Appl Biochem Biotechnol (2009) 153:58–66
DOI 10.1007/s12010-008-8457-z

Bioremediation of Marine Sediments Impacted by Petroleum

Aike C. da Silva · Fernando J. S. de Oliveira · Diogo S. Bernardes · Francisca P. de França

Received: 22 May 2008 / Accepted: 26 November 2008 /
Published online: 16 January 2009

Abstract The aim of this work was to optimize the bioremediation of crude oil-contaminated sand sediment through the biostimulation technique. The soil was obtained in the mid-tide zone of Guanabara Bay, Rio de Janeiro, Brazil and was artificially contaminated with crude oil at 14 g kg^{-1}. Bioremediation optimization was performed using an experimental design and statistical analysis of the following factors: supplementation with commercial biosurfactant Jeneil® IBR 425 and commercial mineral NPK fertilizer. The response variable used was the biodegradation of the heavy oil fraction, HOF. The analysis of the studied factors and their interactions was executed using contour plots, Pareto diagram and ANOVA table. Experimental design results indicated that the supplementation with fertilizer at 100:25:25 C/N/P ratio and biosurfactant at 2 g kg^{-1} yielded biodegradation of HOF at about 30% during 30 days of process. Some experiments were carried out using the experimental design results, yielding 65% of biodegradation of HOF and 100% of *n*-alkanes between C15 and C30 during 60 process days. Intrinsic biodegradation test was carried out, yielding 85% of biodegradation of *n*-alkanes between C15 and C30 during 30 days of process.

Keywords Marine sediments · Petroleum · Bioremediation · Biostimulation · Intrinsic bioremediation

A. C. da Silva (✉) · D. S. Bernardes · F. P. de França
Escola de Química, Universidade Federal do Rio de Janeiro, Centro de Tecnologia, Bloco E, Ilha do Fundão, 21949-900 Rio de Janeiro, RJ, Brazil
e-mail: aikecosta@msn.com

F. P. de França
e-mail: fpfranca@eq.ufrj.br

F. J. S. de Oliveira
Petróleo Brasileiro SA, Avenida Almirante Barroso, 81, 24° andar, Centro, 20031-004 Rio de Janeiro, RJ, Brazil
e-mail: f.oliveira@petrobras.com.br

Humana Press

Introduction

Bioremediation is a technology that is being widely employed due to its low operational cost and the reduced environmental impact when compared to conventional physical and physicochemical cleanup methods [1]. Among the bioremediation techniques, biostimulation is one of the most effective. This process consists of the optimization of the contaminant biodegradation through nutrients and/or surfactant addition [2, 3]. This technique is showing better results than bioaugmentation and natural attenuation techniques [4].

Coastal sites are extremely sensitive environments that are habitats for different species, forming different biomes important to environmental quality. Some oil and oil derivative transport and storage operations are done in coastal sites, aiming cost reduction by using marine transportation logistic. Crude oil and petroleum derivatives are hazardous chemicals, presenting in their composition hydrocarbons, heavy elements, and other compounds [2, 5, 6]. So, coastal environmental protection and sites cleanup processes is an actual concern.

The importance of the present research is related to the fact that approximately 2% of world petroleum production takes place through offshore activity along the coast of Rio de Janeiro state, Brazil, which increases the risk of environmental disasters due to accidents that may occur during oil transportation and/or storage; it is also worth emphasizing the necessity of adequate treatment for the residues from this industry. Therefore, the purpose of this work is to contribute to a better understanding of the bioremediation process on beaches in tropical regions since the majority of research on this issue is done in temperate climates. For that, an oil spillage in marine sediment—obtained from the mid-tide zone—was simulated, and the soil was treated by biostimulation technique using nutrients and commercial biosurfactant.

Materials and Methods

Sediment Sampling and Characteristics of the Oil

The sediments collection was performed in Anil beach, in the northeast portion of Guanabara Bay (S 22° 48′52″, W 42° 08′46″), Rio de Janeiro, Brazil, at 5 cm depth in relation to the surface. This region constitutes an estuary located near a large urban center (metropolitan zone of the Rio de Janeiro city) where an oil refinery is located. The samples were stored at 4 °C prior to physicochemical and microbiological analyses.

An Arabian light crude oil, ALCO, was employed. The oil presented the following composition in percentage terms: saturated compounds 46.50, aromatic compounds 32.30, and polar compounds 21.27. The elemental composition expressed in percentage terms was 82.3 of carbon, 11.2 of hydrogen, and 0.0 of nitrogen.

Physicochemical Analyses of the Sediment

The organic carbon and nitrogen analyses were performed employing the method described and adapted by Verardo et al. [8] and Hedges and Stern [9] using the EA 1110 analyzer from Carlo Erba Instruments. The phosphorus analyses were performed by photometry under UV–*vis* [10].

HOF content was measured by gravimetric method and hydrocarbon quantifications by gas chromatography, according to the methodologies described in the sections 5520D and 5520F of the Standard Methods [11]. The extractions were made with CH_2Cl_2/CH_3OH

(93:7) azeothropic mixture for 6 h. Chromatography assays were done using a gas chromatographer HP 5890 II equipped with an automatic injector HP 7673, flame detector, and a phenyl-methyl-polysiloxane (5:95) column.

Aerobic bacteria enumerations were performed through the most probable number technique, MPN [12], employing nutrient broth (expressed in grams per liter: meat extract 3.00; peptone 10.00; K_2HPO_4 1.00 and NaCl 5.00), and incubating at 30±1 °C/48 h. Fungal quantification was performed through colony-forming units (CFU) using Sabouraud medium (Difco Laboratories, 0109) containing 0.04% of Chloramphenicol (Merk 1023660050) and incubation at 30±1 °C for 5 days.

Counting of indigenous oil-degrading microorganisms was done using the MPN technique, with the mineral medium described by Ridgway et al. [13]. The pH was adjusted to 7.4±0.2, and incubation was set to 30±1 °C for 7 days.

Bioremediation Experimental Design

Tests for the treatment of crude oil-contaminated sediment were performed using a central composite design. The statistical calculations were performed employing the Statistica TM 99 for Windows® version 5.5 software produced by Statsoft®.

The essays of the experimental design were performed in polyvinyl chloride, PVC, rectangular recipients with the following dimensions: 0.32 m×0.22 m×0.12 m in height. Each reactor was filled with 5.0 kg of sediment and contaminated with 70.0 g of the ALCO in order to obtain a proportion of 14.0 g of oil per kg of sediment.

The effects of the oil hydrocarbons' biodegradation were investigated according to the following variables:

1. Commercial biosurfactant concentration, containing 25% of rhamnolipids, from Jeneil® in the values of 0, 1, and 2.0 g/kg of contaminated sediment.
2. C/N/P ratio in the minimum values (100:10:10), medium (100:25:25), and maximum (100:50:50) using the commercial NPK 10:10:10 fertilizer, made by Ouro Verde Co.

Bioremediation Employing Experimental Design Results

From the results obtained in the experimental design, biodegradation experiments were performed with 60 days of duration employing the Jeneil® biosurfactant in the concentration of 2 g of product to 1 kg of sediment and the commercial NPK fertilizer in order to obtain the C/N/P ratio of 100:25:25, keeping the contaminant concentration at 14 g kg^{-1} of sediment.

Results and Discussion

The soil presented low levels of nitrogen and phosphorous, respectively 1.46±0.20 and 0.86±0.30 μg g^{-1}, indicating that it is a non-eutrophied environment. The reason for the low N and P values found is related to the small affinity between the chemical species and the sedimentary matrix [14, 15].

The result of the analysis of the organic carbon concentration in the sediment showed a value of 0.05±0.01 μg g^{-1} and a content of oil and grease of 6.0 mg g^{-1}. These values may not be related directly to contamination by oil hydrocarbons, but may be related to wax and resins present in estuarine environments that show marginal vegetation. In order to confirm

that hypothesis, an oil hydrocarbon analysis of the sediment was performed, and the value was null, indicating that the organic carbon was not related to petroleum contamination.

The quantification of total heterotrophic bacteria present in the sediment indicated the presence of $2.0\pm0.9\times10^5$ cells g^{-1}, and the total fungus analysis was $3.7\pm0.8\times10^2$ CFU g^{-1}. The presence of $2.0\pm0.4\times10^3$ cells g^{-1} of microorganisms able to degrade oil compounds was detected in the test of the Arab light crude oil degradation. Xu et al. [1] reported $2.0\pm0.5\times10^5$ cells g^{-1} of microorganisms present in sandy beach sediment from an area under environmental protection. These authors also found $1.45\pm0.5\times10^5$ cells g^{-1} of oil-degrading microorganisms, showing that 72.5% of the total microorganisms present in the sediment are able to degrade oil. Despite the fact that Xu et al. [1] work with sand beach soil, the percentage of oil-degrading microorganisms verified by these authors are very different to the results obtained in the present studied soil, indicating the importance of researches with local conditions.

Bioremediation Experimental Design

The factorial design is a sequential procedure with the purpose of screening important variables in the bioremediation, by means of which optimum process conditions may be reached.

Table 1 shows the experimental results of biodegradation of the HOF together with the process variables that were studied. Tests 5–6 are the central point repetition that were done to provide the opportunity of detecting measurement errors and to subsequently use the deviation in the calculation of HOF biodegradation to obtain the variance. One can note that there was variation in biodegradation of the HOF depending on the process conditions.

The HOF biodegradation results obtained from the essays were used to develop an empirical model to describe the experimental data. A quadratic model, Eq. 1, was proposed for biodegradation of the HOF.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_{11} + b_{22}X_{22} \quad (1)$$

where Y is the response variable; X_1, X_2 are the studied factors; b is constant of the modeling process.

A hierarchical regression was applied, which means that non-significant interaction effects were sequentially eliminated, and the main significant coefficients were retained, resulting in Eq. 2:

$$Y = 15.001 + 0.528X_1 - 0.007X_1^2 + 2.411X_2 + 0.008X_2 \quad (2)$$

where Y is the biodegradation of the heavy fraction of the oil; X_1 and X_2 are, respectively, the fertilizer and biosurfactant concentration.

Table 1 Experimental design matrix of bioremediation tests.

Experiment	Fertilizer (g kg^{-1})	Biosurfactant (g kg^{-1})	Degradation of the heavy fraction of the oil (%)[a]
1	11.50	0	20.12±1.26
2	11.50	2	25.13±1.45
3	57.50	0	21.34±2.98
4	57.50	2	27.10±1.45
5	28.80	1	27.12±1.56
6	28.80	1	26.55±2.21

[a] Abiotic losses were considered. Abiotic losses were 5.63±1.94%

Empirical model evaluation was done using an analysis of variance ANOVA (Table 1). The values of the constants b_0, b_1, b_2, b_{12}, and b_{11} listed in the table are related to the constant, linear, and quadratic terms of Eq. 2. All of these elements were considered in the mathematic model based on the values obtained in Test-*t* and Test-*p*.

The correlation measurements to test the adequacy of fit of the Eq. 2 are carried out using the multiple correlation coefficient R and the coefficient of determination, R^2. The fact that the value of R (0.9846) for the statistical model is close to 1 indicates a high degree of correlation between the observed and predicted values. The value of the coefficient of determination R^2 (0.9695) suggests that the model explains all, except about 4%, of all the variations. The low values for Test-*p* indicate that these terms are statistically significant for modeling the biodegradation.

Multiple regression analysis was used in order to evaluate the studied variables. Table 2 shows the statistical analysis of the estimated parameters. The p and F values (with 95% confidence interval) were used as tools to check the significance of each studied variable and their interactions. F and p values indicate that the biosurfactant was the most influential on the oil biodegradation. The quadratic term of the fertilizer is also significant, p=0.061, indicating that the indigenous microorganisms needed the inorganic nutrient to increase oil biodegradation.

The analysis of Table 2 data can also be carried out by using the Pareto diagram (Fig. 1). This figure indicates that the biosurfactant supplementation was very important to HOF biodegradation, reaching the estimated effect of 13.36. The other factors are marginal statistically significant results for the studied bioprocess. Biosurfactant addition is widely used in experiments of soil bioremediation and, therefore, is extensively reported in literature. Volkering [16] showed that the addition of a biosurfactant composed of rhamnolipids fostered the removal of 89% of the recalcitrant fractions such as phenanthrene, pristane, and phytane.

Analyzing the three-dimensional surface graphics, Fig. 2, one can observe that at the biosurfactant concentration of 2 g kg^{-1}, the highest values of biodegradation were reached, about 30%. On the other hand, the optimum fertilizer concentration was 35 g kg^{-1}, equivalent to a C/N/P ratio of 100:25:25. The biosurfactant presence increased degradation from 19% to approximately 30%, which represents an increase of 65% in the process efficiency. The fertilizer addition as a factor of biostimulation for microorganisms involved in the bioremediation process is widely used due to its low cost and high efficiency [17]. The increase in the ratio from 100:10:10 to 100:50:50 of C/N/P did not produce significant effects in the crude oil heavy fraction removal (Table 3).

After the selection of adequate biosurfactant and fertilizer concentration to the bioremediation process, 60-day-long experiments were performed by employing sandy sediment contaminated with ALCO in the proportion of 14 g kg^{-1} of sediment. Figure 3 shows the results of the experiments performed under a biostimulation condition. The

Table 2 Regression coefficients of model terms.

Factor	Regression coefficients	Standard error	$t(1)$	p value
b_1	0.528	0.052	10.210	0.062
b_{11}	−0.007	0.001	−10.238	0.062
b_2	2.411	0.363	6.637	0.095
b_{12}	0.008	0.009	0.930	0.523
b_0	15.001	0.795	18.864	0.033

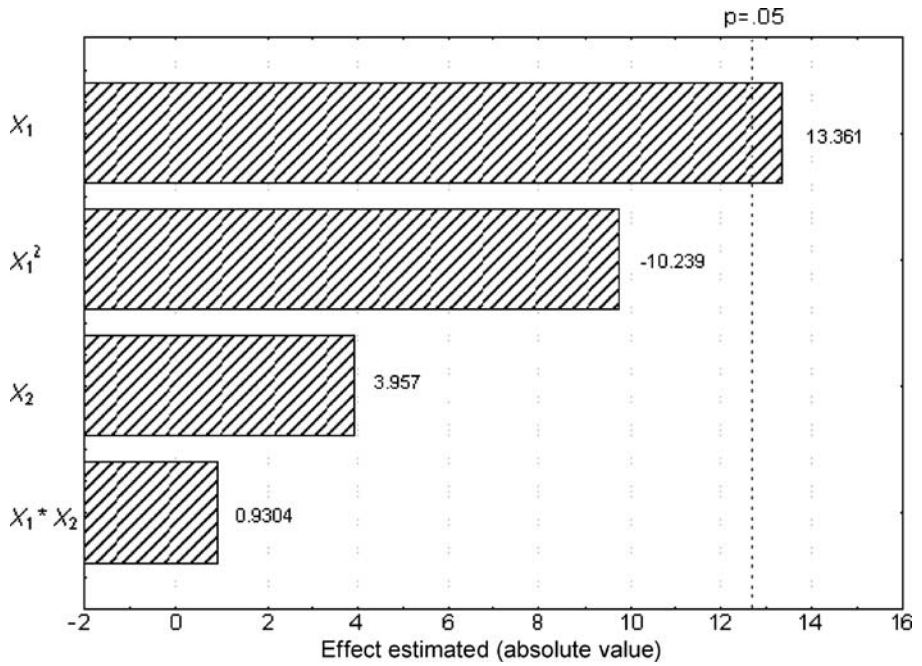

Fig. 1 Pareto diagram

removal of the HOF during 30 days of process was 35%, reaching 65% at the end of the experiments. During 30 days of experimentation, the HOF removal rate was 0.16 g day^{-1} when 5.10×10^{7} CFU g^{-1} of bacteria and 3.20×10^{5} CFU g^{-1} of fungus were detected. At the end of the 60 days, the bacterial concentration was 1.08×10^{8} CFU g^{-1}. Meanwhile, the fungus concentration was reduced, reaching the value of 1.87×10^{4} CFU g^{-1}. This is probably due either to the inability of these microorganisms to degrade the more recalcitrant organic forms present in the sample or to the formation of metabolites that are toxic for certain strains. During this period, the removal rate of the HOF was 0.17 g day^{-1}.

In the control test, the absence of microbial growth was verified, and the variation of the heavy fraction of oil was 4.96%. This value was taken into consideration in the experiments.

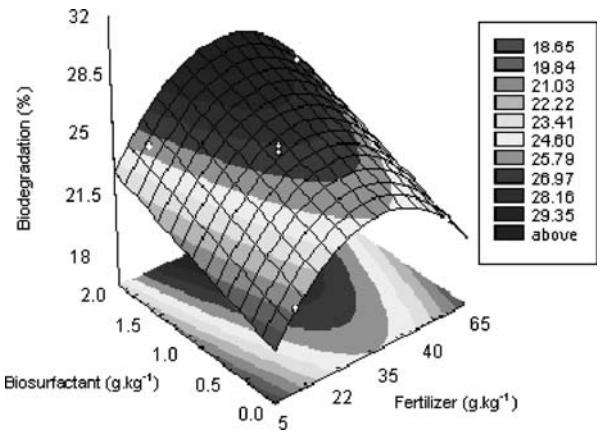

Fig. 2 Contour plots of oil biodegradation in a sandy soil contaminated with Arabian light crude oil

Table 3 ANOVA table.

Factor	Square sum	Degree of freedom	Mean square	*F* value	*p* value
X_1	2.544	1	2.544	15.660	0.158
X_1 by X_1	17.028	1	17.029	104.825	0.062
X_2	28.998	1	28.998	178.505	0.047
X_1 by X_2	0.141	1	0.141	0.866	0.522
Error	0.162	1	0.162	–	–
Total square sum	47.372	5	–	–	–

The results of the chromatographic analyses of soil extract are shown in Figs. 4 and 5. These figures show the behavior of the biodegradation of aliphatic hydrocarbons between C15 and C30 under biostimulation and intrinsic bioremediation conditions. One can note that after 30 days of process, in both situations, the biodegradation was significantly lower when compared to 60 days of process. In the biostimulation process, at the end of 30 days of experimentation, the percentage of hydrocarbon removal was 74%, and in the reactor of intrinsic bioremediation, this value was only 35.5%. In the intrinsic bioremediation tests, the HOF reduction from soil was 21.12% during 30 days of process and 47.30% at 60 days of process, minor than the results obtained from biostimulation tests. These results indicate the nutrient supplementation necessity to maintain the biodegradation of other fractions of the oil after paraffin consumption.

When biostimulation was employed, hydrocarbon metabolization was more efficient, also showing a higher velocity of microbial action due to the appropriate C/N/P ratio. In this case, some monitored alkanes were 100% biodegraded, except the C17 and C18 hydrocarbons that reached values of 95%. The intrinsic bioremediation process is generally used for environments with high sensitivity, where the processes and equipments employed

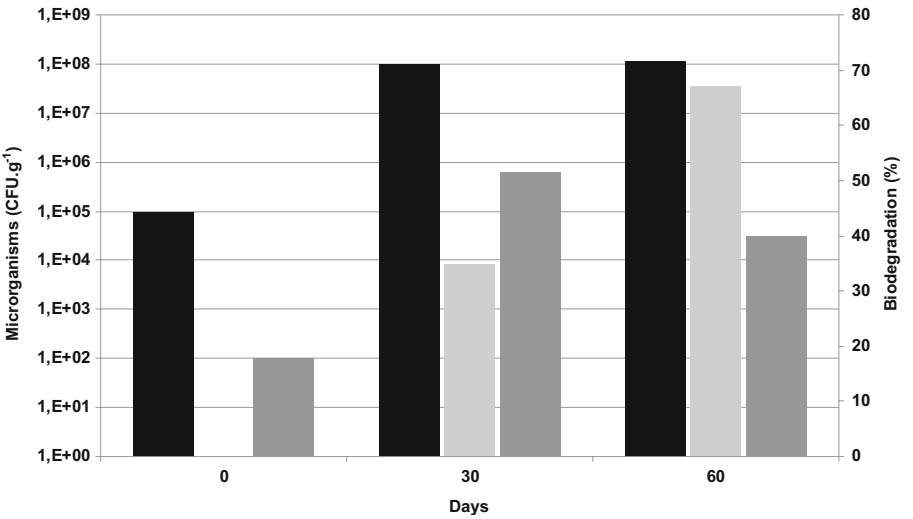

Fig. 3 Microbial population detected and degradation of the heavy fraction of oil during bioremediation of sandy soil under biostimulation condition. *Black bars* Bacteria, *gray bars* fungus, *white bars* oil biodegradation

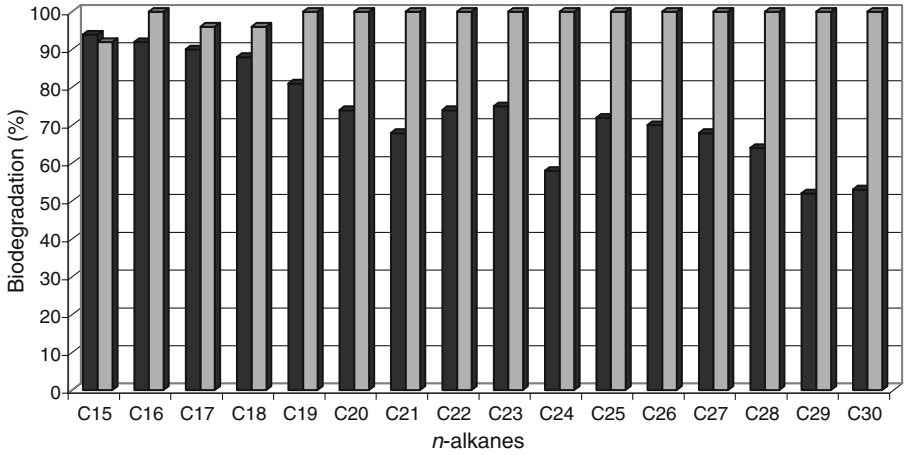

Fig. 4 Alkanes biodegradation in oil-contaminated sandy soil during biostimulation tests. *Black bars* 30 days, *gray bars* 60 days

can be as aggressive to the environment as the introduction of a contaminant agent [18, 19]. Roling [20] reported that an intrinsic bioremediation process will only be efficient if the site shows microorganisms able to degrade the contaminant, enough nutrients, and contaminant bioavailability. In the intrinsic bioremediation tests, these characteristics were present, including the presence of nitrogen and phosphorous. The biodegradation results corroborates those reported by Zhu et al. [7] who reported that the nutrients present in the interstitial water of a sandy beach located at an environment contaminated with 15 g of oil per kilogram of soil were enough for the bioremediation process. Hydrocarbon degradation results can also be related to indigenous microbial population adaptation since Guanabara bay is an estuarine environment with historical of oil spill.

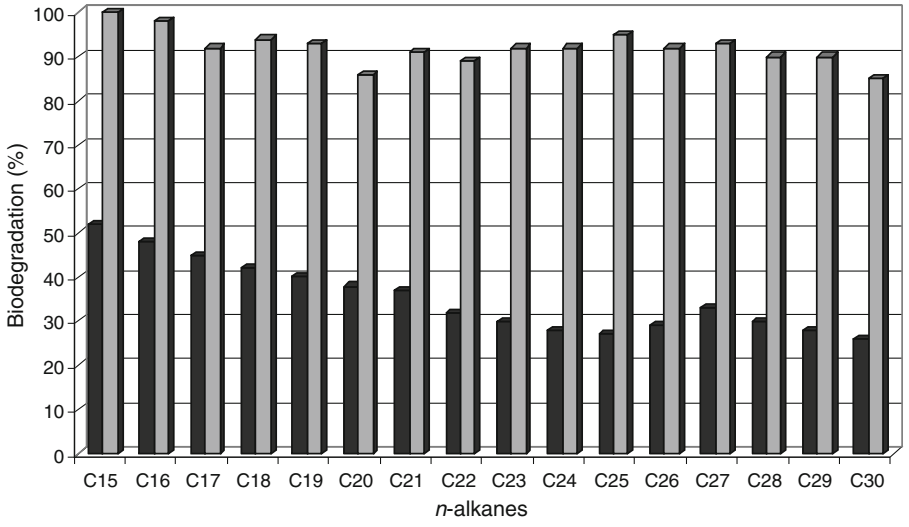

Fig. 5 Alkanes biodegradation in oil-contaminated sandy soil during intrinsic bioremediation tests. *Black bars* 30 days, *gray bars* 60 days

Conclusions

In this paper, the enhancement of oil-contaminated soil bioremediation by the use of biostimulation techniques was verified. Experimental design tools were used to study the effects of commercial fertilizer and biosurfactant on biodegradation of the HOF by indigenous microbiota. A variable screening procedure showed that the oil biodegradation depended primarily on the biosurfactant concentration, while the use of 25 to 45 g kg^{-1} of fertilizer had a slight effect on oil biodegradation. One can conclude that the experimental design tool led to an increase in the contaminants biodegradation—expressed as HOF biodegradation—from 20% to 30% at 30 days of bioprocess. Under the studied biostimulation conditions, the indigenous microbial population in the mid-tide zone of Guanabara Bay was able to degrade 100% of the *n*-alkanes between C15 and C30 and reduce 65% of HOF, at the end of 60 days of process. The intrinsic bioremediation tests showed a lower level of biodegradation, varying from 85% to 100% of the *n*-alkanes between C15 and C30 at the end of the 60 days of experimentation.

Acknowledgments Our thanks to Coordenação de Apoio a Pessoal de Nível Superior, CAPES, Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, and Financiadora de Projetos, FINEP, for the financial support. The authors would like to thank Petrobras for providing the Arabian light oil employed in this research.

References

1. Xu, R., Obbard, J. P., & Tay, E. T. C. (2003). *World Journal of Microbiology & Biotechnology*, *19*, 719–725. doi:10.1023/A:1025116421986.
2. NAS-National Academy of Sciences (2003). *Oil in the Sea: Inputs, Fates and Effects*. Washington DC: National Academy.
3. Cubitto, M. A., Moran, A. C., Commendatore, M., Chiarello, M. N., Baldini, M. D., & Sineriz, F. (2004). *Biodegradation*, *15*, 281–287. doi:10.1023/B:BIOD.0000042186.58956.8f.
4. Prince, R. C., Lessard, R. R., & Clark, J. R. (2003). *Oil & Gas Science and Technology*, *58*, 463–468. doi:10.2516/ogst:2003029.
5. Kennish, M. J. (1996). *Practical handbook of estuarine and marine pollution*. New York: Wiley.
6. Mills, M. A., Bonner, J. S., McDonald, T. J., Page, C. A., & Autenrieth, R. L. (2003). *Marine Pollution Bulletin*, *46*, 877–889. doi:10.1016/S0025-326X(02)00367-3.
7. Zhu, X., Venosa, A. D., Suidan, M. T., & Lee, K. (2001). USEPA., Cincinnati.
8. Verardo, D. J., Froelich, P. N., & Mcintyre, A. (1990). *Deep-Sea Research*, *37*, 157–165. doi:10.1016/0198-0149(90)90034-S.
9. Hedges, J. I., & Stern, J. H. (1984). *Limnology and Oceanography*, *29*, 657–663.
10. Hach Company. (1995). 531–538. Hach Company Loveland, CO.
11. APHA—American Public Health Association. (1992). *Standard methods for the examination of water and wastewater*. Maryland.
12. Oblinger, J. L., & Koburger, J. A. (1975). *Journal of Milk and Food Technology*, *38*, 540–545.
13. Ridgway, H. F., Safarik, J., Phipps, P., Carl, P., & Clark, D. (1990). *Applied and Environmental Microbiology*, *56*, 3565–3575.
14. Oliveira, F. J. S., & de França, F. P. (2005). *Applied Biochemistry and Biotechnology*, *121–124*, 593–603. doi:10.1385/ABAB:122:1-3:0593.
15. Lee, K., Stoffyn-egly, P., Tremblay, G. H., Owens, E. H., Sergy, G. A., Guénette, C. C., et al. (2002). *Spill Science & Technology Bulletin*, *8*, 285–296. doi:10.1016/S1353-2561(03)00042-2.
16. Volkering, F., Breure, A. M., & Rulkens, W. H. (1998). *Biodegradation*, *8*, 401–417. doi:10.1023/A:1008291130109.
17. Lacotte, D. J., Mille, G., Acquaviva, M., & Bertrand, J. C. (1995). *Chemosphere*, *31*, 4351–4358. doi:10.1016/0045-6535(95)00303-P.
18. Atlas, R. M. (1995). *International Biodeterioration & Biodegradation*, *35*, 317–327. doi:10.1016/0964-8305(95)00030-9.
19. Alexander, M. (1999). *Biodegradation and bioremediation*. New York: Academic.
20. Roling, W. M. F., & Verseveld, H. W. (2002). *Biodegradation*, *13*, 53–64. doi:10.1023/A:1016310519957.