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RESEARCH ARTICLE

Effect of Time and Density of Microorganisms on Bioremediation of Mangrove Sediment with Petroleum

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Abstract:

Bioremediation microbial consortium, isolated from mangrove swamp in the northeast region of the Todos os Santos Bay (BTS), Brazil, was tested to assess how time and cell density influence the bioremediation process. In this study, the experiments used a factorial design 22, where the main objective was to correlate the time variable with the consortium density as a function of the degradation of paraffinic oil. For this, an experiment was set up with 7 bioreactors containing mangrove sediment, paraffinic oil and immobilized microbial consortium. Microbial growth was measured using Colony Forming Unit (CFU) counting by serial dilution and total petroleum hydrocarbon (TPH) analysis was performed using the "whole oil" method, using a flame ionization detector - GC/ FID The results indicated that any time has a significant influence on the process and that reactor 2, for the proposed model, explains 93.4% of the bioremediation process data. The hydrocarbon removal was 56% after 30 days of experiment, using 9% of consortium while 3% removed 26%. This study provides a view that consortium density influences the final oil removal and that factorial design can be used to evaluate other important parameters for the bioremediation process.

Keywords: Biodegradation, Oil, Statistical planning, Microbial consortium, Contamination

Introduction

Mangroves are transition areas between the marine and terrestrial environment, playing a key role in climate regulation, coastal region protection and reproduction of marine fauna species. They are bioindicators of the quality and environmental health of any coastal ecosystem, sequester and store carbon over time [17, 13]. The oil spill in these ecosystems generates significant impacts on the environment and human health, due to the inherent properties, such as solubility, volatility and biodegradability [25, 10].

The toxicity of petroleum hydrocarbon can be acute or chronic. Acute toxicity is related to low molecular weight alkanes and aromatic compounds, on the other hand chronic toxicity is linked to polycyclic aromatic hydrocarbons (PAHs), which contains benzene ring, such as (pyrene, chrysene, benzene, anthracene), resistant to microbial degradation, persist in the environment and accumulate in the food chain [23, 7, 11, 25]. Currently, the increase in contamination of these compounds in mangrove sediment has been causing the disappearance of mangrove forests, affecting microorganisms present in the sediment. Fungi and bacteria present in these environments play a fundamental role in cycling nutrients such as carbon, nitrogen, and phosphorus, and are sensitive indicators of environmental changes [24, 8].

Studies indicate changes in microbial composition and diversity due to oil contamination [7]. In this situation, bioremediation is an ecologically and economically viable technique for the restoration of contaminated environments, as it accelerates the process of natural degradation carried out by microorganisms, and can be applied in coastal areas that have suffered oil spills [13, 9]. The use of factorial planning in the bioremediation process in a laboratory experiment contributes significantly to the application of the in situ technique, because it is an analytical tool for determining significant variables of a system, and can be applied in samples of a different nature, reduce the amount of tests and maintaining the quality of information and data accuracy [22, 5].

From the information obtained by planning, the factors that do not influence the process can be eliminated thus reducing time and cost, however, there are few bioremediations experiment work using factorial planning. In a biotechnological process the evaluation of the parameters involving the effectiveness of the process is very important for determining profitability. Therefore, it is necessary to optimize the density of microorganisms used in the bioremediation process, because their production directly affects the costs of the process, and it is extremely important to use microorganisms that in small concentration can remove a large amount of pollutant [2]. Thus, the use of microbial intercropping composed of varied species of fungi and bacteria during bioremediation contributes positively to a greater removal of the pollutant, since each species of microorganism has a preference for different fractions of petroleum hydrocarbons [4].

Bioremediation in a controlled experiment can evaluate the efficiency of the two main approaches of the technique, which can be applied through bioaugmentation, biostimulation and bioventilation, consists in the addition of strains of microbial community specialized in the environment, aiming to accelerate the degradation of the pollutant, the method is more effective when microorganisms are indigenous [4, 16, 25]. The biostimulation consists of the addition of nutrients (C, N and P) essential for the growth of microorganisms, stimulating the increase of the microbial population [4, 16].

Considering that the São Paulo River mangrove, located in the Todos os Santos Bay, Brazil presents chronic contamination by petroleum hydrocarbons, and bioremediation is a technique that can be applied to decontaminate areas with these characteristics, this work aimed to evaluate the influence of the variable consortium density and time on the degradation of petroleum hydrocarbons in bioremediation processes using a factorial design 22. The result obtained in this study can also indicate whether the bioremediation using standardized density (%) of autochthonous microbial consortium, for time, is a promising strategy for cleaning mangrove environments contaminated by hydrocarbons.

Material and methods

A bioremediation experiment of mangrove sediment with oil was mounted in the Laboratory of Excellence in Geochemistry Oil, Energy and Environment (LEPETRO), located at the Institute of Geosciences, at the Federal University of Bahia (UFBA). The sediment sample used was collected 10 cm from the surface with the aid of a stainless steel witness, homogenized in an aluminum basin, stored in aluminum marmites and packed in a thermal box at 4ºC.

Water was collected in the São Paulo river bed, with a plastic bottle of 20 liters. The collection area is located in the northern region of The Bay of All Saints (BTS), between the municipalities of São Francisco do Conde and Candeias, at coordinates 12°43'04.2"S and 38°32'50.4" W (Figure 1), near the Landulpho Alves refinery (RLAM), where oil activities are carried out. The microbial consortium used for bioaction were previously isolated from this area by [13, 6].

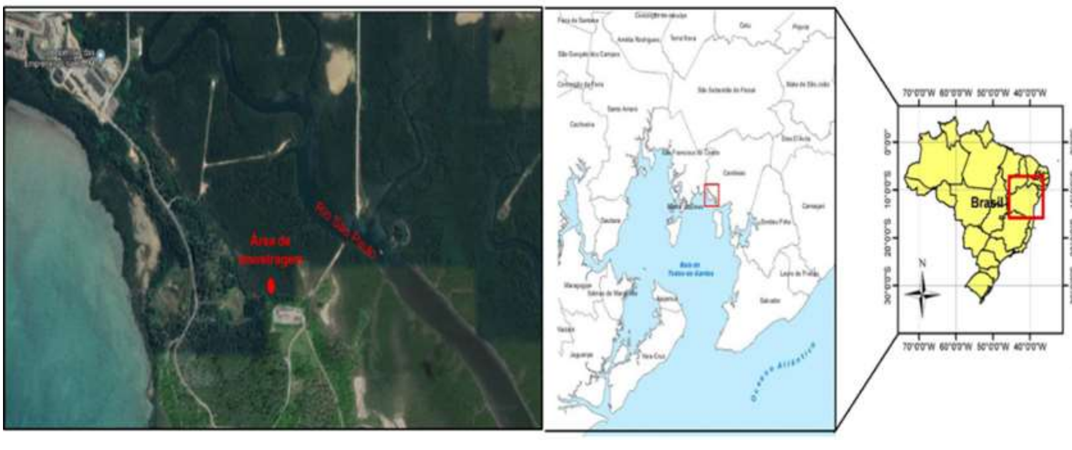

Figure 1 - Map of the location of the collection area in the estuary of the São Paulo River, Candeias - Bahia, Brazil

Source: Adapted from Google and Conder[26].

Microbial Consortium

The GEOMICRO1 microbial consortium (fungi and bacteria) was isolated from mangrove sediment samples from the study area by Lima et al, 2018 (Patent BR 1020210023414). The consortium was encapsulated (figure 2) using natural polymers, based on two substrates, whose constitution provides nutrients and physical support, protecting them against desiccation, predation and competition according to Lima (2014) (Patent BR 1020210033363).

Figure 2 - Microorganisms encapsulated based on two natural substrates
Source: LIMA, 2014

Factorial planning for bioremediation process

Factorial planning 22 was carried out to evaluate the percentage factors of intercropping and time in a bioremediation experiment of mangrove sediment with oil. Table 1 shows the control factors of the bioremediation process with their respective lower (-1), upper (+1) and central point levels in triplicate (0) with real and coded values.

Table 1 - Factors and levels used for factorial planning 2^2, in the study of TPH degradation in mangrove sediment. The -1, 0, and +1 values represent the encoded levels

FActors	-1	0	+1
Consortium (%)	(3%)	(6%)	(9%)
Time (days)	(4)	(17)	(30)

Source: Author, 2020.

Assembly of the experiment for bioremediation process

From the express experimental design (Table 1), a bioremediation experiment was set up. The proposed planning matrix was evaluated in the free software R version 3.6.0, with the Rstudio interface in version 1.1.463, using the quality Tools package (ROTH, 2016). The interpretation of the data and the construction of the Pareto chart were performed with the same package. The experiment consisted of 7 bioreactors as shown in Table 2.

Table 2 - Description of the amount of sediment, oil and consortia contained in the bioreactors and the length of stay in the system

TIME	BIOREACTOR	SEDIMENT	**CRUDE OIL**	CONSORTIUM
4	Bioreactor 1 (R1)	400 g	4 g = 1% sediment	12 g = 3% sediment
4	Bioreactor 2 (R2)	400 g	4 g = 1% sediment	36 g = 9% sediment
17	Bioreactor 3 (R3)	400 g	4 g = 1% sediment	24 g = 6% sediment
17	Bioreactor 4 (R4)	400 g	4 g = 1% sediment	24 g = 6% sediment
17	Bioreactor 5 (R5)	400 g	4 g = 1% sediment	24 g = 6% sediment
30	Bioreactor 6 (R6)	400 g	4 g = 1% sediment	12 g = 3% sediment
30	Biorreator 7 (R7)	400 g	4 g = 1% sediment	36 g = 9% sediment

Source: Author, 2020.

Sediment and water samples were autoclaved at 121ºC for complete sterilization. Two vacuum pumps (Figure 3) were used to move water from one pair of bioreactors to another, every 6 hours under the control of a digital time, the pump remained on for 1 minute, bubbling when reaching the sediment. The water movement aimed to simulate the regime of rising and falling tide.

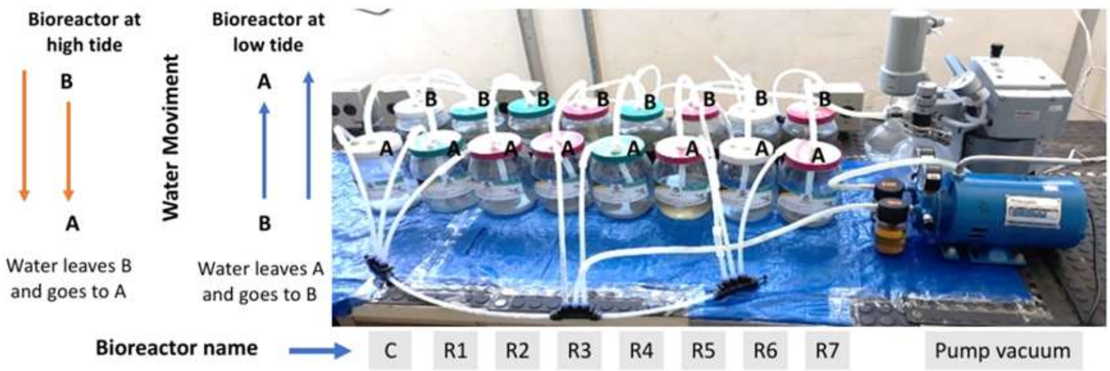

Figure 3 - Description of the movement of rising and lowering of the tide in the experiment of bioremediation of mangrove sediment with oil

Source: Author, 2020.

The experiment was collected at 4 days (R1 and R2), 17 days (R3, R4 and R5), and 30 days (R6 and R7). The procedure was performed in the laminar flow chapel, with the aid of a stainless steel spoon and the physicochemical parameters (temperature, pH and oxygen) were measured with a multiparameter probe in each bioreactor. Subsequently, the sediment was stored in glass pots previously autoclaved at 121 ºC for 15 minutes and decontaminated with dichloromethane (DCM).

Count of microorganisms

For the enumeration of colony forming units (UFCs), the serial dilution method 10-8, in which 25 g of sediment was inserted into a 250 mL Erlenmeyer containing saline solution in the proportion of 2.25 g NaCl and 750μL of Tween 80, the content was homogenized with a glass stick [18]. Subsequently, 0.1mL of dilution was transferred to microtubes containing 0.9mL of the solution that solution, this step was repeated until dilution 10-8. 0.1mL was removed from each dilution and transferred to petri dishes containing nutrient agar culture medium (Acumedia). The plates were stored in the incubator at a temperature of 30 ºC for 24 hours. After this time, the UFCs were counted with the naked eye.

Quantification of total petroleum hydrocarbons (TPH)

For quantification of TPHs, initially the sediment was lyophilized, disaggregated and sifted to 80 mesh. Solid/liquid extraction was performed using the DIONEX ASE 350 equipment, Thermo Scientific brand. At a temperature of 150 ºC, for 2 cycles of 5 minutes, using DCM, merck brand). The oil was filtered in a volumetric balloon, with the aid of a funnel containing calcined sodium sulfate (Merck brand), then the solvent was evaporated in Rotary Evaporator(R-200 Rotary Evaporator, BUCHI),then the oil was taken to the chromatograph.

TPHs analysis was performed using the whole oil method "whole oil" adapted by LEPETRO/UFBA, using a flame ionization detector (CG/FID, model 7890B, Agilent Technologies, California, USA), with the following conditions: Capillary chromatographic column model DB 1 (1% phenyl-polymethylsiloxane, 15 m x 0.25 mm , 0.25 μm); mobile phase flow rate (He): 1.0 mL/min; injector division ratio (split) 20/1, injected volume of 1 μL. The operating temperatures were: injector at 300 °C, detector at 300 °C and column at programmed temperature, starting at 40 °C for 2 minutes, followed by elevation from 10 °C/min to 300 °C, remaining for 12 minutes at this final temperature.

Results

Bioreactors play an important role in the detoxification of hazardous organic contaminants, such as hydrocarbons, as they are structures where living organisms perform biological reactions. Its main advantage is the control of environmental and physicochemical conditions, which affect the kinetics of microbial growth, oxygen, pH and temperature [1]. The mangrove sediment analyzed in this study was probably exposed to chronic contamination by petroleum hydrocarbons, since the sampling site is located near the Landulpho Alves refinery (RLAM).

Multivariate optimization for bioremediation process experiment

Factorial 22 of two-level factorial planning with triplicate at the central point was used to optimize the methodology for TPH degradation. Percentage factors of consortium (3%, 6% and 9%) and time (4, 17, 30). Figure 4 shows microbial growth in bioreactors over the experiment time. The lower, upper levels and the TPH/UCM ratio (analytical signal of the process) were expressed in Table 2.

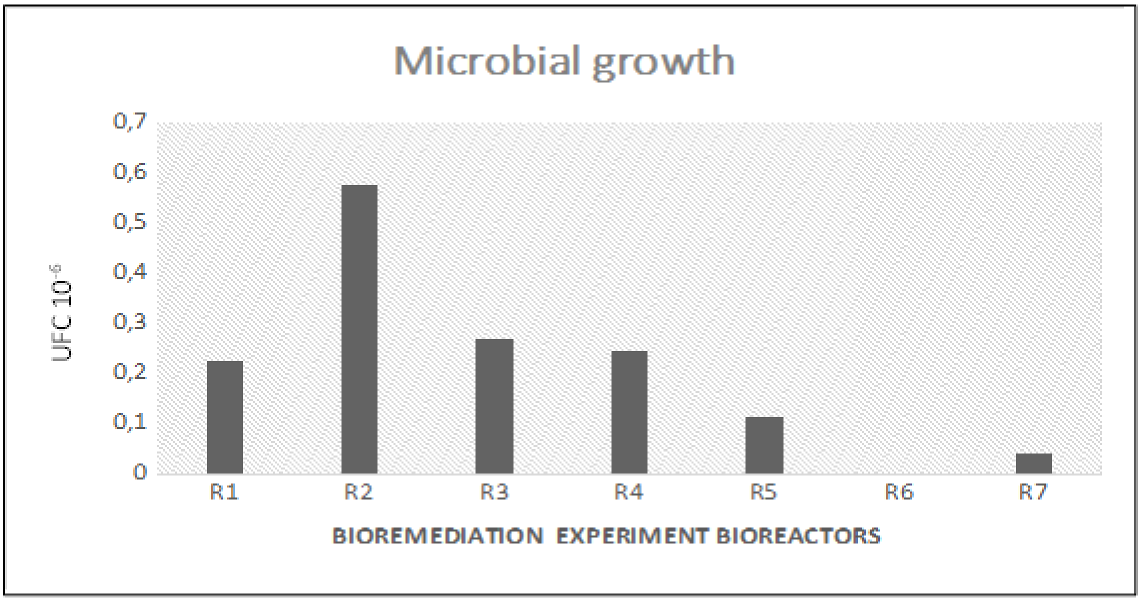

Figure 4 - Growth of the microbial intercropping during the bioremediation experiment
Source: Author, 2020.

The result of microbial growth in the experiment was proportional to the intercropping density inserted in each bioreactor in the initial times. Thus, the largest number of UFCs occurred in (R2), followed by reactors (R3) and (R4). After the seventeenth day, there was a decline of microorganism over time, on the thirtieth day there was a decrease in the growth of microorganisms (R7).

The physicochemical parameters remained in ideal conditions for the bioremediation process, the pH remained neutral around 7. Dissolved oxygen fluctuated significantly, with the lowest minimum percentage of 12.5% in R2, and a maximum of 66.5% in R7 (figure 5). Oxygen is a final acceptor of electrons in microbial metabolism, participating in the main ways of hydrocarbon degradation, and its limitation is one of the reasons for the reduction of oil biodegradation in mangroves [19].

The temperature remained at an average of 29.7ºC (Figure 5). This parameter exerts a strong influence on bioremediation, since it accelerates the chemical and enzymatic reactions of microorganisms, making them grow rapidly. The ideal temperature for biodegradation is 15 to 30°C for aerobic processes and 25 to 35°C for anaerobic processes [19]. Therefore, the temperature remained adequate to the process.

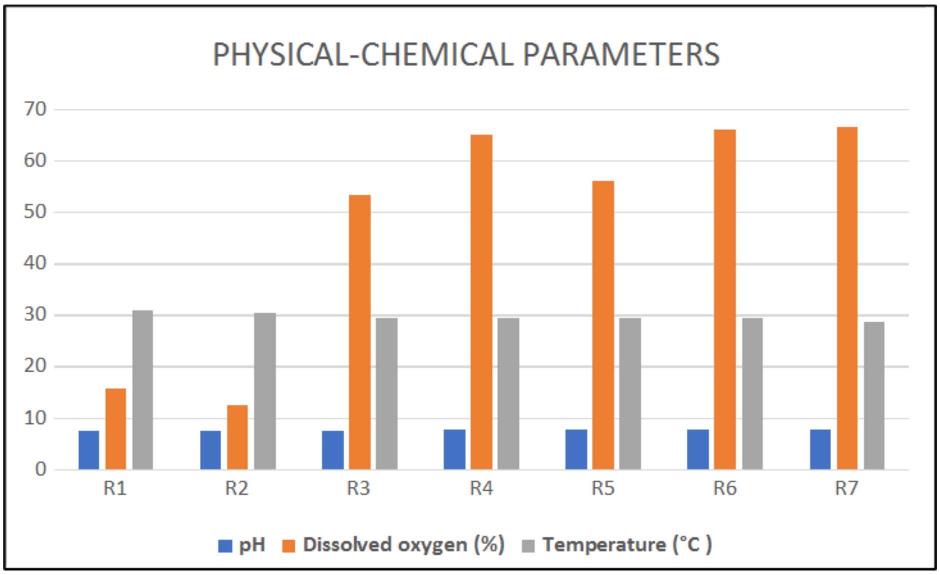

Figure 5 - Variation of physicochemical parameters during the bioremediation experiment
Source: Author, 2020.

The percentage of degradation was calculated taking into account the quantification in ppm of TPHs (Table 3) consumed during the experiment, using the formula:

$$\% \text{ removal} = \frac{\text{Co} - \text{Ce}}{\text{Co}}$$

The results showed that using 9% of consortium, it was possible to remove 56% of the TPHs present in the sediment in 30 days, confirming that higher cell density accelerates the bioremediation process by almost 50% in the same time interval.

Table 3 - % of TPH concentration removal in the simulated bioremediation process for mangrove environment. Co- initial concentration, Ce - final concentration

	TPHs Concentration (ppm) 3% OF CONSORTIUM	TPHs Concentration (ppm) 9% OF CONSORTIUM
Co	3568,28	3437,73
Ce	2528,13	1514,15
Co-Ce	1040,15	1923,57
Removal%	29%	56%

Source: Author, 2020.

UCM is the name given for the unresolved complex mixture, that is, it is the compounds that cannot be detected by the gas chromatography method [20]. The TPH/UCM ratio is used to evaluate the degradation of oil by microorganisms, so that as this ratio increases it means that there is more hydrocarbons, but the decrease in the value of this ratio indicates that the amount of UCM is greater than the amount of hydrocarbons, so TPH is being removed.
This reason has been used in several studies [13, 12, 6, 3], to determine the degree of TPH degradation by microorganisms during bioremediation. The effects plot is used to evaluate the influence of factors on the optimization process. The amount of intercropping is influenced by time, since the smallest percentage of consortium

(3%), requires more time for biodegradation (30 days), as shown in Table 4. Similarly, the higher amount of intercropping (9%), requires less time (4 days) for the degradation of oil in mangrove sediment. Table 4 corroborates this statement, because the experiments (R2, R3, R4, R6) presented the lowest TPH/UCM ratio, indicating higher degradation.

Table 4 - Coded levels, real values and TPH/UCM ratio obtained in factorial planning 22, with triplicate of the central point, to evaluate TPH degradation in the simulated bioremediation process for mangrove environment

Bioreactor	Consortium (%)	Time (dias)	TPH/UCM
Bioreactor 1 (R1)	-1 (3)	-1 (4)	2,32
Bioreactor 2 (R2)	1 (9)	-1 (4)	2,20
Bioreactor 3 (R3)	0 (6)	0 (17)	2,21
Bioreactor 4 (R4)	0 (6)	0 (17)	2,21
Bioreactor 5 (R5)	0 (6)	0 (17)	2,25
Bioreactor 6 (R6)	-1 (3)	1 (30)	2,20
Bioreactor 7 (R7)	1 (9)	1 (30)	2,24

Source: Author, 2020

According to this figure 6 (a), the time factor has a negative influence on biodegradation. With pareto graph analysis, it was possible to evidence that the interaction of factors (intercropping and time) is significant with positive effect, as well as the time factor was more significant with negative effect exerting greater influence on the bioremediation process, presenting a significance level of 95% ($p < 0.05$). The analysis of variance (ANOVA) (Table 5) was used to verify the significance of the factors and the linear model at a significance level of 95% ($p < 0.05$) for factorial planning 2^2.

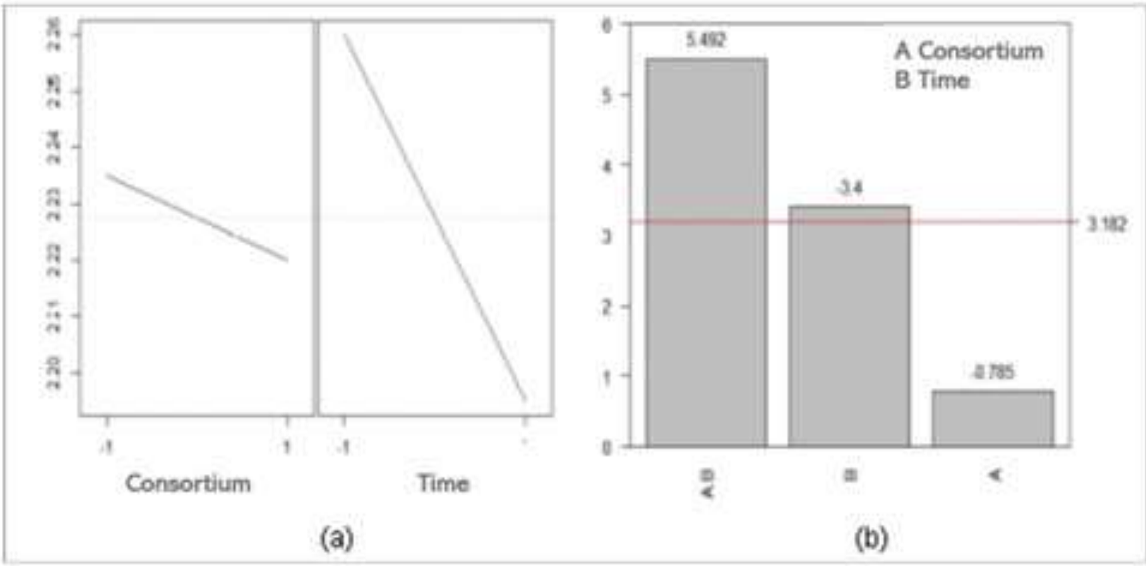

Figure 6 - Effect Plot (a) and Pareto (b) for percentage ratio of consortium and time
Source: Author, 2020.

Table 5 - Variance analysis for the linear model adjusted the multiple response to a 95% confidence level

Parâmeters	G.L.	Sq	Mq	Fcalc	Ftab	p-value
A	1	0,0002	0,0002	0,62	10,13	0,49
B	1	0,0042	0,0042	11,56	10,13	0,04
A:B	1	0,011	0,011	30,17	10,13	0,01
Resídue	3	0,001	0			
Linear regression						
A, B	2	0,004	0,002	0,73	6,94	0,54
Resídue	4	0,012	0,003			
Lack of adjustment	2	0,011	0,006	10,36	19,00	0,09
Pure error	2	0,001	0,001			

Through A = Consortium, B = Time, G.L. = number of degrees of freedom, Sq = quadratic sum, Mq = quadratic mean, Fcalc = calculated F test value, Ftab = tabled F test value.
Source: Author, 2020.

Complete factorial planning provides a lot of information about the factors identifying the interactions between them and the effects that different interactions can have on the experimental response (APARÍCIO et al., 2018). In this study, it was observed through Table 4 that the time factor and the interaction of the factors were significant for the bioremediation process, thus corroborating the Pareto graph. ANOVA also pointed out that there was no lack of adjustment for the regression model proposed in factorial planning 2^2 at a significance level of 95%. In order to measure the contribution of each factor and their interaction to describe the simulation of the bioremediation process, a linear model was constructed (Table 6).

Table 6 - Coefficient of the linear regression model for the set of planning tests 2^2

Parameters	Coefficient	standard Error	p-value
Intercept	2,23	0,01	7,55x10-8
A	-0,01	0,01	0,49
B	-0,03	0,01	0,04
A:B	0,05	0,01	0,01

Source: Author, 2020.

Discussion

A study conducted by [20] evaluated the spatial distribution of TPHs in surface sediments of the BTS tidal zone, and found that the determining factor for the distribution of these organic pollutants is their proximity to the sources of contamination, thus, the study divided BTS into three areas, of which the highly contaminated area with higher concentrations of TPH in mangrove sediment samples is associated with the São Paulo River near the refinery. The second area located in the Mataripe River is also influenced by the refinery, and can be considered moderately contaminated, and the third area is slightly contaminated by hydrocarbons. According to [23], research on the degradation of hydrocarbons by autochthonous microorganisms in mangrove sediments is very important, because knowledge about biodegradation rates and removal efficiency helps in the development of sustainable bioremediation technologies.

This may have occurred due to not biostimulation of the consortium with insertion of essential nutrients such as Nitrogen, Phosphorus and Potassium. Or with the bioavailability of oil, because in the initial days it was more accessible to microorganisms and over time, the oil was adsorbed in the clay minerals present in the mangrove sediment, thus decreasing its bioavailability for microorganisms. Studies conducted by [13, 14, 6, 3], evaluated the oil degrading potential of the microbial consortium isolated from the mangrove sediment of the São Paulo River, through microcosm experiments, these studies showed that the consortium used in this work degrades different fractions of oil.

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The effectiveness of bioremediation of a given matrix depends on the influence of several physicochemical factors and their interactions [2], the use of bioreactors is extremely important for controlling physicochemical conditions, making microbial activity improved [21]. The bioremediation study performed [21] showed that the use of higher temperatures (35ºC) in a bioreactor containing soil sample with diesel oil, impaired biodegradation causing a negative effect. The study concluded that the best condition for oil removal occurred with the temperature at its lowest level (25°C).

The regression model has the function of investigating the relationship between random variables of interest (response variable, Yi, i = 1, 2..., n) with a set of explanatory or exploratory variables (xi, i= 1, 2..., n). Thus, it is possible to predict values for the variable of interest, as well as evaluate the influence of exploratory variables on the response variable. For this to occur the regression model must present a good fit, so R2 indicates the best fit for the model, because the closer to one that value, the better the model fit. Thus, R2 for the proposed model was 0.934, i.e., the model explains 93.4% of the data from the bioremediation process.

Factorial planning has been successfully applied to screening of significant factors for further optimization biodegradation process of oil and petroleum products. The work carried out by aimed to determine the most favorable conditions for the biodegradation of a marine fuel by a microbial consortium, through a bioremediation experiment. A complete factorial planning 23 was applied to determine the best conditions. At the end of the experiment, the results showed that the consortium used degraded a total of 93.5% of the hydrocarbons in the experimental project and reached optimized degradation levels in the bioreactor test for the fuel constituents, and can be used as a bioremediation agent of environments polluted by this fuel [1].

A job used the complete factorial planning 24 in a bioremediation experiment of soil contaminated with chromium and lindane to evaluate four factors (temperature, humidity, initial concentration of Cr (VI) and lindane), each at two levels, plus a central point. The evaluated responses were the final concentrations of lindane and Cr. According to the response optimizer, the ideal humidity level was 30% for all bioremediation processes. The factorial planning was adequate for the study of bioremediation, allowed to predict with great precision the result of the activity of the consortium during the bioremediation of contaminated soils, knowing the initial conditions of the system [2].

The study with a bioremediation experiment carried out by (SILVA et al., 2015) used the experimental factorial design with 3 factors and two levels (2^3), to evaluate the effects of nitrogen concentration in the medium, temperature (25°C or 35°C), and the conditions of the C: N ratio more favorable to biodegradation. As a result, factorial planning showed that the ratio C: N and temperature were the factors that had a significant effect on the bioprocess. The increase in ratio C: N and the amount of inoculum produced a positive effect, while the increase in temperature caused a negative effect on biodegradation.

Thus, the cited studies find that statistical planning is a useful tool to evaluate the best conditions in various experiments, including bioremediation experiments, reducing time, costs with the experiment and improving process yield. In the present study, it was possible to verify the statistical significance of the variables time and percentage of intercropping during the bioremediation experiment, obtaining the response more quickly, at a confidence level of 95%.

Conclusion

Factorial planning is a useful tool for bioremediation experiments, in this work it proved satisfactory for evaluation of the variables time and density of consortium, explaining 93.4% of the data, showing as a result that time exerts greater influence than the percentage of consortium in the bioremediation process. The relationship between time and percentage of consortium was significant, as, with the increase in the percentage of consortium, the degradation of oil occurs in a shorter time. Other important parameters for the bioremediation process, such as pH, temperature, nutrient content, should be evaluated in future works, as well as the concentration of petroleum hydrocarbons, so that other parameters can be optimized and thus improve the bioremediation process.

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