degradation of linear hydrocarbons was verified by comparision to analyzed PAHs. Furthermore, biodegradation of aromatic compounds was increased with total *n*-paraffin consumption.

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