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Effect of surfactants on the removal of total petroleum hydrocarbons and microbial communities during bioremediation of a contaminated mining soil

Efecto de los surfactantes en la remoción de hidrocarburos totales de petróleo y las comunidades microbianas en la biorremediación de un suelo minero contaminado

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Abstract
The objective of this study was to evaluate the effect of ionic and nonionic surfactants on the biodegradation of total petroleum hydrocarbons (TPH) and the abundance and diversity of microbial communities. The experiments were conducted in two stages. In the first stage at microcosm level with Tween 80, Triton X-100, and SDS at 0, 1, 5, and 9 critical micelle concentrations (CMC) at 10, 20, and 30% moisture. The TPH removal rates, in decreasing order were 42.97 ± 0.70% for Tween 80 at 5 CMC and 30% moisture; 27.71 ± 0.62% for SDS at 1 CMC and 30% moisture; 27.33 ± 1.47% for Triton X-100, 5 CMC and 30% moisture; and 13.97 ± 0.38% the for negative control (no surfactant added) at 30% moisture. In the second stage, the best conditions of microcosm experiments were replicated in the biopile system. In this stage, the highest values of abundance and diversity of microbial communities and TPH degradation (49.89 ± 0.62%) were obtained for Tween 80 treatment with 5 CMC and 30% moisture. Consequently, the result shows that surfactant addition and moisture content influenced the microbial communities and TPH degradation suggesting that this method could be used to remove hydrocarbons from contaminated soils.
Keywords: Surfactants, bioremediation, bioavailability, metabolites, TPH.

Resumen
El objetivo de este estudio fue evaluar el efecto de surfactantes iónicos y no iónicos en la biodegradación de hidrocarburos totales de petróleo (HTP), abundancia y diversidad de comunidades microbianas. La primera etapa fue realizada a nivel microcosm empleando Tween 80, Triton X-100 y SDS a concentraciones micelares críticas (CMC) de 0, 1, 5 y 9, a 10, 20 y 30% de humedad. El consumo de HTP fue de 42.97 ± 0.70% para Tween 80 a 5 CMC y 30% de humedad; 27.71 ± 0.62% para SDS a 1 CMC y 30% de humedad; 27.33 ± 1.47% para Triton X-100, 5 CMC y 30% de humedad y 13.97 ± 0.38% para el control negativo (sin surfactante) al 30 % de humedad. En la segunda etapa, se replicaron las mejores condiciones a nivel microcosmo, empleando un sistema de biopila, obteniendo los valores más altos de abundancia y diversidad de comunidades microbianas y degradación de HTP (49.89 ± 0.62%) para el tratamiento Tween 80 con 5 CMC y 30% de humedad. Se confirmó que la adición de surfactante y la humedad influyeron en las comunidades microbianas y degradación de HTP, sugiriendo que este método podría usarse para tratar suelos contaminados con hidrocarburos.
Palabras clave: Surfactantes, biorremediación, biodisponibilidad, metabolitos, HTP.

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1 Introduction

One of the main problems of environmental pollution is the hydrocarbon spills and derivatives such as gasoline, diesel, and motor oil, caused by anthropogenic accidents, maritime disasters, pipeline failures, persistent pollution, and natural oil leaks (Wang *et al*., 2022). The main adverse effects of petroleum hydrocarbons spills are the high toxicity and carcinogenicity associated with polycyclic aromatic and polycyclic aromatic hydrocarbons (PAHs) compounds, making its remotion a critical need (Dai *et al*., 2022). Various physical and chemical technologies can be used to remove hydrocarbon pollutants from soils; however, these technologies are still not capable of the complete removal of these pollutants (Ossai *et al*., 2020). On the other hand, biological treatments such as natural attenuation, biostimulation, and bioaugmentation are well-known alternative techniques used to biodegrade many of these environmental pollutants up to carbon dioxide, water, and other less complex and harmless metabolic products (Nwankwegu *et al*., 2022). The biopile process is a conventional bioremediation technology used to remove hydrocarbons from contaminated soils (Liu *et al*., 2021). The stability and efficiency of this technology depend on physical, chemical, and biological conditions of the system, such as the moisture content of the soil (Velázquez *et al*., 2022), which is one of the main factors that influence the survival of microbial communities in contaminated soils since it affects the activity of indigenous microorganisms in oil-contaminated soil, which has low water holding capacity for its hydrophobicity (Liu *et al*., 2021). However, to the best of our knowledge, the information of the effects of moisture content on the soil microbial community and hydrocarbon degrading microorganisms in petroleum-contaminated soil is still not clear (AlKaabi *et al*., 2020).

Bioavailability is another important factor involved in the slow biodegradation of hydrophobic organic compounds in soil and an alternative to increase this bioavailability is the addition of surfactants (Yesankar *et al*., 2023). Surfactants are amphiphilic molecules that contain both hydrophobic and hydrophilic moiety and therefore can reduce surface and interfacial tensions, which helps to accumulate PAHs and increases the aqueous solubility. The concentration at which micelles first begin to form is known as critical micelle concentration and corresponds to the point where the surfactant exhibits the lowest surface tension (Ling *et al*., 2023). Bioremediation of petroleum hydrocarbons contaminated soils depends on surfactant type and its concentration, contaminant concentration, moisture content, and microorganisms present in the contaminated soil (Vázquez *et al*., 2022). However, some reports indicate that nonionic surfactants inhibit TPH biodegradation within concentrations above their critical micellar concentration (CMC) and it is related to the toxicity effect of these compounds to the microbial cell membrane. Although surfactants have been studied in complex water-soil systems, the effects are not well understood (Borah *et al*., 2021). It is known that through traditional microbiology techniques only 0.1% of the microbial diversity can be cultivated; however, for a greater understanding of the role of microorganisms in the bioremediation process it is necessary to explore other microorganisms that are participating in the process. Some molecular biology techniques allow us to reveal new microorganisms and carry out a detailed study of the microbial populations participating in bioremediation processes. Major molecular techniques include polymerase chain reaction (PCR), fluorescent in situ hybridization, denaturing gradient gel electrophoresis (DGGE), ribosomal intergenic spacer analysis, amplified ribosomal DNA restriction analysis, terminal-restriction fragment length polymorphism, single-strand conformation polymorphism, and ribosomal intergenic spacer analysis. DGGE is a technique that has gained great importance, and it is used to evaluate the dynamics of populations and their diversity in time and space. This technique is based on the electrophoretic separation of PCR amplicons which have the same length but have a different primary sequence. The DGGE technique introduced by Muyzer and de Waal (1994) has been widely used for the analysis of the composition of microbial communities during the bioremediation process. The DGGE technique is based on the differences in the sequence-dependent melting behavior of double stranded DNA (Shekhar *et al*., 2020). Different studies have reported the effective use of the DGGE technique for monitoring microbial communities in heavy metal and petroleum hydrocarbon remediation processes (Kunito *et al*., 2023; Das & Panda, 2022). Currently, there are methods such as Next Generation Sequencing (NGS), based on 16S ribosomal sequencing that allows greater resolution and sensitivity for the quantification of operational taxonomic units (OTUs), including those underrepresented in the environmental sample analyzed. However, DGGE is a technique that is traditionally used to quickly observe the fingerprint of microbial population dynamics, it is affordable and has good resolution, according to scientific reports (Brito *et al*., 2020; González *et al*., 2022).

The aim of the present study was to evaluate the effect of moisture content and addition of nonionic and anionic surfactants on the biodegradation of TPHs and the structure and diversity of the microbial community in a biopile remediation system, to understand the role

of microorganisms in the bioremediation process.

2 Methodology

2.1 Soil

The contaminated soil used in this investigation came from a confinement warehouse of a mining company located in Tayoltita Durango, Municipality of San Dimas, in the state of Durango, Mexico. The soil was contaminated with TPHs, from accidental spills, derived from the maintenance operations carried out on the heavy machinery used in the mine. Soil was sieved and soil that passed through sieve number 10 (2 mm diameter) was collected, characterized according to the techniques described by Margesin and Schinner (2005), and used to conduct the experimental phase.

2.2 Chemicals

Surfactants: Tween 80 (nonionic) with a CMC value of 0.012 mM, Triton X-100 (nonionic) with a CMC value of 0.2-0.9 mM, and sodium dodecyl sulfate (SDS, ionic) with a CMC value of 7-10 mM were obtained from Sigma - Aldrich. CMC values were taken from each bottle of surfactant, CMC values are reported for a temperature range of 20-25°C.

Reactants: $(NH_4)_2SO_4$ (batch 16-0703-11A57) was purchased from the Jalmek Company (Monterrey, Mexico); $FeSO_4{\cdot}7H_2O$ (batch 46) was obtained from CIVEQ Company (Guadalajara, Mexico).

2.3 Microcosm experiments

The microcosm experiments (Figure 1) were carried out using 120 mL glass batch reactors by duplicate, containing 90 g of contaminated mining soil, the addition of the surfactants to be evaluated (1, 5, and 9 CMC), mineral solution (MS) described for López *et al*., (2018) in g/L (2 of $(NH_4)_2SO_4$, 4 of KH_2PO_4, 6 of Na_2HPO_4, 0.2 of $MgSO_4{\cdot}7H_2O$, 0.05 of $CaCl_2{\cdot}2H_2O$, and 0.01 of $FeSO_4{\cdot}7H_2O$), and moisture content was adjusted to the desired percentage (10, 20, and 30%). Batch reactors were sacrificed at different established times (0, 5, 10, 15, 30, 60, and 90 days) to measure TPH removal, according to a blocked 2^3 factorial design for each surfactant for 1, 5, and 9 CMC at 10, 20, and 30% moisture content, as shown in Table 1.

Reactors lids were loose to ensure the presence of oxygen in the entire process, which is used as an electron acceptor (ea^-) for TPH degradation. To determine TPH abiotic loss, negative controls were used consisting in sterile soil autoclaved at 120°C for 30 min (Burmeier 1995).

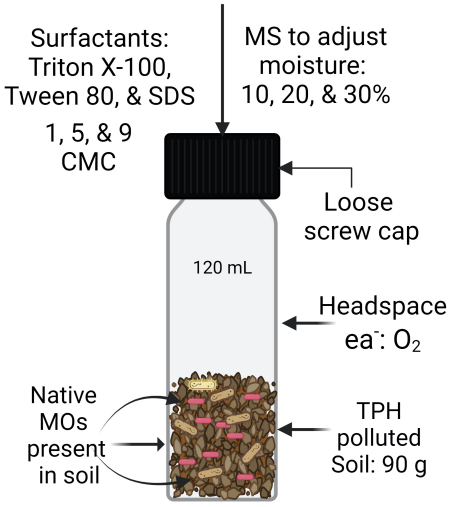

Figure 1. Microcosm diagram: Treatment of TPH contaminated soil (with native microorganisms, MOs), mineral solution (MS) to provide macro and micronutrients and to adjust moisture, to degrade TPHs with the addition of surfactants and using oxygen as electron acceptor (ea^-) present in the headspace of batch reactor. Created with Biorender.com.

2.4 Biopile treatment

Biopile experiments were conducted in polypropylene-plastic containers (4 mm thickness) of 22.5 L (30 x 30 x 25 cm) with 2.5 kg of contaminated soil (duplicate). The best moisture content and surfactant conditions obtained at microcosm level (SDS: 30% moisture & 1 CMC; Tween 80: 30% moisture & 5 CMC; Triton: X-100: 30% moisture & 5 CMC) were tested. The biopile experiments were conducted in a temperature-controlled room at 25°C for 90 days with a flow air addition of 300 mL/h using an Evans® compressor (180 L capacity and 2 HP).

2.5 TPH quantification and identification of its chemical composition

Samples at 0, 5, 10, 15, 30, 60, and 90 days were collected and analyzed to determine its TPH content following the 3540C and 821-B-94-004 US EPA methods. Samples of 10 grams of soil were mixed with 10 g of anhydrous Na_2SO_4 and extracted with 180 mL of 1:1 ratio of n-hexane/acetone mixture in a Soxhlet extractor. The solvent was evaporated at 70 rpm and 60°C in an IKA® rotatory evaporator. The extract was dried at 45°C until a constant weight was reached to determine TPH removed, relating the difference in weight of extract and weight of soil sample, and expressed as removal percentage (Cisneros *et al*., 2016). Hydrocarbon components of TPH extracts were measured with gas chromatography/mass spectrometry (GC/MS) analysis.

Table 1. Experimental design to test the influence of surfactant, CMC, and moisture of contaminated soil. T28, T29, and T30 were the abiotic controls used.

Conditions		**Treatments**			
Moisture (%)	CMC (mg/L)	SDS	Tween 80	Triton X -100	Abiotic control
10	0	---	---	---	T28
	1	T1	T10	T19	---
	5	T2	T11	T20	---
	9	T3	T12	T21	---
20	0	---	---	---	T29
	1	T4	T13	T22	---
	5	T5	T14	T23	---
	9	T6	T15	T24	---
30	0	---	---	---	T30
	1	T7	T16	T25	---
	5	T8	T17	T26	---
	9	T9	T18	T27	---

*CMC: Critical micelle concentration
*SDS: Sodium Dodecyl Sulfate

Extracts were mixed with 5 mL of a 1:1 hexane-acetone mixture and shaken for two minutes. Samples of three milliliters were syringe filtered (0.45 μm Millipore Millex-HN Nylon), eluents were collected into 2-mL amber chromatography vials and were loaded into GC/MS equipment. Analysis was performed with a GC/MS system (Agilent Hewlett Packard model 7890A) in splitless injection mode with injector and detector temperatures of 290 and 300°C, respectively. A HP-5 fused-silica column was used (30 m length, 0.25 mm inner diameter, 0.25 μm film thickness), and helium (1.0 mL/min) was the carrier gas. An initial temperature of 45°C was maintained for 1 min before it was increased to 100°C at a rate of 5°C/min. Then, the temperature was increased from 100 to 275°C at a rate of 8°C/min; the final temperature was 275°C, and it was maintained for 5 min (López *et al*., 2018).

2.6 Molecular genetic methods

The methodology described by Cisneros *et al*. (2016) was used for DNA extraction, described as follows:1 g of soil sample from each experimental treatment at 0, 30, 60, and 90 days was suspended with 1 mL of phosphate buffer (10 mM, pH 7.5) in Eppendorf tube with 200 mg of glass beads and vortexed for 1 min. Then, 30 μL of Tris/HCl extraction buffer, 20 μL of lysozyme (10 mg/mL; USB Corporation, Cleveland, USA), and 15 μL of proteinase K (20 mg/mL; Invitrogen, Germany) were added to the tube.. The tube was left to incubate at 37°C for 30 min, added 30μL sodium dodecyl sulfate (10 %) and 200μL sodium acetate (5 M, pH 8), and then incubated it for another 10 min at 60°C. An equal volume of phenol:chloroform (24:1) to then vortexed for 1 min and centrifuged at 7000×g for 10 min. The supernatant was taken and placed in a new tube and an equal volume of phenol:chloroform (24:1) was added, vortexed for 1 min, and centrifuged for 20 min at 7000×g. Again, the supernatant was taken and placed in a new tube and an equal volume of isopropanol was added and incubated for 12 h at -20°C; afterward, it was centrifuged for 20 min at 7000×g to precipitate the DNA. The precipitate formed was washed twice, first with absolute ethanol and then with 70% ethanol. In each wash, it was centrifuged for 20 min at 4°C at 7000×g.The formed pellet was re-suspended it in 50 μL of deionized sterile water. The presence of DNA was detected by electrophoresis (90V, 30 min) on 1% agarose gel and visualized in a UVP transilluminator (Bio-Rad).

The V3 hypervariable region of the bacterial 16S rRNA was amplified by PCR using the 356f (5'-CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC CCC TAC GCG AGG CAG AGC CTA CGC GGG CA-3 and 517r (5'-ATT ACC GCG GCT GCT GG-3') primers the forward primer included

a GC clamp (Cisneros *et al*., 2016). A sample of 0.5 μL (or 38.7 ng/μL) of metagenomic DNA was added to a 24.5 μL of PCR mixture containing 0.25 μL Taq DNA polymerase (5 u/L) (Promega, USA), 0.5 μL of each primer, 12.5 μL of master mix (Promega, USA) and 10.75 μL of nuclease-free water. The PCR analysis was conducted in a Thermocycler (Eppendorf) programmed as follows: first DNA denaturation at 95°C for 1 min followed by ten cycles of denaturation at 95°C and 30s, annealing at 65 to 60°C for 30 s. The temperature was reduced to 0.5°C in each cycle during the first ten cycles, followed by an extension at 72°C for 1min. Additionally, 20 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. Finally, an extension at 72°C for 7min, was performed. The PCR products were analyzed by agarose gel electrophoresis (2%) stained with ethidium bromide and run at 90V for 30min. Electrophoresis in denaturing gradient gel was performed using a Dcode^{TM} Universal Mutation Detection System (Biorad, Hercules, California, USA) onto 8% polyacrylamide gel with denaturing gradient of urea-formamide ranged from 30 to 60%. Electrophoresis was carried out at 60°C at a constant voltage of 39 V during 14 h in 1 x TAE buffer (20 mM Tris base, 10 mM sodium acetate, 0.5 mM EDTA, pH 7.4), this gel was silver stained (Cisneros *et al*., 2016).

2.7 Determination of maximum biodegradation rate (R_{max})

The maximum degradation rate constant in the treatments performed in biopile (R_{max}) was calculated using the Modified Logistic Model Eq. according to equation 1 (Wang and Wan 2009):

$$H = \frac{H_{max}}{1 + \exp[4R_{max}(\lambda - t)/H_{max} + 2]} \quad (1)$$

Where H_{max} is the cumulative amount of degraded TPH, R_{max} is the maximum degradation rate, λ is the lag time, and t is the experimental bioremediation time.

2.8 Diversity of bacterial communities

The optical densities (OD) of the gel bands were analyzed with the Image J software, each OD intensity of gel DGGE band represents the concentration of one type of microorganism. The Shannon-Weaver diversity index (H') was estimated using equations 2 and 3:

$$p_i = \frac{n_i}{N} \quad (2)$$

$$H' = -\sum[p_i \times \ln(p_i)] \quad (3)$$

Where p_i is the proportion of the community, represented by i species; n_i is the area of each peak of each band and N is the sum of all peak areas (Hedrick *et al*., 2000; Zucchi *et al*., 2003). The relative abundance (Ap) of each distinctive band on DGGE gel was calculated by equation 4 (Grace *et al*., 2013).

$$Ap = \frac{n_i}{N} \times 100 \quad (4)$$

A dendrogram was constructed by the clustering algorithm of Ward using Minitab 18 software to graphically represent the hierarchy of clusters formed according to the degree of similarity and shared characteristics between the different treatments (Zucchi *et al*., 2003).

2.9 Statistical analysis

The TPH degradation data obtained from the experiments in microcosms were analyzed by means of a one-way analysis of variance (ANOVA) by Tukey's LSD test (least significant difference, α = 0.05) to find out if there were significant differences between treatments for each surfactant. To find the significant effect of the type of surfactant, percentage of moisture, and CMC, as well as their interaction effect on the degradation of TPH a factorial analysis was conducted. Minitab software version 7.0 was used for both analyses.

3 Results and discussion

3.1 Soil characteristics

The contaminated soil to be used in the experimental phases was characterized and the results are summarized in Table 2.

Table 2. Physicochemical properties of contaminated mining soil.

Parameter	Values
Total petroleum hydrocarbons (mg TPH/kg dry soil)	71,416
True density (g/cm^3)	2.50
pH	7.34
Moisture (%)	5.53
Total Nitrogen (%)	0.05
Organic matter (%)	3.04
Phosphorus (mg/Kg)	112
Texture	Sandy-Silty soil
	Sand (%) 82.20
	Silt (%) 10.40
	Clay (%) 7.40
Porosity (%)	44.08

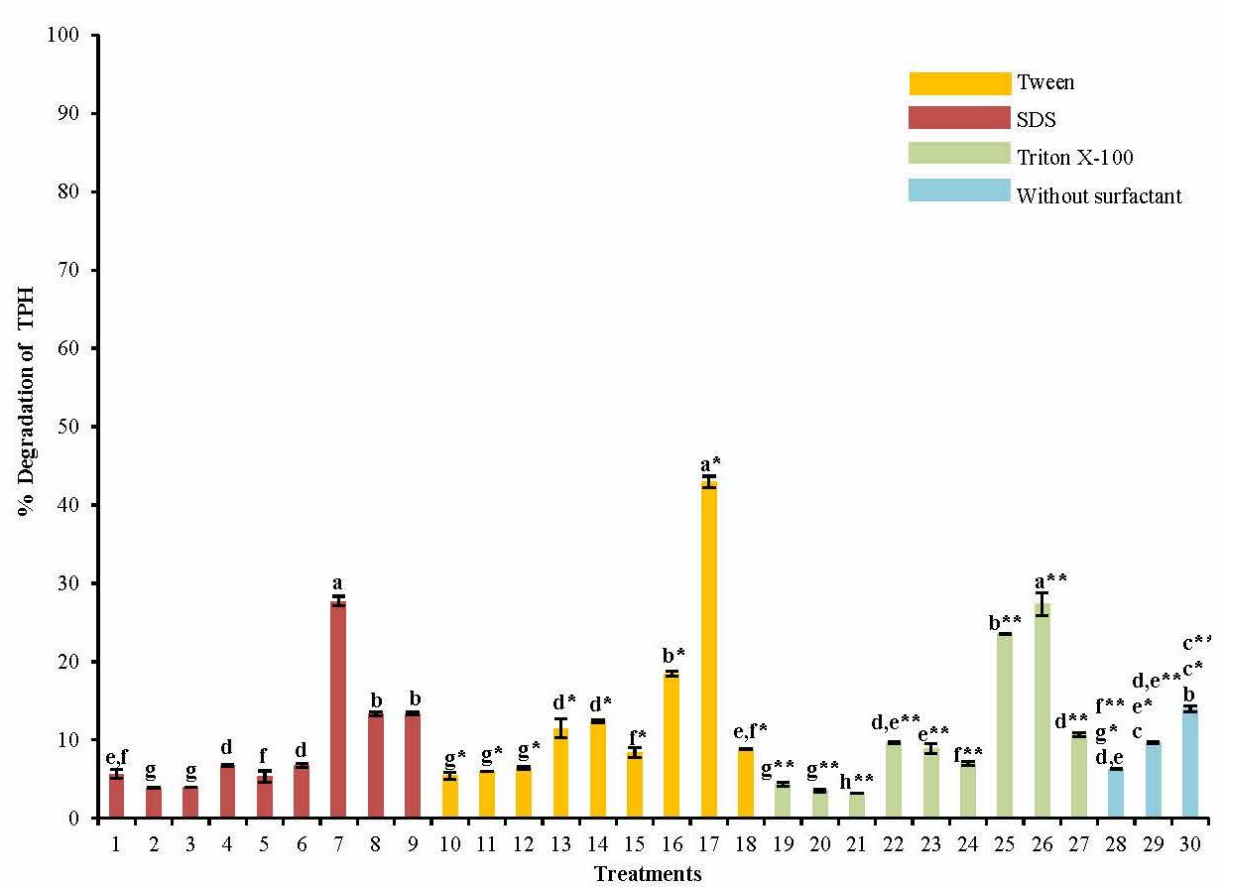

Figure 2. TPH removal in experiments carried out at microcosm level in the presence of surfactants (SDS, Tween 80, and Triton X-100), and without surfactants. Letter a corresponds to the treatment with the highest TPH removal for each specific condition evaluated. Different letters (b, c, d, e, f, and g) indicate statistical differences among treatments. The asterisks represent the differences of each treatment when compared to controls (without surfactant). Where: (*) = treatments with Tween 80 added and (**) = treatments with the addition of Triton X-100.

Table 3. Factorial ANOVA showing the significant effect of surfactant type, moisture (%), and critical micelle concentration (CMC).

Source	dF	AMS	ASS	F	p value
Model	35	77.5387	2.2154	514.20	0.000
Surfactant	2	1.7854	0.8927	207.19	0.000
Moisture	2	49.5418	24.7709	5749.38	0.000
Critical Micelle Concentration	3	5.4751	1.8250	423.60	0.000
Surfactant*Moisture	4	0.5545	0.1386	32.18	0.000
Surfactant*Critical Micelle Concentration	6	5.4920	0.9153	212.45	0.000
Moisture*Critical Micelle Concentration	6	9.3551	1.5592	361.89	0.000
Surfactant*Moisture*Critical Micelle Concentration	12	5.3347	0.4446	103.18	0.000
Error	36	0.1551	0.0043		
Total	71	77.6938			

dF = Degrees of freedom AMS =Adjusted mean squares ASS = adjusted sum of squares

3.2 Biodegradation of TPH at the microcosm level

Results of TPH degradation obtained in experiments at microcosm level are plotted in Figure 2, this bar graph shows the TPH degradation % and the significant differences according to an ANOVA analysis when comparing the treatments of each surfactant individually.

The best TPH removal results were for the treatments with Tween 80 (5.41 to 42.97%), being T17 treatment (Tween 80: 5 CMC and 30% moisture) the highest TPH removal, significantly

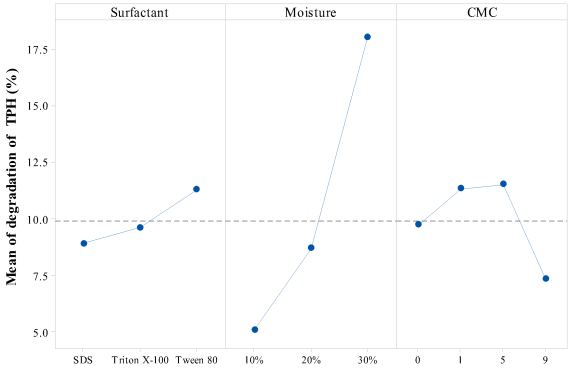

Figure 3. Main effect for the different surfactants, % moisture, and critical micelle concentration (CMC).

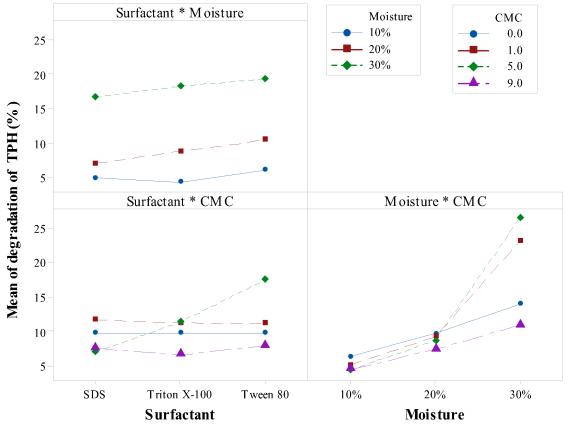

Figure 4. Interaction effect for surfactants, % moisture, and critical micelle concentration (CMC) and its influence on TPH degradation.

higher compared to all other treatments. Treatments with the addition of SDS showed lower TPH removal (3.88 - 27.71%) than for Tween 80, being T7 (SDS: 1 CMC and 30% moisture) the treatment that showed the highest TPH removal according to the ANOVA. TPH degradation results for Triton X-100 surfactant ranged from 3.2% (T21) to 27.3% (T26), corresponding to 5 CMC and 30% moisture conditions. These results indicate that the best removal conditions were obtained in T17 treatment; however, the factorial analysis clearly revealed the effect of each of the variables and their interaction on the degradation of TPH present in the soil. Factorial analysis is summarized in Table 3, it shows that all the variables and their interaction were significant accordingly with $p \ll 0.05$.

Figure 3 shows the main effect of surfactant, moisture, and CMC on the percentage of TPH degradation. Surfactant Tween 80 had the greatest effect whereas SDS had the least effect on the percentage of TPH degradation. As for moisture, the graph shows that TPH degradation increases as moisture increases too, achieving the greatest effect at 30%. The importance of the moisture content in contaminated soil (TPHs in this case) is crucial to obtain high removal rates or total mineralization of TPHs and their metabolites. It has been reported that it is necessary to have moisture content in a range such that the microorganisms present in the soil (native or not) can carry out their metabolism. If the presence of moisture is limited (dry soil) the degradation stops, even if oxygen is present, or if it is in excess (waterlogged soil) it would limit the diffusion of oxygen which would counteract the presence of the vital electron acceptor (aerobic process) and not degradation can take place (Bahmani *et al*., 2018; Hernández *et al*., 2021). Skopp *et al*., (1990) reported that microbial activity is maximum in soil when the water content of the soil is 60% of its water holding capacity. Haghollahi *et al*., (2016) reported that maintaining a moisture content of 20% in sandy soil for 270 days would result in a TPH removal rate of 70%. Other studies by Wang *et al*., (2016) confirmed that moisture adjusted to 33% increased the community diversity of microbes in soils contaminated with diesel and lubricants. The effect of the different levels of CMC shows that the greatest TPH degradation was at 5 CMC while the least at 9 CMC. The inhibition of hydrocarbon biodegradation at a concentration of 9 CMC may be due to the fact that increasing the concentration of a synthetic surfactant increases the levels of chemical compounds present in the soil while reducing microbial diversity during the bioremediation process (Cheng *et al*., 2018).

The interaction of surfactant, moisture content, and CMC in the degradation of TPH is shown in Figure 4. The interaction of surfactant with moisture shows that the greatest degradation of TPH was reached using 30% moisture with Tween 80, while 10% moisture with Triton X-100 gave the least TPH degradation. The interaction of surfactant concentration and moisture percentage shows that the moisture interaction of 30% at 5 CMC had the greatest effect on TPH degradation, while the least degradation of hydrocarbons was achieved at 10% moisture for 1, 5, and 9 CMC surfactant concentration. About the interaction between surfactant and CMC, the greatest degradation was achieved for Tween 80 at 5 CMC. On the other hand, the lowest TPH degradation was found for Triton X-100 at 9 CMC followed by SDS at 5 CMC, as shown in the graph. We attribute that the inhibitory effect of TPH degradation in SDS concentration at 5 CMC may be due to the formation of structural chemical complexes that inhibit microbial growth that affects the biodegradation of TPH, a behavior that has also been reported in studies by Zarei and Fazaelipoor (2022). The inhibitory effect at 9 CMC of Triton X-100 may be due to the dissolution process being affected as well as that the bacterial biodegradation activity probably due to an excess of micelles of the contaminant (Zdarta *et al*., 2020). Other studies have reported that the addition of Triton X-100 at high concentrations (360 mg/L) to the system with diesel oil affected the hydrophobicity of the cell

surface, causing the microorganisms to be unable to adhere to the surface of the hydrocarbon (Kaczorek *et al*., 2010).

3.3 Biodegradation of TPH in biopile and kinetic parameters

The best conditions of TPH removal at microcosm level were tested in a biopile system (Table 4). Figure 5 shows the degradation kinetic profiles of the different biopile treatments (TB1, TB2, TB3, and TB4). In general, an inverse L-shaped curve is exhibited, characterized by a high phase of TPH degradation (0 - 45 days) followed by a phase of decrease in biodegradation (45-90 days). A possible explanation of this behavior is that the contaminants are bioavailable for microbial attack in the first stage. In contrast, in the second stage, the microorganisms have poor ability to degrade the remaining contaminants because they may be strongly adhered to humic substances and clay particles of the soil, making them unavailable to soil microorganisms (Huesemann *et al*., 2004). TPH removal on the biopile treatments varies between 20.13 ± 0.36 to 49.89 ± 0.62%. According to these values, the biostimulation in the biopile improved the hydrocarbon biodegradation in comparison with those in the microcosm level. When comparing TPH degradation in the microcosm level vs biopile system (TB1, TB2, TB3, and TB4) the removal of the hydrocarbons increased by 6.92, 6.96, 0.84, and 6.16%, respectively.

According to the ANOVA, the TPH removal % obtained in the TB1 treatment were significantly higher than TB2, TB3, and TB4. In this sense, the TPH removal values obtained in the TB2 treatments were significantly higher than TB3 and TB4 treatments (Table 4). These results suggest that Tween 80 addition improves the hydrocarbon bioavailability and favors its degradation.

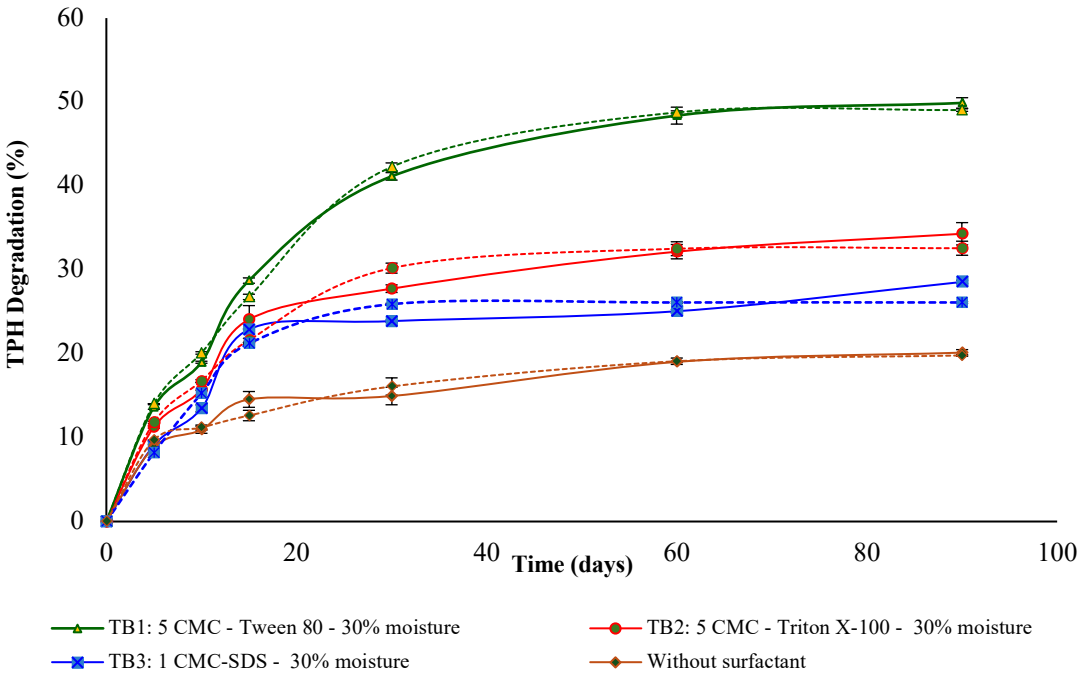

Figure 5. TPH removal in soil samples evaluated in biopile treatments with different surfactants vs negative control. The dotted lines belong to the kinetic behavior obtained from the corresponding modified logistic model for each of the different treatments.

Table 4. TPH removal (%) and biodegradation rates ($R_{\max}^{TPH}$) for the different biopile treatments.

Surfactant	CMC	Moisture (%)	Treatment	TPH Removal (%)	TPH Degraded (mg/kg)	Biodegradation rate (R_{max}^{TPH}) (mg/kg-day)
Tween 80	5	30	TB1	**49.89 ± 0.62[a]**	35,361.50 ± 441.27[a]	955.85 ± 24.91[a]
Triton X-100	5	30	TB2	34.29 ± 0.21[b]	24,307.09 ± 943.71[b]	718.95 ± 1.65[b]
SDS	1	30	TB3	28.55 ± 1.33[c]	20,238.70 ± 145.38[c]	1,037.29 ± 28. 94[a]
Control	0	30	TB4	20.13 ± 0.36[d]	14,268.78 ± 252.48[d]	213.10 ± 52.34[c]

Table 5. Bioremediation studies of TPH contaminated soil using surfactants to promote degradation.

Reference	Scale	TPH initial concentration (mg/Kg)	Experimental time (days)	TPH Removal (%)
Iturbe *et al*. (2004)	Industrial	4,600	56	85
Dias *et al*. (2015)	Pilot	21,909	50	71
Ma *et al.* (2016)	Pilot	35,200	90	87
Kim *et al*. (2018)	Pilot	5,196	260	58
Micle et al. (2018)	Microcosm	7,600	126	76
Mohsen *et al*. (2019)	Pilot	2,140	35	76
Žeradjanin *et al*. (2020)	Pilot	21,600	150	87
This study	Microcosm	71,416	90	42
This study	Semi pilot	71,416	90	49

Table 4 also shows the biodegradation rates $R_{\max}^{TPH}$ calculated with the modified Logistic model (Eq 1). The TB1 and TB3 treatments (955.85 ± 24.91 and 1,037.29 ± 28.94 mg/kg-day, respectively) were significantly higher than the obtained with TB2 and TB4 treatments. These results indicate that the conditions of TB1 and TB3 treatments favored the TPH utilization rate; however, the TB1 treatment TPH removal was significantly higher than the TB3 treatment (Table 4). This behavior is attributed to the abundance of microbial communities being very similar at 30 days in TB1 and TB3. However, at 60 and 90 days, the abundance of microbial communities in the TB3 treatment was significantly lower than in the TB1 treatment, indicating that the surfactant had an inhibitory effect on microbial growth, affecting the TPH biodegradation. Results also show that the removal rate for TB3 treatment was significantly higher than the obtained for TB2 treatment; however, the removal rate for TB2 treatment was significantly higher than the obtained for TB1 treatment. This behavior could be because during the entire bioremediation process in the TB3 treatment there was a greater abundance of microbial communities compared to the TB2 treatment; however, despite a lower abundance of communities in the TB3 treatments, the greater degradation of TPH may be due to the prevalence of microbial communities with a greater capacity for degradation of TPH (Figure 6). The abiotic controls showed only 0.4% TPH removal, indicating that the hydrocarbons present in the soil were removed mainly by microbial activity. When comparing the TPH removal achieved at conditions in TB1 with other studies carried out in a biopile system (Table 5), it can be noted that the results were satisfactory even though the removal percentage obtained was lower. One reason could be because in this study we worked with a soil with a much higher concentration of TPH compared to the studies shown in Table 5.

3.4 Diversity of microbial communities

The structural changes of the microbial community in the biopile treatments were analyzed by the DGGE method. Figure 6 shows the polyacrylamide gel, where the DNA bands of different molecular weights represent the composition of the dominant microbial communities are highlighted, including culturable and non-culturable microorganisms presented in the soil samples of the different evaluated treatments (TB1, TB2, TB3, and TB4) at 0, 30, 60, and 90 days. A total of 13 bands highlighted by their high intensity distributed throughout the gel were taken as a reference to compare the bands obtained. For the control sample at time zero 13 bands were detected (B1 to B13); at 30 days most of the bands were detected in treatments TB1, TB2, TB3, and TB4 (B1 to B15, except band B11). At 60 days the number of bands decreased in treatments TB2, TB3, and TB4 (B1, B2, B3, B4, B12, B13, B14 and B15 disappeared); however, in treatment TB2 the appearance of a new band was observed (B11), whereas in TB1 treatment, bands from B1 to B13 were detected. Finally, at 90 days, in most treatments TB1, TB2, TB3, and TB4 bands B7, B9, and B11 were detected, as well as the appearance of two new bands (B16 and B17). Finally, at 90 days, in all treatments (TB1, TB2, TB3, and TB4) bands B7, B9, and B11 were detected, as well as the appearance of B16 and B17 new bands (Figure 6).

The behavior of the microbial communities in the different treatments at 30 days could be because during this period there were TPH compounds that were metabolically more assimilable for the microorganisms, which allowed the proliferation of a greater number of microbial communities. It is believed that the decrease in the number of bands in treatments TB1, TB2, TB3 and TB4 at 60 and 90 days, compared to 30 days and at the initial time, was because as the bioremediation time elapsed, the TPH compounds produced were structurally more difficult to degrade. These results show that the changes in the behavior of the microbial community are closely related to the bioavailability of hydrocarbons and to the interactions of multiple substrates. Consequently, the remaining microbial populations present at the end of the bioremediation process had the metabolic capacity to degrade the most recalcitrant hydrocarbon compounds.

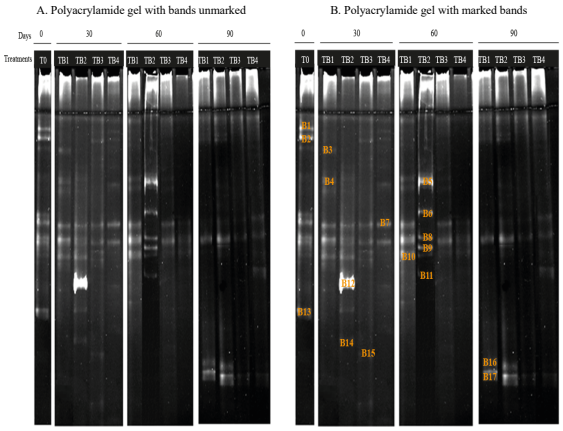

Figure 6. Denaturing gradient gel electrophoresis (DGGE) of the V3 region of 16S ribosomal DNA of PCR products from soil samples obtained from different bioremediation treatments after the 90th day of incubation time. B1-B17 are the bands obtained with the highest intensity.

Other studies show a similar behavior during the petroleum hydrocarbon degradation process (Zhang *et al*., 2021; Zhen *et al*., 2021).

The highest values of diversity index were obtained in 30 days for TB1 treatment and the lowest values for TB4 treatment. The diversity of the microbial communities at 60 days had an increase compared to the values obtained at 30 days for TB1, TB2, and TB4 treatments, obtaining the highest relative abundance for the TB1 treatment, while in the TB3 treatment the diversity values decreased (Figure 7). The decrease in microbial diversity is attributed to the fact that the stationary phase of biodegradation of TPH was reached at 60 days, so it is expected that microbial communities will decrease, and a reduced number of microorganisms remain. This indicates a clear inhibition of the development of microbial communities attributed to an effect caused by the lack of bioavailability of TPH, as well as by the formation of toxic metabolic by-products for some species of microorganisms.

The non-degraded hydrocarbons at 90 days (Tables 6, 7, and 8) are compounds metabolically more difficult to degrade by microbial communities, reducing proliferation and diversity. It has been reported that the expression of microbial communities depends on the metabolic capacity to biodegrade and remain in the presence of recalcitrant hydrocarbons (Bidja *et al*., 2020; Mahjoubi *et al*., 2021). Other studies reported that during the TPH degradation process there are variations in microbial communities due to the expression of different catabolic genes (Menezes *et al*., 2005). The treatments were grouped and are shown in different dendrograms (Figure 8), such grouping was based on the percentage of similarity of the different treatments and discussion follows.

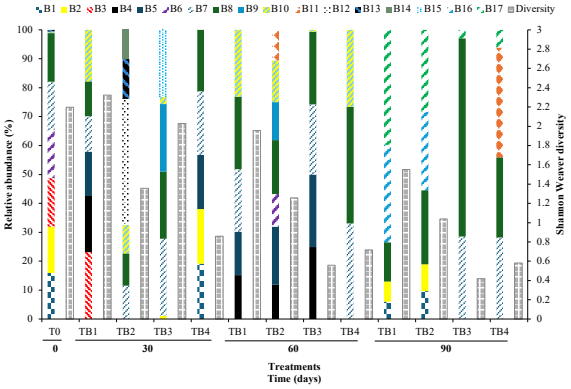

Figure 7. Values of relative abundance and diversity index obtained for the soil samples at the initial time T0 and the different treatments TB1, TB2, TB3 and TB4 for the times 30, 60 and 90 days.

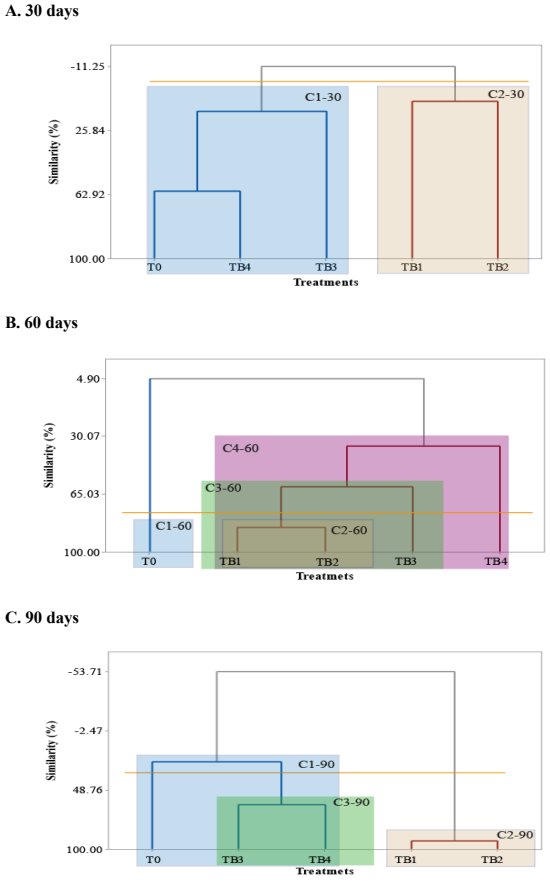

Figure 8. Dendrograms of the microbial communities from samples taken at time zero (T0) and for the different experimental treatments (T1, T2, T3, and T4), at 30, 60, and 90 days (A, B, and C, respectively). The yellow line indicates the cut of the dendrogram to group the different treatments into clusters.

TB1 and TB2 treatments of cluster C2-30 (Figure 8A), showed an approximate 10% similarity sharing 3 bands in common B7, B8, and B10. The initial time T0 sample and treatments TB3 and TB4 of cluster C1-30 showed a similarity of approximately 15%, sharing only 3 bands in common B2, B7, and B8 (Figure 6).

Treatments TB1 and TB2 of cluster C2-60 showed a similarity of approximately 80%, which indicated that the microbial communities were very similar between treatments, sharing bands B4, B5, B8, and B10 in common. The grouping of treatments TB1, TB2, and TB3, cluster C3-60, have an approximate similarity of 60% sharing in common the bands B4, B5, B8, and B9. Treatment TB4 and cluster C3-60 have a similarity of approximately 40%, having in common the bands B8 and B10. The cluster C1-60 and cluster C4-60 showed a similarity of approximately 5%, having in common the bands B8 and B10 (Figure 8B).

Treatments TB1 and TB2 of subcluster C2-90 showed an approximate 90% similarity, sharing the B1, B2, B8, B16, and B17 bands (Figure 6). Treatments TB3 and TB4 of subcluster C3-90 showed a similarity of approximately 60%, sharing bands B7, B8, B11, and B17 in common. The T0 sample with the treatments of the C3-90 cluster were grouped into the C1-90 cluster, with an approximate similarity of 10% sharing in common the bands B7 and B8 (Figure 8C). The microbial communities from 0 to 30 days had a greater similarity with those of the T4 treatment, which indicates that the addition of surfactant induced greater changes in the structure of the soil microbial communities than without surfactant. At 60 and 90 days in the different treatments there were low similarity values compared to the initial time samples (T0), this can be attributed to the fact that very selective microbial physiological conditions were formed, as well as intermediate metabolites compounds difficult to break down were formed. Therefore, microorganisms with the metabolic capacity to survive under these conditions prevailed and developed.

Table 6. Alkanes detected (✓) by GC/MS analysis in samples at zero-time (T_0) and end time for (T_{90}) experiments realized for the (TB1, TB2, TB3, and TB4) treatments.

Compound	Formula	MW (g/mol)	T0	TB2	TB1	TB3	TB4
Hexane	C_6H_{14}	86.1	✓				
Octane	C_8H_{18}	114.2	✓				✓
Nonane	C_9H_{20}	128.2	✓				
Decane	$C_{10}H_{22}$	142.2	✓				✓
Undecane	$C_{11}H_{24}$	156.3	✓				✓
Dodecane	$C_{12}H_{26}$	170.3		✓	✓		
Tridecane	$C_{13}H_{28}$	184.3	✓	✓	✓		✓
Tetradecane	$C_{14}H_{30}$	198.3	✓	✓	✓		✓
Pentadecane	$C_{15}H_{32}$	212.4	✓	✓	✓	✓	✓
Hexadecane	$C_{16}H_{34}$	226.4	✓	✓	✓	✓	✓
Octadecane	$C_{18}H_{38}$	254.5	✓	✓	✓		✓
Nonadecane	$C_{19}H_{40}$	268.5	✓	✓	✓	✓	✓
Eicosane	$C_{20}H_{42}$	282.5	✓	✓	✓	✓	✓
Heptadecane	$C_{17}H_{36}$	240.5	✓	✓	✓		✓
Heneicosane	$C_{21}H_{44}$	296.6	✓	✓	✓		✓
Docosane	$C_{22}H_{46}$	310.6	✓				
Tricosane	$C_{23}H_{48}$	324.6	✓	✓			✓
Tetracosane	$C_{24}H_{50}$	338.7	✓				
Hexacosane	$C_{26}H_{54}$	366.7	✓			✓	
Heptacosane	$C_{27}H_{56}$	380.7	✓			✓	
Octacosane	$C_{28}H_{58}$	394.8	✓				
Nonacosane	$C_{29}H_{60}$	408.8	✓				
Triacontane	$C_{30}H_{62}$	422.8	✓				
Hentriacontane	$C_{31}H_{64}$	436.8	✓				
17-Pentatriacontene	$C_{35}H_{70}$	490.9	✓				✓
Hexatriacontane	$C_{36}H_{74}$	507		✓			
Tritetracontane	$C_{43}H_{88}$	605.2				✓	

Table 7. Substituted alkanes and cycloalkanes detected by CG/MS analysis in samples at initial time (T_0) and at the end of the experiment (T_{90}) for T1, T2, T3, and T4 treatments.

Compound	Formula	MW (g/mol)	T0	T2	T1	T3	T4
Trimethylene oxide	C_3H_6O	58.1	✓				
Silane, dimethyl-	C_2H_6Si	58.2	✓				
Trimethylamine	C_3H_9N	59.1	✓				
Cyclopentane, 1,2-dimethyl-	C_7H_{14}	98.2	✓				
Pentane, 2,4-dimethyl-	C_7H_{16}	100.2	✓			✓	
1-Butanamine, N, N-dimethyl-	$C_6H_{15}N$	101.2	✓				
Pentane, 2,3,4-trimethyl-	C_8H_{18}	114.2	✓				✓
Heptane, 4-methyl-	C_8H_{18}	114.2	✓			✓	✓
Octane, 3-methyl-	$C_{10}H_{20}$	140.3	✓				
Heptane, 2,2,3,5-tetramethyl-	$C_{11}H_{24}$	156.3	✓				
Decane, 3,3,4-trimethyl-	$C_{13}H_{28}$	184.4	✓				
Dodecane, 2,6,10-trimethyl-	$C_{15}H_{32}$	212.4		✓	✓	✓	
Tetradecane, 3-methyl-	$C_{15}H_{32}$	212.4		✓			
Pentadecane, 2-methyl-	$C_{16}H_{34}$	226.4		✓			
Undecane, 4-cyclohexyl-	$C_{17}H_{34}$	238.5		✓			
1-Decanol, 2-hexyl-	$C_{16}H_{34}O$	242.4		✓			
Heptadecane, 2-methyl-	$C_{18}H_{38}$	254.5		✓			
Pentadecane, 2,6,10-trimethyl-	$C_{18}H_{38}$	254.5	✓	✓	✓		
Heptadecane, 2,6-dimethyl-	$C_{19}H_{40}$	268.5		✓			
Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl)-	$C_{20}H_{40}$	280.5	✓				
Hexadecane, 2,6,10,14-tetramethyl-	$C_{20}H_{42}$	282.5	✓	✓	✓		✓
Octadecane, 1-chloro-	$C_{18}H_{37}Cl$	288.9	✓		✓		
1,1,3-Tricyclohexylpropane	$C_{21}H_{38}$	290.5		✓			
Octadecanoic acid, ethenyl ester	$C_{20}H_{38}O_2$	310.5	✓				
Silane, trichlorooctadecyl-	$C_{18}H_{37}Cl_3Si$	387.9	✓				
Hexasiloxane, tetradecamethyl-	$C_{14}H_{42}O_5Si_6$	458.9			✓	✓	
Dotriacontyl pentafluoropropionate	$C_{35}H_{65}F_5O_2$	612.9	✓				
Tetratriacontyl heptafluorobutyrate	$C_{38}H_{69}F_7O_2$	690.9	✓				
Azulene	$C_{10}H_8$	128.1	✓				
Cyclohexane, methyl-	C_7H_{14}	98.1	✓				
Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl)-	$C_{20}H_{40}$	280.5	✓				
Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl)-	$C_{20}H_{40}$	280.5	✓				
Heneicosane, 11-cyclopentyl-	$C_{26}H_{52}$	364.6	✓				
1,3-Dicyclopentyl-2-n-dodecylcyclopentane	$C_{27}H_{50}$	374.7	✓				
Cyclooctasiloxane, hexadecamethyl-	$C_{16}H_{48}O_8Si_8$	593.2	✓	✓	✓		✓
Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$	667.3		✓	✓	✓	✓

3.5 Identification of TPH metabolites during the remediation process

The chemical composition TPH extracted from contaminated soil at initial time (T0) and at 90 days for the different treatments was analyzed by Gas Chromatography-Mass Spectrometry (GC/MS). Among the compounds identified there were alkanes, substituted alkanes, cycloalkanes, alkenes, and aromatics compounds (Tables 6, 7, and 8). The

identified compounds at 90 days for treatments TB1, TB2, TB3, and TB4 were compared with the detected compounds in T0 samples to determine the chemical compounds degraded and formation of new compounds. Table 6 shows that n-alkanes with C_{6-11} carbon chains at 90 days were not detected in treatments TB1, TB2, and TB3. These results indicate that the conditions of the TB1, TB2, and TB3 treatments favored the degradation of C_{6-11} chain alkanes. This behavior is because the metabolic activity of degradation of these compounds by microorganisms is favored, attributed to their low molecular weight (Chaudhary *et al*., 2020; Yang *et al*., 2019).

The C_{12-20} chain n-alkanes in treatments TB1, TB2, TB3, and TB4 were detected, which indicates that these compounds were not degraded. On the other hand, most of the C_{21-43} chain n-alkanes were not detected in the four treatments, so it can be concluded that these were biodegraded. The lack of degradation of C_{12-20} and C_{21-43} chain n-alkanes may be because C_{12-20} chain alkanes are a product of the degradation metabolism of structurally more complex compounds since it is assumed that the microorganisms began to degrade the short chain compounds, creating the conditions for a diauxic growth which induced the long carbon chain compounds to degrade, thus leaving the C_{12-20} alkanes as remnants. Table 6 also shows the presence of 3 new alkanes (Hexatriacontane, Tritetracontane, and Dodecane) for treatments T1, T2, T3, and T4; it is attributed that the new alkanes formed were from the degradation of the higher molecular weight compounds. Table 7 shows the substituted alkanes C3-38 and cycloalkanes C7-27, which were degraded principally on treatments TB1, TB2, TB3, and TB4. However, treatment TB1 shows the least quantity of these compounds. Also, new compounds are formed in different treatments since one cycloalkane, and nine substituted alkanes were detected on the TB2 treatment.

Table 8. Alkenes and aromatic compounds detected by CG/MS analysis in samples at initial time (T0) and at the end of the experiment (T90) for T1, T2, T3, and T4 treatments.

Compound	Formula	MW (g/mol)	T0	T2	T1	T3	T4
3-Heptene	C_6H_{12}	84.1	✓				
1-Docosene	$C_{22}H_{44}$	308.6				✓	✓
1-Hexacosene	$C_{26}H_{52}$	364.7	✓				
Squalene	$C_{30}H_{50}$	410.7					✓
Pentadec-7-ene, 7-bromomethyl-	$C_{16}H_{31}$	303.3	✓				
Toluene	C_7H_8	92.1	✓			✓	✓
p-Xylene	C_8H_{10}	106.1	✓			✓	✓
Ethylbenzene	C_8H_{10}	106.1	✓			✓	✓
Indane	C_9H_{10}	118.1	✓				
Benzene, 1-ethyl-2-methyl-	C_9H_{12}	120.1	✓			✓	✓
Benzene, 1,2,3-trimethyl-	C_9H_{12}	120.1	✓				✓
Naphthalene, 1,2,3,4-tetrahydro-	$C_{10}H_{12}$	132.2	✓	✓			
Benzene, 1-methyl-2-(2-propenyl)-	$C_{10}H_{12}$	132.2	✓				
Benzene, 1-methyl-3-propyl-	$C_{10}H_{14}$	134.2	✓				✓
Benzene, 1-ethyl-3,5-dimethyl-	$C_{10}H_{14}$	134.2	✓				
Benzene, 4-ethyl-1,2-dimethyl-	$C_{10}H_{14}$	134.2	✓	✓			✓
Benzene, 1,2,4,5-tetramethyl-	$C_{10}H_{14}$	134.2	✓				✓
Benzene, 1-ethyl-2,3-dimethyl-	$C_{10}H_{14}$	134.2	✓				✓
Benzene, (2-methyl-1-butenyl)-	$C_{11}H_{14}$	146.2	✓				
Benzene, 1,3-dimethyl-	$C_{11}H_{16}$	148.2	✓				
1,2-Benzenedicarboxylic acid	$C_8H_6O_4$	166.1	✓	✓	✓	✓	✓
N-Benzyl-2-phenethylamine	$C_{15}H_{17}N$	211.3	✓				✓
Benzene, 1,3,5-trimethyl-	$C_{12}H_{12}O_6$	252.2	✓			✓	✓
Mono(2-ethylhexyl) phthalate	$C_{16}H_{22}O_4$	278.3	✓	✓	✓	✓	✓
Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.6	✓	✓	✓	✓	✓
Di-n-octyl phthalate	$C_{24}H_{38}O_4$	390.6	✓	✓	✓	✓	✓

Table 8 shows the C_{6-35} alkanes and C_{7-24} aromatic compounds, having the least amount of these compounds detected in TB1 and TB2 treatments compared to TB3 and TB4 treatments. This indicates that the conditions of the TB1 and TB2 treatments stimulated the microbial communities to degrade C_{6-35} alkanes and C_{7-24} aromatic compounds, which is very important since these compounds are the most recalcitrant and difficult to degrade. Three aromatic compounds belonging to Phthalate with C_{16-24} carbon chains were detected in the four treatments, which indicates that the conditions in the different treatments did not induce the degradation metabolism of these compounds. We attribute that the lack of degradation of phthalate compounds with C_{16-24} carbon chains is due to their complex structure, as well as the toxicity they present during microbial growth (Boll *et al*., 2020; González *et al*., 2020).

It is worth mentioning that most of the alkanes, substituted alkanes, cycloalkanes, alkenes, and aromatics compounds of the TPH were mostly degraded in the TB1 treatment, which indicates that the conditions of this treatment stimulated the microorganisms to metabolize the aromatic compounds to products such as CO_2, H_2O, and other organic chemical compounds with lower number of carbons. Based on the above, it is suggested that the conditions of the TB1 treatment turn out to be the best and could be used in biopile on a pilot scale with a larger amount of soil to treat prior to move to industrial scale for the bioremediation of TPH contaminated soils.

Conclusions

The highest TPH biodegradation at microcosm level (42.97 ± 0.70 %) was reached with Tween 80 addition at a concentration of 5 CMC, followed by SDS (27.71 ± 0.62) at 1 CMC, and Triton X-100 (27.33 ± 1.47 %) at 5 CMC and with no surfactant addition (13.97± 0.38 %), where the remarkable issue was that for all treatments the moisture content was 30%. These results support the fact that moisture content in the system is crucial and plays a very important role in the degradation of TPH. In biopile treatments, all experiments showed a marked reduction of hydrocarbon contaminants during the first 30 days of the bioremediation process. Notably, aeration improves hydrocarbon removal mainly with the addition of surfactants. The maximal removal of TPH (49.89 ± 0.62) was obtained with the addition of Tween 80 at 5 CMC and 30% of moisture. Tween 80, moisture, and presence of oxygen as electron acceptor through air addition improved the abundance and microbial diversity. This treatment showed the reduction of most alkenes and aromatic compounds of highly polluted soil. Consequently, conditions from this investigation may be implemented to remove the hydrocarbon contaminants on impacted soils; however, we suggest further studies at a larger scale should be conducted.

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