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Oil bioremediation in soils contaminated with oil spills in tropical environments

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Abstract: Bioremediation techniques like bioaugmentation and/or biostimulation are an economical and environmentally friendly procedure which emerged as the most advantageous methodology for treatment of contaminated sites by oil spills pollutants. This research uses a tropical soil contaminated with oil based drilling fluids (OBMs) and drill cuttings were evaluating at laboratory scale. Seven treatments were implemented separately: (C) control; (A) natural attenuation; (B) compost (Bs) nutrients; (BsT) nutrients and tween 80; (BsTL) nutrients, tween 80, leonardite, and (BL) nutrients, tween 80, leonardite and d-limonene. For three months, changes in Total Petroleum Hydrocarbons (TPH) soil microbial counts and activity were monitored as indicators of biodegradation. In order to evaluate the efficiency of treatments in the microcosm experiments. After 90 days of incubation hydrocarbon biodegradation is 76.2% (C), 28.6% (A), 76.2% (B), 66.7% (Bs), 83.3% (BsT), 69% (BsTL) and 88.1% (BL), respectively. Scanning electron microscopy (SEM) of OBMs evidenced absence of heavy metals. Biodiversity analysis showed a decrease in bacterial diversity and a rise in tolerant genus of hydrocarbons such as *Nocardiodes*, *Streptomyces*, *Dietzia* and *Advenella*. The co-substrate and stimulants had synergistic effect on the biological degradation of hydrocarbons. This research suggests that the implementation of bioaugmentation and biostimulation methods will be used a larger scale in contaminated sites.

Key words: Biodegradation, bioaugmentation, biostimulation, oil based drilling fluids (OBMs), total petroleum hydrocarbons (TPH).

INTRODUCTION

Soil is one of the most important parts of the natural environment and largely non-renewable. World-wide, all economies depend on the goods and services provided by the natural environment. Soils as a natural resource perform a number of key environmental, social and economic functions (Blum 2005). Soil degradation is considered a major issue of the modern era because it poses a serious threat to human well-being, and must be viewed in terms of its adverse effects on present or potential soil functions (Lal et al. 1998). The soil is currently a final destination of waste from several industries such as: agriculture, construction, and petrochemicals among others (FAO 2015) . The petrochemical industry makes the largest ecological footprint, because its operation stages have a big interaction within the environment; notwithstanding drilling phase presents a variety of impacts, with high responsibility on muds used and final waste. The resulting oil spill that could be into the soil (onshore) environments are very toxic and hazardous to the environmental ecosystem and could adversely affect the well-being of living organisms, air, water, and soil processes as well as the potential of fire hazards (Akhundova & Atakishiyeva 2015, Ojewumi et al. 2018). These fluids generate negative effects on human health

(Adekunle et al. 2013), due to their carcinogenic and mutagenic properties (Koul & Fulekar 2013). In the last years, use of petrochemical compounds are rising in drilling processes. In Ecuador and Mexico since 2001 have drilled more than 13,000 wells (AIHE 2017, PEMEX 2013) and more than 1100 wells in Colombia (ANH 2014) which produce (2012-2015 period) 3.354.886 barrels of cut and solids (ECOPETROL 2017). Drilling fluids and residues are aqueous colloidal phase formed by fluid compound oil (hydrocarbons), water, clays and several chemical additives (Adekunle et al. 2013). Analyzing the large volume of wastes produced and inefficient final disposal with the latent risks to human health is a priority intervention (Tahhan & Abu-Ateih 2009, Kogbara et al. 2016). There are a larger number of published studies (Balba et al. 1998, Alavi et al. 2014 , Akhundova & Atakishiyeva 2015, Ojewumi et al. 2018) that focused on microbiological methods in oil contaminated soils and strategies of decontamination with relation to drilling fluids and cuttings. Surveys such as those conducted by (Steliga et al. 2012, Akhundova & Atakishiyeva, 2015) have a shown a significant decrease in oil concentration and high biodegradation through bioaugmentation. Previous studies (Riojas et al. 2011, Steliga et al. 2012, Akhundova & Atakishiyeva 2015, Organiksa & Narpes 2016, Cheng et al. 2017) showed that non-ionic surfactants such as Tween 80, natural oils like D-limonene, biosurfactants, humic and fulvic substances promote degradation of contaminants. The present study intends to evaluate the effectiveness of aerobic bioremediation at laboratory scale from: (i) the Total Petroleum Hydrocarbons (TPH) biodegradation from native microbial communities residing in soil contaminated with oil based drilling fluids via biostimulation and/or bioaugmentation, (ii) the effect of specific treatments on intrinsic microbial community amount, activity and biodiversity. Three methods of biodegradation are employed: natural attenuation, bioaugmentation and biostimulation. For the experimental plots periodic additions of stimulants; Tween 80, Leonardite, D-limonene and Molasses were implemented. The combination of treatments and stimulants attempt to improve TPH removal efficiency of native organisms in contaminated soils with oil based drilling fluids (OBMs). The effect of different treatments on the biological degradation of hydrocarbons provided a powerful insight on nutrient-induced native microorganisms dynamics community in soil contaminated by sludge during TPH removal, which might be useful in designing bioremediation strategies for the treatment of oil spills.

MATERIALS AND METHODS

Soil sampled

Soil used in the experimental phase was collected at Universidad Nacional de Colombia – Campus Medellin according to IDEAM protocol (IDEAM 2007). The soil type is an incipient development soil (Inceptisols) classified as Typic Dystrudept. This soil type belongs to soil association unit Tequendamita. The soil has a low soil moisture retention and well drained, low aluminum toxicity, low fertility, low bases saturation, high acidity and cation exchange capacity. These soils may likely have high rates of mineralization of organic matter, and the predominant soil textures are clay loam and loam (IGAC 2007). Soil physicochemical characteristics are showed in the Table Ia. Samples were taken at three different points and the top layer (0–40 cm) of the soils was removed. To ensure homogeneity of pollutants in samples, the dry soil sample was crushed and then passed through a 2-mm sieve. Soil properties were measured using

standard methods for soil analysis. Soil texture was characterized using the Bouyoucos method (Bouyoucos 1962). Soil water content was determined by placing samples in an oven at 105 °C for 24 h (Jackson 1964). Organic matter (Walkley & Black 1934), pH, ammonia, nitrate (Yuen & Pollard 1954) and phosphorus content (Bray & Kurtz 1945) were assessed previously. The soil sample was mechanically incorporated with OBMs and a cutting (Contaminated Soil (CS)) was obtained from the Floreña Oil Field located in the Municipality of Yopal, Departament of Casanare in Colombia, see Figure 1. To guarantee homogeneity of the resulting contaminated soil, it was sieved with a 2 mm mesh.

Experimental setup

In the laboratory experimental microcosms plots were used in aluminum trays. Each sample had 2kg of mixed soil with three replicas by treatment. Seven treatments were evaluated: (C) soil + HCl (2M), (A) soil + H_2O, (B) soil + H_2O + compost

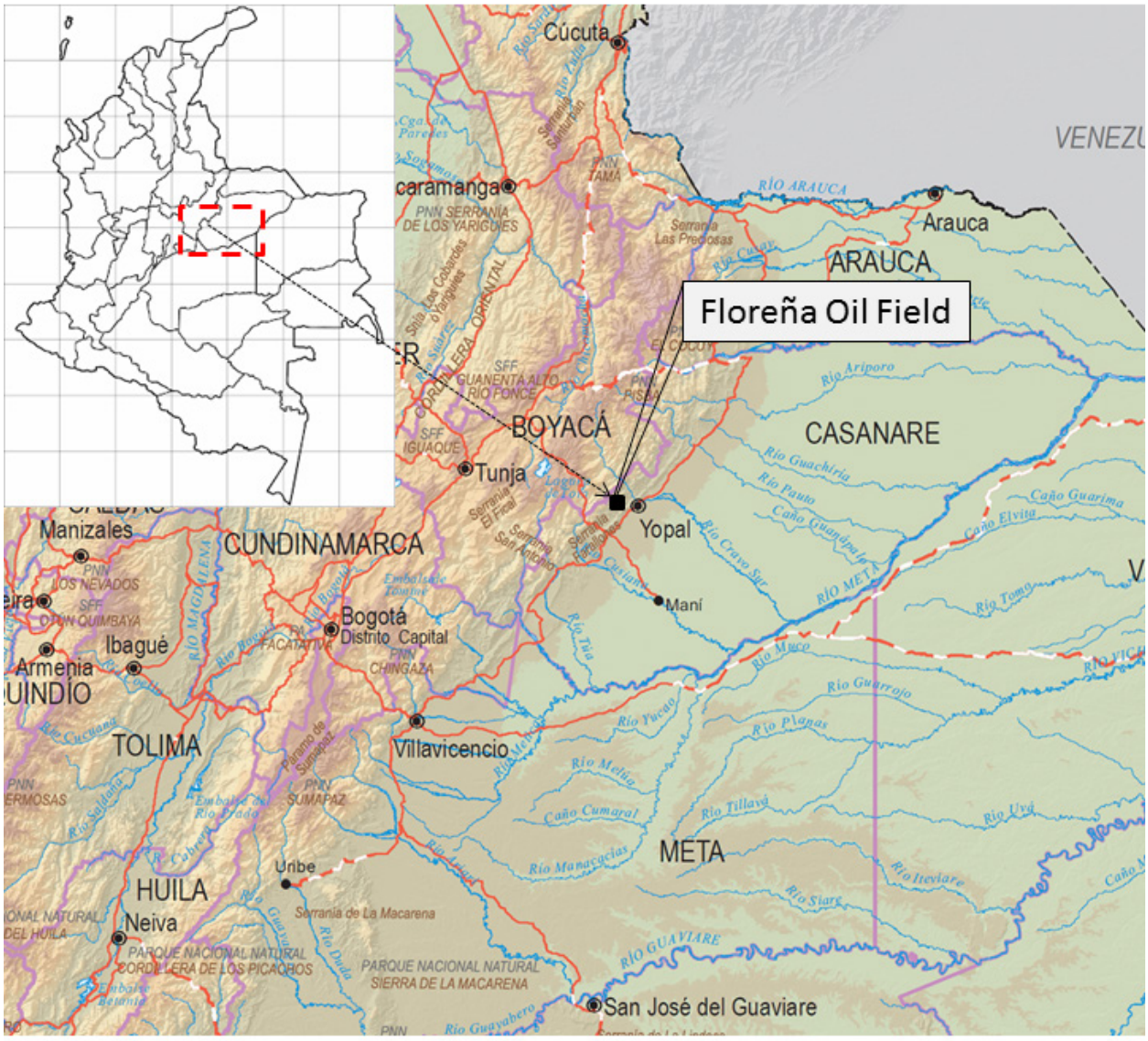

Figure 1. Localization of soil, OBMs and cuttings sampled sites.

(10%), (Bs) soil + H_2O + urea + $Na_5P_3O_{10}$, (BsT) soil + H_2O + urea + $Na_5P_3O_{10}$ + Tween 80, (BsTL) soil + H_2O + urea + $Na_5P_3O_{10}$ + Tween 80 + Leonardite and (BL) soil + H_2O + compost (10%) + urea + $Na_5P_3O_{10}$ + Tween 80 + Leonardite + D-limonene. The components were mixed and kept for 90 days in a closed room without direct sunlight at 20% substrate humidity, adding compost ($1.8 * 10^8$ CFU/g) to the treatments B and BL to increase bacterial density. Nutrients addition was made by the equation proposed by Rittman & McCarty (2001) based on the physical-chemical characterization (table Ia), giving a C:N:P ratio of 100:12:2. The amount of Leonardite was added at 3% p/p according to Trejos (2017). The molasses was been used in treatments Bs, BsT, BsTL and BL, and the amount of Tween 80 and D-limonene was obtained from a pretreatment performed for 5 concentrations in the CS soil with MCC – 25 MCC – 50 MCC – 75 MCC – 100 MCC (Micellar Critical Concentration) according to (Orantes 2002) to increase the bioavailability of the contaminant, the more efficient concentration of the stimulants to promote bacterial activity was 392.25 mg T80/L each 14 days. The control (C) treatments contains HCl (2M), the main function to the control is to show the inhibition of microorganisms populations in the soil at the same period of time than the other experimental plots.

Analytical methods

The pH values were measured weekly using an HQ 40d multi-parameter (HACH®), through dilution method (1:10) mixing 2g of dry soil with 20ml of distilled water, which was later taken to a vortex mixed (FALC®) for 90 seconds, and the measurement was made introducing the electrode in the final mixture. Substrate moisture content was periodically evaluated using a hand-held digital thermometer in order to keep it constant at 20%. The calibration of digital thermometer (OMEGA®) was using the Reemt et al. (2017) methodology.

The measurement of respiratory activity of the substrate was carried out following the methodology used by Celis et al. (2009), an indicator to evaluate the carbon dioxide production of the microorganisms present in the soil, which is related to the efficiency of biodegradation process (Celis et al. 2009, Riveroll-Larios et al. 2015).

The dynamic of populations of heterotrophic and hydrocarbon oxidizing bacteria was evaluated weekly by surface planting on nutritive and selective agar respectively (Sieuwerts et al. 2008), using hexane as a carbon source (Steliga et al. 2012). A dilution was composed of 1g of soil in 9 ml of sterile distilled water prepared in a tube (10^{-1}); 1 ml of this solution was transferred to another tube with 9 ml of sterilized distilled water (10^{-2}). The process was repeated until reaching 10^{-5} dilution for the seeding of heterotrophs and hydrocarbon-oxidizing (Trejos 2017). Plates were incubated at 30°C for a period of 2 days and 5 days respectively, according to the methodology developed by Atlas & Bartha (2001).

Hydrocarbon extraction from soil: Recovery of the Total Petroleum Hydrocarbons (TPH) in the sample was carried out by agitation and centrifugation method Schwab et al. (1999) approved by the United States Environmental Protection Agency (EPA). In propylene vials of 50 ml, 1g of soil and 2.5 g of anhydrous Na2SO4 were added to centrifuge MR22 (JOUAN ®). Afterwards 5 ml of dichloromethane was placed in each vial. The mixture was placed in stirrers Vortex vibration, and mixed (FALC®) for 90 seconds and an electrode was inserted next in the solution. Soil temperature was measured using a hand-held digital thermometer (OMEGA®). Later the samples were centrifuged in the MR22 centrifuge (JOUAN ®) at 7000 rpm for 20 minutes,

subsequently; washing process was performed 3 times on the remaining solid residue until reaching a supernatant volume of 15 ml. A rotary evaporator (Heidolph®) was employed, working at a temperature of 40ºC and pressure of 740 mm Hg to break the bond between the organic extract and the solvent. Samples were stored in the laboratory refrigerator (Revco®) at a temperature of -5ºC before analysis.

Gas chromatography coupled to mass spectrophotometry was used (US EPA, 1996), an 6890N series chromatograph (Agilent®) was employed with an Agilent Technologies mass selective detector 30 m long, 0.32 mm in diameter and a packaging film of 0.25 µm. The initial temperature was 60ºC during 2 minutes; later temperature was increased 8ºC per minute reaching a limit of 300ºC, remaining there for 8 minutes. The injector temperature was maintained at 250ºC and detector temperature was stored at 340ºC; hydrogen was used as a stripping gas at a constant flow rate of 2 ml per minute. The contaminant concentration in the samples was identified and quantified with a standard containing mixture of C8 to C40 aliphatic hydrocarbons S–4149–500–MX (Chiron AS®) with a total of 35 analyses resuspended in carbon disulphide dichloromethane (3:1).

Scanning Electron Microscopy analysis: The OBMs and cuttings sample placed in Scanning Electron Microscopy (SEM) were initially performed using a Carl Zeiss EVO MA10 SEM microscope. After that samples were coupled to the Oxford x-Act detector, using the AZtecEnergy software, where they were covered with gold due to its non-conductive nature. Evaluation was carried out to verify mainly high concentration of heavy metals. Scanning electron microscopy was performed to demonstrate the presence of heavy metals associated with OBMs and cuttings (Kisic et al. 2009). From the previously dried random sample, 3 spectrograms were made for each zone (A, B, C) as shown in figure 2.

Analysis of bacterial diversity

A total of 3 samples were analyzed; the extraction and purification of total bacterial DNA were performed using PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA). This DNA was used as a template for the partial amplification of the 16S ribosomal gene by oligos 27F: 5'AGAGTTTGATCCTGGCTCAG3' and 1492R: 5'TACGGYTACCTTGTTACGACTT3'. For the sequencing of this gene, Bakt_341F CCTACGGGNGGCWGCAG, Bakt_805R ACTACHVGGGTATCTAATCC oligos were used to amplify an approximate fragment of 490 bases. Amplicons analysis were performed with the MOTHUR program version 1.39.5 of Department of Microbiology & Immunology at The University

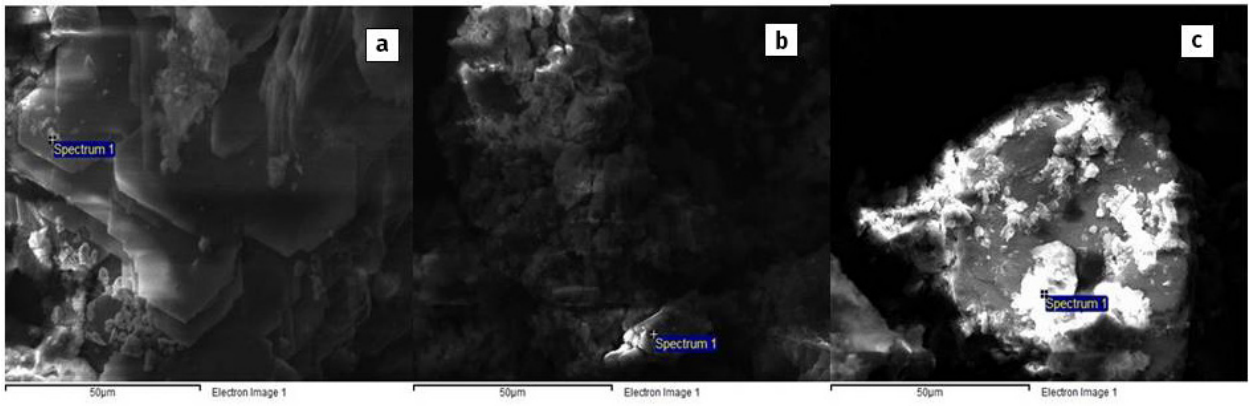

Figure 2. SEM spectrograms in 3 different zones (a, b, c) of the sample.

of Michigan , and the classification was done with the Ribosomal Database Project (RDP) classifier program, it is a the Creative Commons Attribution-Share Alike 3.0 .

RESULTS AND DISCUSSION

Soil analysis

Physical and chemical parameters of BsT and BL treatments were evaluated after 90 days of incubation due to their high rates of degradation. The results are showed in the Table Ia. The treatment does not affect the soil texture that is according to Khaleel et al. (1981) and Lal et al. (1998). However, an increase of clays and silt (%) in BsT and BL is related to the decreased of hydrocarbons. The results obtained from Martínez & López (2001) evidence this tendency in relation to interfered in the final lecture of this property. Total phosphorus, ammonium and nitrate ions increased in the treatments BsT and BL (see Table Ia). This response is due to the addition of urea as a source of nitrogen and $Na_5P_3O_{10}$ like source of phosphorous, enabling to reach the soil C:N:P ratio of 100:12:2 (from McCarty equation). This fertilizer addition also influenced the molasses periodically implemented with the objective of promoting the biodegradation of hydrocarbons, stimulating the growth of the microbial population due to rise of nitrogen and phosphorus content, as mentioned in Safdari et al. (2018). As a result of the volatilization of the urea an increase the pH in the treatments is shown, results agree with Chaillan et al. (2006). The SEM results showed high concentrations of compounds with calcium, magnesium and sodium influenced the CIC, and are consistent with CUCE (2007). The organic matter in BsT increase 5.3% for BL was 22.6% this behavior was related to the addition of compost and Leonardite (Rich in humic and fulvic acids); this finding is consistent with Trejos (2017). Studies of Weand et al. (2010) suggest that the interactions between microbial community composition, enzyme activity, substrate chemistry, and nutrient availability are influenced by substrate composition.

Table Ia. Soil physicochemical properties after 90 days of treatment.

Properties	Control (CS)	BsT	BL
Texture Class	Sandy loam	Sandy loam	Sandy loam
Sand (%)	60	36	38
Silt (%)	30	36	30
Clay (%)	10	28	32
Organic matter (%)	7.5	7.9	9.2
pH	7.7	8.4	8.5
Water content (%)	18	21	20
CEC (cmol/kg)	26	31.1	36.7
Total Phosphorus (mg/kg)	26	229	160
N-NH4 (mg/kg)	13	278	167
N-NO3 (mg/kg)	1	27	44
Total TPH (mg/kg)	14000	2333.3	1666.7

The SEM study confirms high presence of elements such as Ca, Na, C, Mg, K, Si, and large amount of Ba was obtained between 4% and 34%. The results of Adekunle et al. (2013) and Steliga et al. (2012), corroborated that finding. Heavy metals like Pb and Nb were found in less than 1%, which are located in the uncertainty degree of the equipment. Therefore, it is possible to affirm that these poor presence of metals have a low influence in the bioremediation process, according to Vullo (2003) and Steliga et al. (2012).

Development of microcosms

Control (C) presented a pH value of 7.7. This value had a tangible reduction, and reached a range

of values between 6.0 to 6.5 due to the addition of HCl, used as an inhibitor of microbial growth. Treatments A and B exhibited low variations in the behavior pattern with slight variations between 7.5 to 8.0 with a maximum value of 8.2. The pH variations in the treatments evaluated are shown in Figure 3, the pH fluctuated between moderately acid (5.5) to moderately alkaline (8.0). Chang et al. (2014) related such behavior with the nature of solid matrix. Tahhan & Abu-Ateih (2009) found these values to be optimum pH range. In these conditions there isn't inhibition in microbial dynamics. Other treatments with addition of nutrients and other stimulators experimental plots Bs, BsT, BsTL and BL obtained higher pH values 8.6 (strongly alkaline) at the end of the first week of the experimentation. The treatment presented a maximum pH values between moderately alkaline to strongly alkaline with values of 8.4, 8.5, 8.6 and 8.6 respectively. These pH results correspond to the volatilization of urea. Prior studies by Chaillan et al. (2006) that have noted the importance of volatilization process in alkaline soils. Increases in pH for BsTL and BL treatments could be influenced by the Leonardite pH 8.5 (Organiksa & Narpes Colombia 2016).

In the heterotrophic and hydrocarbon-oxidizing plate count three main phases of microbial grown have been identified. The first one corresponded to the adaptation phase until day 15. The second is the exponential phase until day 49, characterized by an increasing and variable behavior in the microbial communities. This phase corresponded to the continual stimulant additions and growth of the bacterial density, the last one is the finally stationary phase, Figure 4 illustrates three main phases of microbial grown in the experimental plots. This finding is consistent with that of Trejos (2017), who found that periodic addition of stimulants every 14 days promote bacterial density and finalize in the stationary phase where the dynamic is stable until treatments are finished. Weand et al. (2010), found that in response to N additions, both microbial community

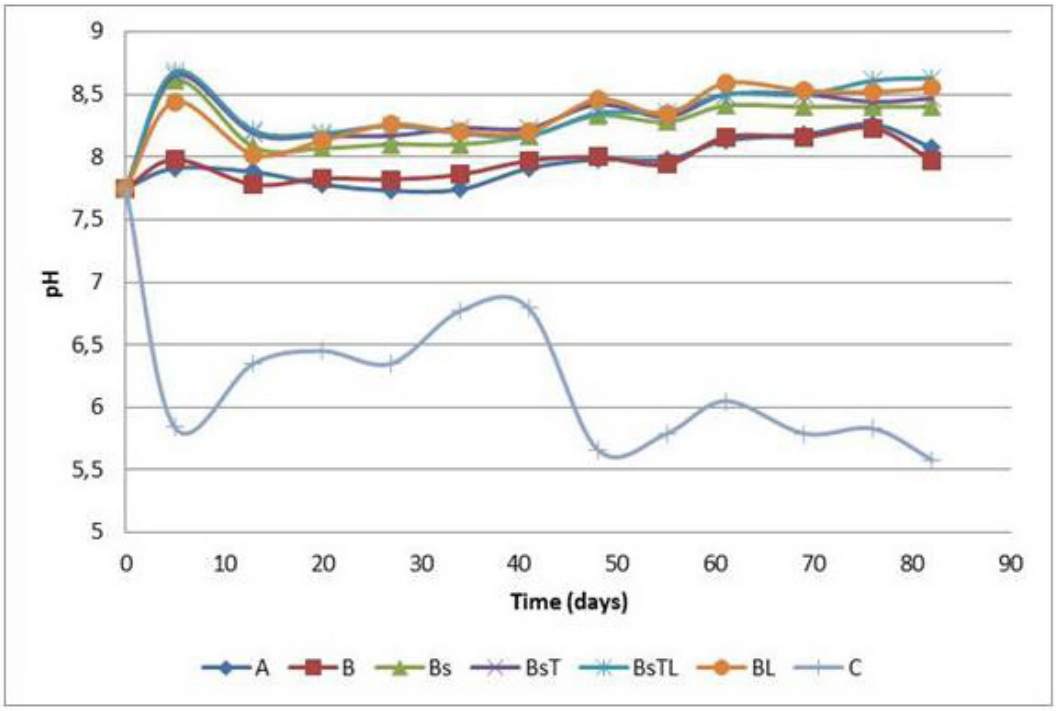

Figure 3. pH values for each treatment.

composition and enzyme activities changed and caused a significant overall increase in fungal biomass, and reduced hydrolytic enzyme activities.

Monitoring of heterotrophic and hydrocarbon-oxidizing bacteria is presented in Figures 4 and 5 respectively.

The initial population of heterotrophic bacteria was $1.8*10^5$ CFU/gr, this data is not shown in Figure 4. Hydrocarbon-oxidizing bacteria exhibited slow growth compared to the other treatments. Trejos (2017) associated this response with the influence of the used selective medium. Nevertheless, an initial phase of adaptation can be appreciated until day 15 where abrupt decrease in the Colony Forming Units (CFU) was observed. The treatments present a variable growth phase until the end of experiments influenced by the periodic addition of stimulants. For heterotrophic bacteria and hydrocarbon-based strains HCl (2M) showed effectiveness in inhibiting microbia growth, however periodic addition of stimulants especially molasses promoted growth of bacteria populations. A strong relation between biodegradation process and growth of bacteria density has been reported by Safdari et al. (2018). The more intense production of carbon dioxide (CO2) is observed during days 0 to 30. This production was stabilized at the end of the experiment. This finding is consistent with that of Wang et al. (2017, 2018); it was reported that in microbial respiration experiments, the addition of N generate overestimation of CO2 derived from added urea, later organic N may provide additional available C for soil microorganisms resulting in lower microbial activity and synthesis of oxidative and hydrolytic enzymes. The presence of two reaction mechanisms is evident, associated to slopes in CO2 curves. The double slope of the first section is equivalent to the second-time lapse. This behavior is

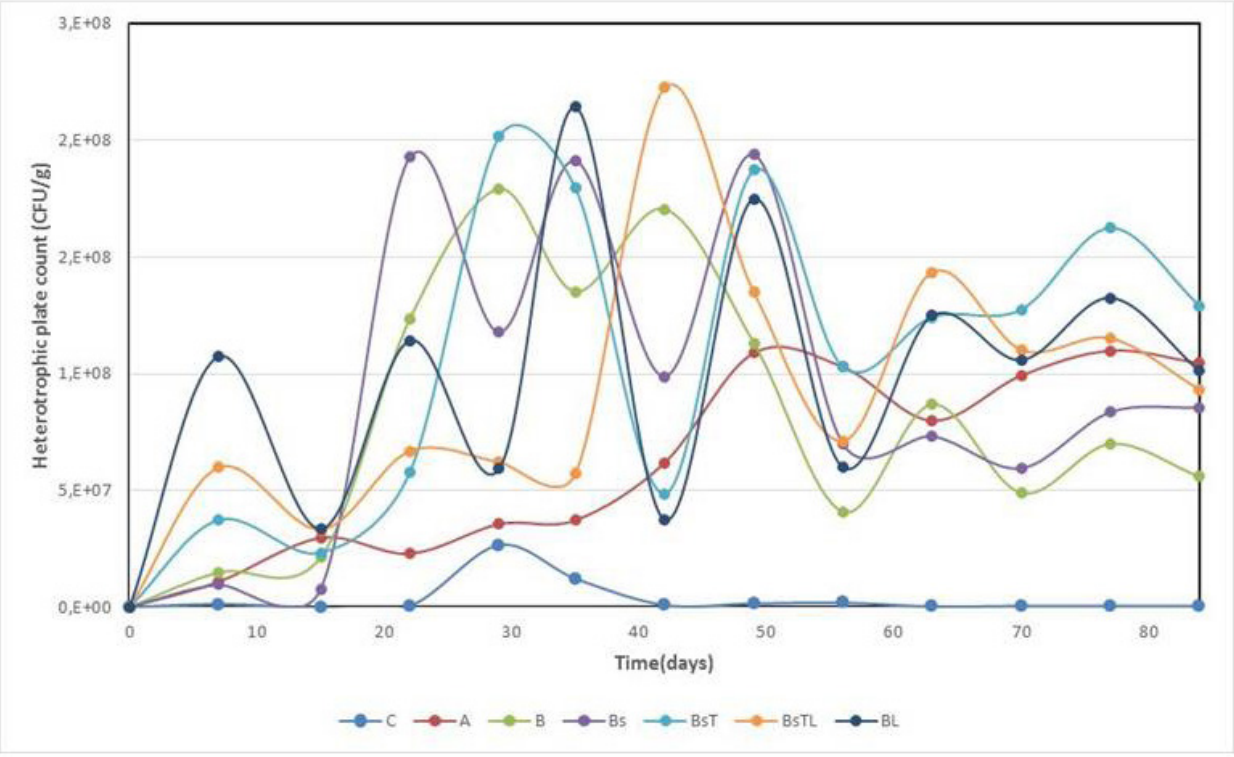

Figure 4. Heterotrophic plate count for each treatment during incubation time.

a stationary phase in the first 30 days of experimentation. Many studies demonstrated that the available nutrient in soils is related to microbial respiration (Li et al. 2015, Wang et al. 2018). A detailed illustration of the results of carbon dioxide production of the treatments is shown in Figure 6.

Final concentration of TPH

The chromatography for contaminated soil (CS) has a shown presence of aliphatic hydrocarbons with carbon numbers between 12 to 28. The first representative hydrocarbon was calculated according to Agudelo (2010), resulting in $C_{17}H_{36}$. Table Ib shows the information of the remaining hydrocarbons in the microcosms after 90 days. The treatment A showed the lowest biodegradation with values of 28.6%. The above confirm that organic N is associated with low microorganism activity (Wang et al. 2017). Control treatment (C) reached high values of degradation with final values of 3333.3 mg/kg, this result overall corroborates the findings of Trejos (2017), who reported the linked between HCl (2M) and performance of chemical transformation. The concentration of hydrocarbons were reduced in treatments B, Bs and BsTL due to the addition of compost and stimulants, that improved the C:N:P ratio. Several reports have shown that addition of nutrients, compost and stimulants increase the biodegradation of hydrocarbons. A number of investigations show great effectiveness of the implementation of bioaugmentation in contaminated sites (Steliga et al. 2012, Organiksa & Narpes Colombia 2016). The biggest degradation percentages were obtained by treatment BsT

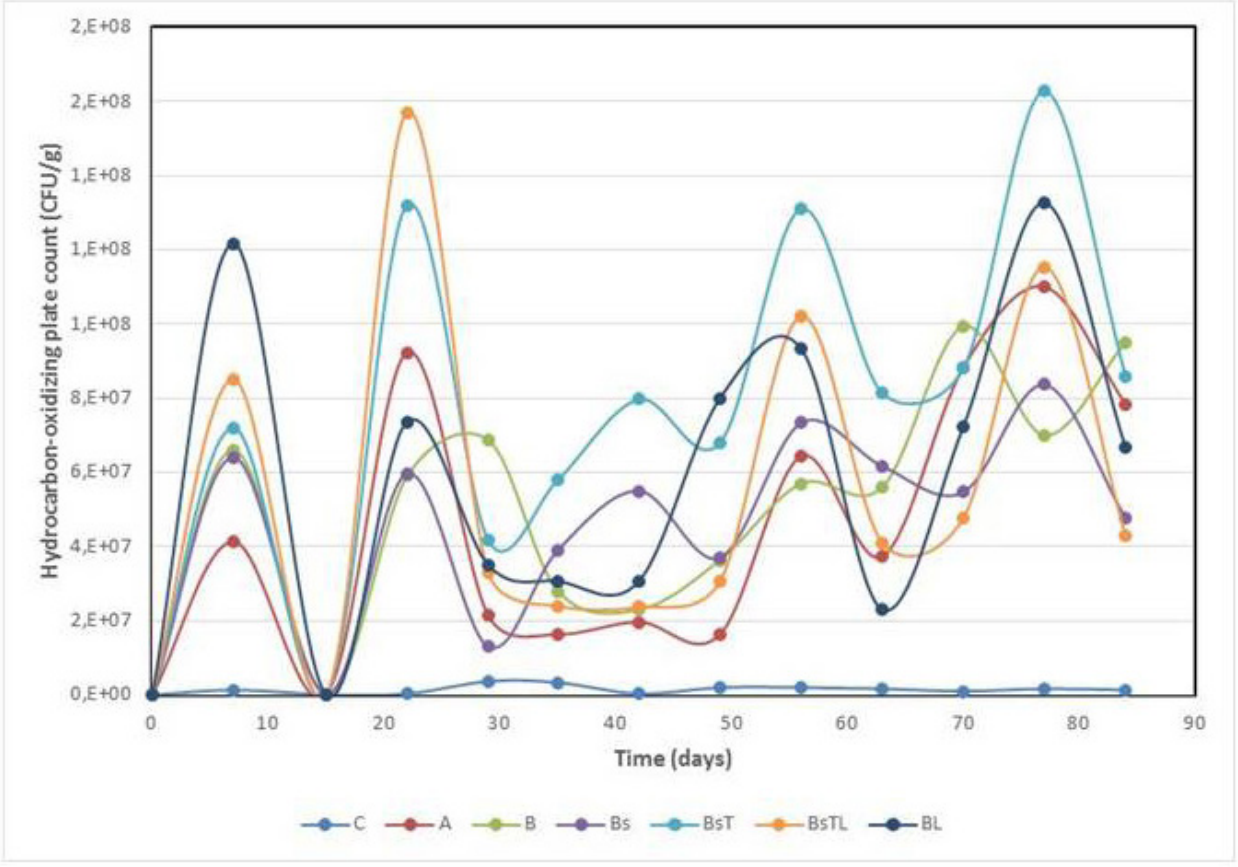

Figure 5. Hydrocarbon-oxidizing plate count for each treatment during incubation time.

and BL, with higher values of 80%. These results confirm that the activity of Tween 80 performing a process of desorption of hydrocarbons favoring its bioavailability, corroborate the findings of Cheng et al. (2017). However, BL treatment was evidencing a synergistic effect between Tween 80 and D-limonene. This result has been reported by the study of Riojas et al. (2011). These results reflect those of Safdari et al. (2018) who also found that the implementation of stimulated bioaugmentation result in greater biodegradation. The current study found that degradation agreed with the average CO_2 production shown in the figure 5. A strong relationship between microorganisms respiratory activity and contaminants mineralization has been reported by Riveroll-Larios et al. (2015).

Statistical analysis

The analysis of variance was used to analyze the relation between degradation percentages of the treatments and statistically significant differences between them. The variance (ANOVA) analysis was performed for the Period I (0 to 30 days) and Period II (31 to 90 days) to find the statistical significance between treatments ($p<0.05$). The F values for stimulants (Tween 80, Leonardite, D-limonene, and Molasses) in the evaluated periods were 209 and 90.4, respectively. A possible explanation for these results might be the influence of stimulants in the carbon dioxide production related to the mineralization of hydrocarbons (Riveroll-Larios et al. 2015). The Period I showed two largest slopes related to BsT (0.063 $mgCO_2/g$ soil*day) and BL (0.064 $mgCO_2/g$ soil*day) values. In accordance with the present results, previous studies of Tahhan et al. (2011), reported values less than 50% in a treatment with two microbial consortia in soil contaminated with drilling fluids. The slope behavior in Period II are consistent with the literature (Trejos 2017, Wang et al. 2017). The F value for the application

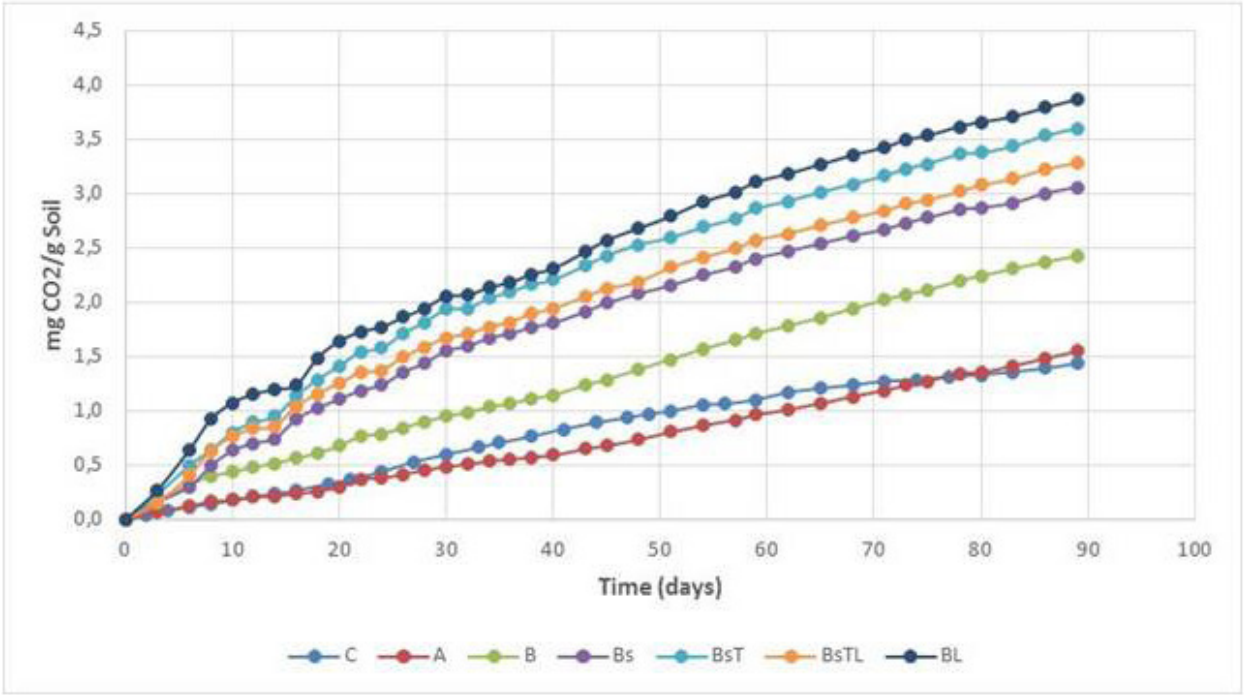

Figure 6. Carbon dioxide production for each treatment.

of nutrients, compost and stimulants was 31.69. This result may be explained by the effect on the percentage of TPH degradation in relation to proposed microcosms. The statistical results provide a valuable insight into the relevant difference between treatments.

Analysis of bacterial diversity

The DNA analysis of CS, BsT and BL samples were done at the end of the incubation (90 days) processes. The table II showed the results for the amplification of the 16S gene and sequencing, and alpha diversity analysis. One interesting finding is a 97% of similarity for more than 54,000 sequences per treatment. The samples for the treatments BL, BsT and CS were given a OTUS (Operational Taxonomic Unit) of 5932, 5032 and 7765, respectively. The treatment of BL and BsT show a reduction of 36% and 24% respect to CS, further hold the idea that the soil in natural conditions support high microorganism diversity than modified soil by changes in ecosystem conditions or anthropogenic perturbations (Singh & Gupta 2018). The Shannon diversity index corroborates the reduction of diversity in treatments BsT and BL. This response may be associated to the addition of stimulants. In accordance with the present results, previous studies of Atlas & Bartha (2001) have demostrated a redistribution of the bacterial population intervening in their antagonistic and synergistic relationships. The Figure 6 illustrates the most abundant phyla present in the samples. This study confirms that Actinobacteria and Proteobacteria are associated with soils. This result supports evidences from analysis of microbiota in the soil across the world (Shah & Subramaniam 2018) that found these organisms in the soil general microbiota. This finding is consistent with recent investigations of Baoune et al. (2018), who obtain *Actinobacteria* as a dominant phylum followed by *Proteobacteria*. The reduction of the Shannon diversity index was correlated to a pronounced decrease diversity for every phylum except for *Actinobacteria, this previous results are in concordance with Pla (2006),* results (table II). The general results at the genus´ level showed an increase in the abundance of *Nocardiodes, Streptomyces, Dietzia, Advenella, Gordonia, Brevibacterium, Rhodococcus, Bacillus, Pseudomonas* and *Arthrobacter* bacteria in BL and BsT microenvironments. According to Baoune et al. (2018) and Roy et al. (2014), this behavior is explained by the microorganism's tolerance to hydrocarbons that influence the degradation of TPH.

Table Ib. TPH degradation for each treatment for each treatment.

Treatments	Initials TPH (mg/kg)	Final TPH (mg/kg)	Degradation percentage
C	14000	3333.3	76.2
A		10000	28.6
B		3333.3	76.2
Bs		4666.7	66.7
BsT		2333.3	83.3
BsTL		4333.3	69
BL		1666.7	88.1

Table II. Alpha diversity analysis.

Group	Nseqs	Coverage	Sobs	Shannon diversity index
BL	54066	0.928	5932	4.76
BsT	54673	0.939	5032	5.13
SIC	56722	0.920	7765	6.88

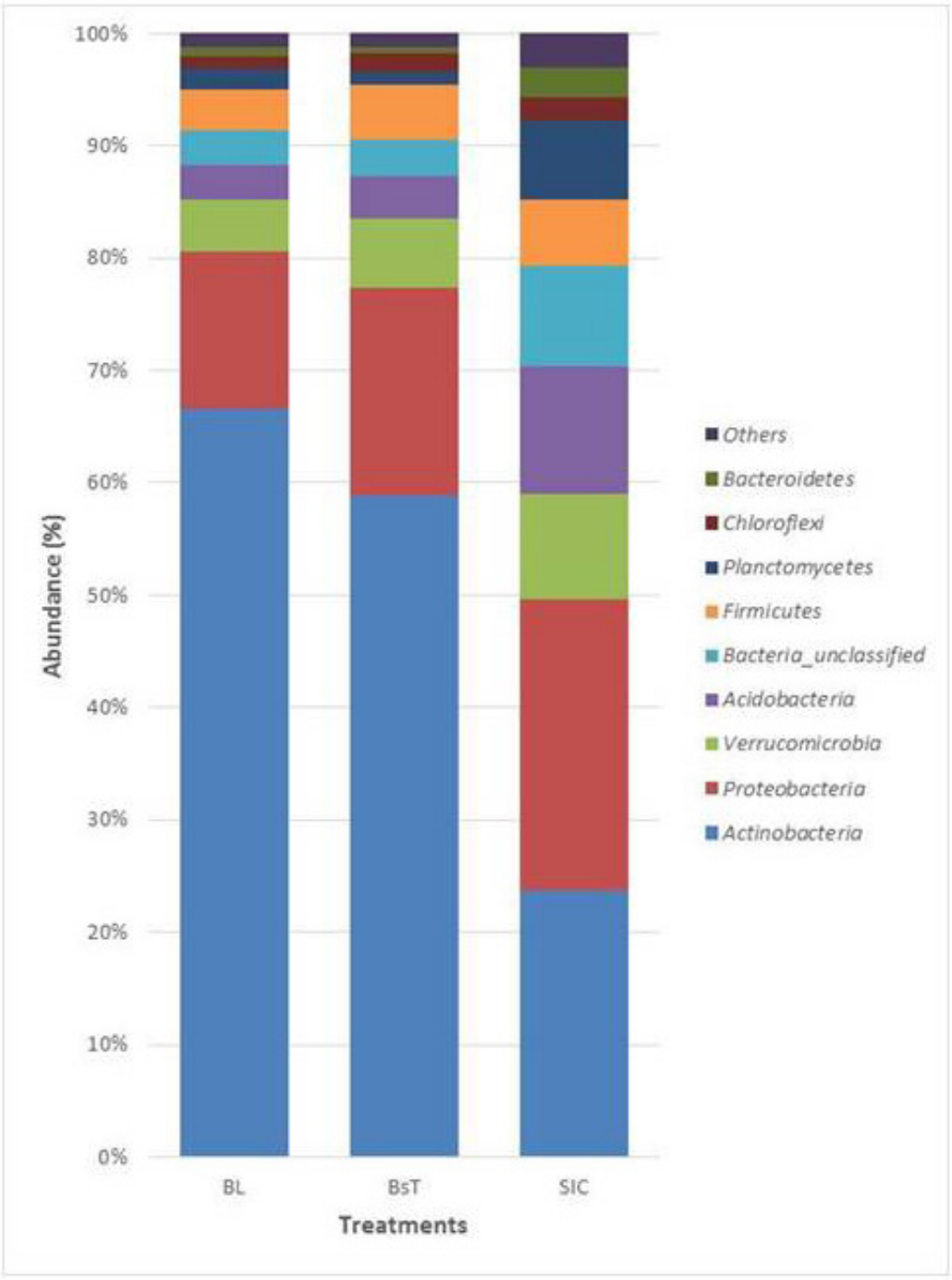

Figure 7. General Results of classification and abundance analyses at phyla level.

CONCLUSIONS

The highest biodegradation of TPH correspond to BL treatment followed by BsT that showed similar values than B. The current data highlight the importance of the use of stimulants, nutrients and compost in oil spill bioremediation programs, and demonstrate the positive effect on TPH biodegradation and improving soil conditions, at the same time promoting the growth of bacterial population. The SEM finding results of this study confirmed the low presence of heavy metals in the OBMs and cuttings. The Alkaline conditions in the soil did not affect the microbial activity that could be observed in respirometry results. Taken together, these findings suggested a role of bioaugmentation and biostimulation in the mineralization of the hydrocarbon and the high biodegradation. This behavior has a close relation with the high CO_2 productions in BsT and BL treatments. The presence of aliphatic hydrocarbons with more than 12 carbon atoms reduces the loss associated to volatilization. In the other hand the more efficient treatments redistributed the soil bacterial efficiently carrying out a process of desorption of hydrocarbons in the soil. The use of biostimulants favored the increase of microbial genera tolerance of hydrocarbons. These results contribute to our understanding of the effects of biotechnological solutions on soil ecosystems and extend our knowledge regarding the composition of, and shifts in, the soil microbiota in restored and polluted soil ecosystems. The principal implication of this research is validate to the possibility of intervening contaminated sited at the reactor scale with a high range of assertiveness at the laboratory scale. A further study could assess the successful treatments in a contaminated site by oil spills.

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