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BIOREMEDIATION OF SOILS CONTAMINATED WITH AUTOMOTIVE OIL USED BY BIOPILE SYSTEM

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— *Abstract*—

In the investigation was made the bioremediation of contaminated soil with waste oils from automobile service by biopiles technique. The study was made taking four samples of soil, from the UMA "La Huella" of Pedro Méndez town in the municipality of Chiapa de Corzo, Chiapas, Mexico; being characterized in laboratories at the Universidad de Ciencias y Artes de Chiapas and El Colegio de la Frontera Sur, analyzing parameters such as moisture, organic matter, texture, pH, temperature, total nitrogen and available phosphorus. Four biopiles were built, developing three treatments with three replicates and a control, which were contaminated with oils used in different concentrations, 30,000 ppm to biopile witness, for the first treatment, 10,000 ppm, for second 30,000 ppm and 50,000 ppm third of HTP. The strains used were *Acinetobacter Sp, Stenotrophomona Sp and Sphingobacterium Sp*; each bacterium was inoculated at a concentration of 2.25 × 108 CFU for each of the treatments. Counts of viable microorganisms was performed the fifteenth day during the experiment. Treatments were monitored for three months on days 1, 15, 30, 60 and 90 in the biotechnology laboratory of the Universidad Autónoma del Carmen, Campeche. With this work considerable removal percentages of the fractions were achieved, in the aliphatic removal 93.7 to 87.1% was achieved and the aromatic hydrocarbon fraction, removals from 0 to 94.8 in 90 days of treatment with the application of bioremediation bacteria and identification of bacterial strains native soil, who managed to resist change in their environment.

Keywords

Bioremediation, automobile waste oils, biopiles technique, Acinetobacter Sp, Stenotrophomona Sp and Sphingobacterium Sp.

The inadequate management of hazardous waste has generated a global problem of contamination in soils, the air and water. Among the most severe contamination is that produced by the extraction and management of oil and its by-products. In Mexico, one of the countries with the highest oil production worldwide, there are a considerable number of sites impacted by these pollutants. In southeast Mexico there are sites with different levels of environmental impact by oil activity from over fifty years of activity (Randy et al . , 1999; Roldán and Iturbe, 2005).

The traditional physical-chemical methods for the remediation of contaminated sites have been: incineration, landfills, soil washing or some chemical treatment. These are costly and can produce different toxins that are able to stay on the ground, migrate into surface or groundwater, or are emitted to the atmosphere (Eweis et al . , 1998; cited by Volke and Velasco, 2002; Ifeanyichukwu, 2011) .

According to studies conducted in the United States and the United Kingdom, the bioremediation market for contaminated soil treatment has increased because costs can be reduced by 65-80% over physicochemical methods (Zechendorf, 1999; By Volke and Velasco, 2002). Acceptance of bioremediation as a viable strategy depends on its cost and efficiency on a contaminated matrix (Semple et al . , 2001; cited by Volke and Velasco, 2002).

In the United States, around 3.2 billion liters of used motor oil is recycled annually. In Mexico, their use and management are less adequate and has become a contamination factor (Ifeanyichukwu, 2011).

This study aims to bioremediate automotive oil contaminated soil by Acinetobacter Sp, Sphingobacterium Sp and Sp Stenotrophomona through a system of biocells, analyzing the physical-chemical soil characteristics and identifying the contribution of bioremediation bacteria during processing.

Background

The concept of bioremediation describes a variety of systems using live organisms to remove, degrade or transform toxic organic compounds into less toxic or safe metabolic products (Van Deuren et al., 1997). Biological processes involving enzymes as catalysts can alter organic molecules to produce changes in their structure and toxicological properties, resulting in the conversion of these compounds into inorganic products such as water, CO2 or inorganic forms of N, P and S, cellular components and products of metabolic pathways (Alexander, 1994; cited by Volke and Velasco, 2002).

Used oils are composed of total petroleum hydrocarbons (TPH), polychlorinated biphenyls (PCBs), polycyclic aromatics (PAHs), metals and other pollutants that cause deterioration in the environment and human

health, are toxic and poisonous, are substances that are difficult to degrade (Vasquez et al., 2010) and are hazardous waste NOM-052-SEMARNAT-2005. Motor oil is a lubricating agent and auxiliary fuel, refined and incorporated into asphalt. Many components of the oil are toxic and may be carcinogenic and endocrine disruptors. The aliphatic components are removed from the environment through natural attenuation; however, the recalcitrant fractions remain persistent. Polycyclic aromatic hydrocarbons (PAHs) are hazardous components of used motor oil (Ifeanyichukwu, 2011).

In California, in situ bioremediation in soils contaminated by diesel oil was done by indigenous microorganisms, nutrients, oxygen and inoculation of an enriched bacterial consortia extracted from the same soil mix soil. It allowed the identification of hydrocarbon degrading bacterial consortia identified by 16S-RNA gene sequencing, with Bacilluscereus, Bacillusphaerus, Bacillusfusiformis, Bacilluspumilis, Acinetobacterjunii, and Pseudomonas Sp (Volke and Velasco, 2002).

Perez et al. (2007) in Cuba studied biodegradation by isolated strains of soils contaminated with petroleum, by sequential enrichment using petroleum as a source of carbon and energy. They isolated bacterial strains, five Gram negative and four Gram positive, from the Bergey's Manual (1994). Pseudomonas aeruginosa was selected because of its higher growth with oil as a source of carbon and energy, degrading 57%.

In a study in Colombia, there was an evaluation of the native microbial consortia added to dewatering sludge biopiles from the primary treatment of domestic wastewater, sludge from car washings and sewage sludge from the industrial area of Bucaramanga. In these tests there were isolated, identified and preserved microbial strains degrading capacity of total petroleum hydrocarbons (TPH) like Pseudomonas spp., Pseudomonas aeruginosa, Acinetobacter spp, enterobacterial rcloacae, Citrobacter spp., Bacillusbrevis, Micrococcusspp and Nocardiaspp. It was inoculated at a concentration of 3x10 8 CFU / ml bacterial and fungal microorganisms as Aspergillus., Fusarium spp., Trichodermaspp., At a concentration of 1x106 spores / ml. Removal percentages were obtained up to 94% of HTP in 120 days and 84% in 40 days (Vasquez et al., 2010).

Garcia et al. (2012) in Venezuela, studied the biodegradation of a medium oil in two soils of different textures, with and without the addition of a structuring agent (litter). Soils that were tested were sandy (quartz and ilite), and sandy loam (quartz and kaolinite). Four treatments in triplicate in microcosms with an initial crude content of 5% (m / m) were evaluated and fertilizers were added to obtain the C / N = 60 and C / P = 800 ratios, and the piles were aired and the Humidity between 40 and 60% was maintained. The total hydrocarbon concentration was determined at 1, 15, 30, 45 and 90 days. The clay-sandy-clay texture favored the biodegradation process of

the total hydrocarbons in the saturated components. The structuring agent influenced biodegradation.

Acuna et al. (2012), worked with humidity of 3, 10, 15 and 20% and temperatures of 5, 15, 28 and 37 ° C, and followed with hydrocarbon mineralization. To optimize the nutrient ratio, microcosms monitored for oxygen consumption and gas chromatography were designed. The results indicate that the mineralization was optimal for hydrocarbon humidity of 10 to 20% and temperatures of 25-37 ° C with production values of CO 2 from 3.000 to 4.500 mgCO2 kg-1. The optimum C: N: P ratio was 100: 1: 0.1 in which the highest oxygen consumption and removal of 83% of the total hydrocarbons were observed, with 78 and 89% removal of n- Alkanes and polyaromatic hydrocarbons.

Biochemical basis of bioremediation

It is based on oxide-reducing reactions that occur in the respiratory chain, or electron carrier of the cells. The chain is initiated by an organic substrate external to the cell that acts as an electron donor, where the metabolic activity of the cell degrades and consumes the substance. The acceptors used by microorganisms are oxygen, nitrates, iron (III), sulfates and carbon dioxide (Maroto and Rogel, 2013).

Biodegradability depends on factors such as:

Humidity (depends on soil texture and porosity).

Nitrogen is necessary for protein and cell wall synthesis and can be lost by the leaching of ammonium and nitrates and by soil denitrification. Phosphorus is useful for forming nucleic acids and ATP and is limited by low solubility and availability. The presence of minimal amounts of nitrogen and phosphorus allows the biodegradation at low speeds (Roldán and Iturbe, 2005).

Activity of microorganisms and oxygen supply. When the soil pores are occupied by water, oxygen is lower and anoxic conditions occur. For aerobic degradation it is necessary to have 10% of pores free (Roldán and Iturbe, 2005).

The pH affects microbial activity- the greater the diversity of microorganisms, the greater tolerance. The growth of most microorganisms is maximum at pH between 6 and 8 (Dibble and Bartha, 1979, cited by Torres and Zuluaga, 2009).

The temperature in mesophyllic conditions between 20 and 30 ° C allows bacteria to grow, decreasing at temperatures above 40 ° C and inhibiting below 0 ° C (Torres and Zuluaga, 2009).

Organic pollutants are a source of carbon that microorganisms require for biodegradation. When environmental conditions and colony forming units (CFUs) are not in adequate quantities, they are established through engineering processes. At high concentrations of contaminants, there is toxicity in microbial populations and nutrient insufficiency (Roldán and Iturbe, 2005).

Bioremediation technologies include:

- Bio stimulation: addition of oxygen and/ or nutrients to stimulate activity of autonomous microorganisms and biodegradation of contaminants (Van Deuren et al. , 1997; Manacorda and Pictures, 2005).
- Bio augmentation: addition of microorganisms to degrade contaminants (Riser and Roberts, 1998; Manacorda and Pictures, 2005).
- Bio styling: a mixture of volume and nutrient agents, is styled to favor aeration , mixing clean soil with contaminated soil (Van Deuren Et al. , 1997; cited by Volke and Velasco, 2002).
- Bio venting: stimulate biodegradation of a pollutant by a supply of air. Aeration promotes degradation by: volatilization, migration of the volatile phase of contaminants and biodegradation, stimulating bacterial activity (Maroto and Rogel, 2013; Manacorda and Pictures, 2005).
- Phytoremediation: using plants to remove, transfer, stabilize, concentrate and / or destroy contaminants in soils (Van Deuren Et al., 1997; cited by Volke and Velasco, 2002; Manacorda and Pictures, 2005).

Biopiles

Biopiling are ex situ bioremediation in unsaturated conditions, and reduces the concentration of petroleum contaminants from soil (Benavides et al., 2006). The choice of biopiles depends on climatic conditions and structure of volatile organic compounds in the soil. Biopiles are designed as closed systems to maintain temperature, avoid saturation of rainwater, and decrease evaporation of water and volatile organic compounds (Eweis et al., 1998, cited by Volke and Velasco, 2002).

Criteria for the design of biopiles:

- Extension of the contaminated soil (volume and site data) (Roldan and Iturbe, 2005).
- TPH in concentrations less than 50, 000 ppm and higher at 10, 000 ppm (Vasquez et al . , 2010; Roldan and Iturbe, 2005; Benavides et al., 2006).
- Heterotrophic bacteria in concentrations greater than 1 000 CFU / lg of dry soil (Roldan and Iturbe, 2005).
- PH between 6 and 9 (Roldan and Iturbe, 2005).
- Humidity content between 70 and 95% of field capacity.
- Low clay content and / or silt (Roldan and Iturbe, 2005).
- C, N, P and K about 100: 15: 1: 1 (Roldan and Iturbe, 2005).
- Toxic metals less than 2 500 mg / kg (Roldan and Iturbe, 2005).

According to the characteristics of the composting process, in the initial stage aeration is necessary due to an accelerated microbial activity. This increase in activity causes an increase in metabolic heat, producing temperatures in the thermophyllic range (50 to 60 ° C) (EPA, 1998, cited by Volke and Velasco, 2002).

Then the microbial activity decreases because the biodegradable components are consumed. At this stage the oxygen and temperature requirements decrease, so the compost requires less aeration. Once the cell cools and the temperature approaches the environment, the composting period is considered complete, including change of texture and odor. Depending on the amount of organic material mixed with soil, mass is reduced 40% (., Cited by Volke and Velasco, 2002 Eweis et al, 1998).

Optimum composting conditions depend on parameters in three categories: soil characteristics, climatic conditions and characteristics of pollutants. A key factor is the selection of additives, which leads to an increase in the rate of biodegradation of contaminants (Eweis et al . , 1998; cited by Volke and Velasco, 2002).

Regions with warm weather between 20 and 40 ° C are more efficient for bioremediation. The operating temperature of a biopile is between 30 and 40 ° C (mesophyllic stage) and depends mainly on the heat generated by the metabolic activity of the microorganisms in the compost and the climatic conditions of the place (Volke and Velasco, 2002).

Mexico is a region suitable for these technologies since 50.9% of the territory has climates with temperatures between 18 to 26 ° C. The average temperatures range from 25 to 28 ° C (maximum 41 ° C) in regions where most of the sites are contaminated by hydrocarbons (PEMEX, 2001) in

coastal and low regions (Chiapas, Oaxaca, Tampico, Hidalgo, etc.) , Cited by Volke and Velasco, 2002).

Bioremediative Bacteria

Bioremediation accelerates biodegradable processes that naturally occur in contaminated ecosystems, where microbial communities are often dominated by adapted microorganisms capable of using toxic compounds from the contaminated location. Oil contaminated environments are often dominated by Gram negative bacteria (Perez et al . , 2007; Manacorda and Pictures, 2005) system.

If the contaminants are synthetic and new in the environment, the microorganisms do not have the capacity to degrade them, since the biodegradable genes have not evolved. Even if genes are present, the functional expression of genes is essential so that appropriate degrading enzymes can be produced (Daubaras and Chakrabarty, 1992).

MATERIALS AND METHODS

Soil characterization

EIn the laboratory of Environmental Engineering of UNICACH, the following parameters were evaluated: texture using sieves 4, 8, 10, 20, 50, 100 and 200 for a sample of 400 g of dry soil. The pH considered the NOM-021-SEMARNAT-2000, using a potentiometer. The field capacity was done by saturation and allowed to drain for twelve hours, and it was then weighed, kiln dried and weighed without moisture. The percentage of organic matter was obtained by weight difference.

Total nitrogen was calculated using the method of Micro Kjeldahl and available phosphorus with the Olsen method done by the Laboratory of Analysis of Soils and Plants in the Colegio de la Frontera Sur (ECOSUR), according to NOM-021-RECNAT-2000. Temperature was taken with a Taylor digital model 9842 thermometer.

Preparation of the inoculum

Samples of *Stenotrophomonas sp,Acinetobacter sp.* and *Sphingobacterium* strains were identified, which were isolated from soil contaminated with hydrocarbons in Tuxtla Gutierrez, Chiapas (93 ° 10'36.86''W 16 ° 45'43.92''N) identified by the Technological Institute of Tuxtla (Cisneros, 2011). Strains

were maintained in 1.6 ml eppendorf tubes with LB medium and 30% glycerol at -20 ° C.

Bacteria were transferred to test tubes with approximately 3 ml of Luria broth and incubated at 37 ° C for 72 hours. Three sterile Petri dishes were seeded in three sterile boxes for each bacterium, and Agar Infusion culture medium was added and incubated at 37 ° C for 24 hours for identification.

The bacterial count was performed using the Machelle nephelometric scale prepared by the State Public Health Laboratory, for which threaded test tubes numbered 0.5 and 1 to 10 were organized. Each tube was filled with 1% anhydrous barium in aqueous solution and a cold solution of 1% sulfuric acid PA (V / V) in different concentrations according to the desired turbidity.

The bacteria were inoculated into petri dishes previously seeded in a test tube with 4 ml of 0.9% saline solution. The transfer was performed by taking bacteriological loop portions of inoculum and homogeneously mixing in test tubes to desired turbidity compared to the tube 0.5 of the scale, which represents a concentration of 1.5 x 108 CFU / ml.

Genetic analysis

Two genetic analyzes were performed- the first to corroborate the bacteria used and the second to know which bacteria were able to adapt. The extraction of deoxyribonucleic acid (DNA) was performed by cell lysis / phenol-chloroform-isoamyl alcohol method (Espinoza and García, 2003). Three inoculates were placed from a solid culture medium (heart brain infusion agar), for the first analysis and 10 for the second. To each tube added 360 µl of 0.5 M EDTA (pH 8.0); 19 µl of 10% SDS; 25 µl 0.1 M Tris HCl (pH 8.0) and 25 µl Proteinase K (10 mg / ml) was added. They were agitated for 20s and placed in a water bath of 12 to 18 hr at 55 ° C to dissolve tissue.

The samples were submitted to an RNAase treatment. They were removed from the water bath and allowed to cool to room temperature for 5 minutes. To each tube, 2.2 µl of RNAase at a concentration of 10 mg / ml was added and left in a water bath for one hour at 37 ° C. The tubes were removed and allowed to cool to room temperature for 5 minutes. 400 µl of ammonium acetate was added to each tube and vortexed for 20 s. The tubes were centrifuged for 5 minutes at 14,000 rpm.

The supernatant was removed and placed in tubes with 600 µl of alcohol at -20 ° C. Samples were centrifuged for 6 minutes at 14,000 rpm. The alcohol was removed from the tubes and 125 µl of 80% ethyl alcohol was added. They were allowed to stand for 1 to 2 minutes to rehydrate the DNA. The samples were centrifuged for four minutes at 14,000 rpm and the supernatant removed. The tubes were left uncovered for 30 minutes and 100 µl of sterile water was added to hydrate the DNA until the button disappeared.

DNA visualization

Visualization of the extracted DNA was performed by 1% (w / v) agarose gel electrophoresis. To prepare the gel 0.3 g of agarose was dissolved in 38 ml of 1X TAE buffer. 3 µl of 6X blue per sample was placed in a parafilm, and 5 µl of DNA was re suspended with a micropipette. The samples were then placed in the wells of the gel, the chamber was connected to a voltage of 120 volts, for one hour.

When the gel finished running, it was removed from the tray, then stained with fluorescent ethidium bromide (3.8-diamino-6-ethyl-5-phenylphenanthridium bromide, 10 mg / ml for 15 minutes, (Valadez and Kahl, 2000) which, when intercalating with DNA molecules, emits radiation by employing an transilluminator ultraviolet light, where the bands of total DNA are evidenced. DNA amplification was performed by the Polymerase Chain Reaction (PCR), a process consisting of synthesizing a DNA fragment with polymerase at elevated temperatures (Espinosa, 2007).

To prepare the polymerase chain reaction samples (PCR) the Green Master Mix kit was used, placing12 µl of master mix in tube for microcentrifuging, 10 µl of water for master mix, 1 of each primer and 1.5 µl of DNA , With a volume of 25.5 µl, they were placed in the TECHNE TC-3000 thermocycler, according to the following temperature conditions: a four-minute cycle at 94 ° C, followed by 32 cycles of 1 minute at 94 ° C, one minute At 53.5 ° C, one minute 30 seconds at 72 ° C and 7 minutes at 72 ° C.

Amplified DNA visualization was performed by 2% (w / v) agarose gel electrophoresis. For this, 0.6 g of agarose was dissolved in 38 ml of 1X TAE in an Erlenmeyer flask. The loading buffer was 2 µl of the amplified DNA and 1 µl of bromophenol blue and 1 µl of 100-base-pairs DNA ladder consisting of 11 fragments with exact increments of 100 base pairs from 100 to 1500 where the 500 bp band has a triple intensity compared to the other fragments. The gel bleeding schedule was 60 to 120 minutes at 120 volts. For visualization of the amplification the gel was stained with ethidium bromide and the image taken by the camera directly from the ultraviolet light transluminator. The photographs were edited with Picture Manager . The purification and sequencing of samples was performed by the MACROGEN company in Seoul, Korea with an Perkin Elmer ABIPRISM sequencer.

Preparation of biopiles

For the construction of the biocells, four samples of soil from 0.013m3 from the Management Unit and Conservation of Wildlife "La Huella" located in the ejido Pedro Mendez in the municipality of Chiapa de Corzo, Chiapas, Mexico were taken, At a latitude N of 495757 and longitude W of 1840501,

with a height above the mean sea level of 420 m, which presents subhumid warm climate with summer rains (Awo) and average annual temperature of 26.3 ° C. The results of the soil characterization indicated that it has physio-chemical properties for bioremediation: pH between 6 and 8 units, low clay and / or silt content and high nutrient content, a temperature between 25 and 35 ° C is expected during the treatment (Roldán and Iturbe, 2005).

Four wooden crates, 35 cm long by 25 cm wide and 15 cm high, were constructed by calculating that the amount of soil would be adequate to carry out the analyzes during the process. The boxes were designed with slope of 2 to 3% to allow leakage of leachate (Roldan and Iturbe, 2005). They were lined with a plastic layer to avoid leachate absorption and allow it to run off.

In case leachates are generated, these would be collected in an impermeable base placed in the lower part.

Pollution and conditioning

5 kg of dry soil were weighed, then were contaminated by motor oil in three different concentrations of 10,000 to 50,000 ppm of TPH (Vasquez et al, 2010;. Roldán and Iturbe, 2005, Benavides et al . , 2006). The first treatment was contaminated with 10,000 ppm of TPH, the second with 30,000 ppm, the third with 50,000 ppm and the control biopile with 30,000 ppm. The quantities of oil were measured in a graduated cylinder. Finally, the soil was mixed with the oil.

The treatments were labeled: control biopile C1; Biopile with concentration of 10,000 ppm, C2; Concentration of 30,000 ppm, C3 and concentration of 50,000 ppm C4. The conditioning was performed biocell adding water until a humidity of 60 to 70% of field capacity (Vasquez et al., 2010), it was verified that the pH is between 6 and 8. found bacteria were inoculated to a concentration of 1.5 x 10 8 CFU / ml compared to the MacFarland scale 1.5 ml saline inoculated per bacterium (Roldan and Iturbe, 2005) were added inoculating a higher concentration of 1000 CFU / g of dry soil, the was aerated by half of manual mixes, the temperature was taken and the humidity was controlled by fist method every two days and humidity in the oven and pH every 15 days. It was covered with a plastic layer to avoid erosion and rainfall runoff.

Identification of removal levels

Three soil samples were taken from the bottom of each biopile, sectioning the box in three parts lengthwise, per box for each treatment and

the control. The samples were placed in 120 ml plastic tubes with a screw cap and wide mouth filling them completely and finally freezing them to preserve them for analysis. Samples taken were oven dried at 60 ° C for 72 hours, the pellet was homogenized by stirring the three simple samples from the samples of each treatment and the control, thus having four samples to be analyzed for each sample, passed through a sieve of 0.5 µm.

To extract the hydrocarbons, a sub-sample of 20 g of sediment was weighed and the blank, for which sand was used, was weighed in an extraction thimble. 200 ml of methylene chloride was added to a 250 ml round bottom flask and boiling bodies were added. The soxhlet was then assembled and refluxed for 8 to 12 hours. The methylene chloride fraction was evaporated in a water bath using a rotary evaporator; hexane was added three times until the solvent was replaced without allowing the sample to dry. The sample was stored in the flasks to pass through column chromatography.

Column chromatography

For the preparation of the sample, glass chromatographic columns were packed with a Teflon key with glass wool stopper. 10 g of alumina and 20 g of partially deactivated silica gel were weighed into 50 ml beakers. 1 cm of partially deactivated sodium sulfate and dry alumina were added using funnel. 20 g of silica gel and sodium sulfate (1 cm) were added. 30 ml of methylene chloride was then poured through a funnel and keeping the key open, allowed to drain until the level was above sodium sulphate in order to add 1 cm of copper powder to contact with the chloride Methylene chloride.

When the solvent level was found on the surface of the copper powder, the sample was added along with three flushes with 0.5 ml hexane. The key was opened for the sample to enter and elution was initiated for fraction 1 aliphatic hydrocarbons with 100 ml of hexane and for fraction 2 aromatic hydrocarbons with 100 ml of hexane and 100 of methylene chloride mixed. The fractions were evaporated using rotary evaporator to a volume of 1 ml. The sample was transferred to a graduated tube, along with two 2 ml hexane rinses, and concentrated by flowing nitrogen to 2 ml. They were quantitatively transferred to 2 ml vials.

Fraction 1 of saturated aliphatic hydrocarbons was analyzed by gas chromatography using flame ionization detector. Fraction 2 of aromatic hydrocarbons and polychlorobiphenyl pesticides added prior to the injection of TCMX standards (100 µl to each sample) and O-terphenyl (5 µl to each sample) was analyzed by gas chromatography using flame ionization detector and capture de electrones para cada grupo.

Biopiles were monitored by Benavides et al. (2006) and Roldan and Iturbe (2005), five parameters: aeration, temperature, moisture content, carbon / nitrogen (C / N) ratio and pH. The viable cell count was carried out to determine the microbial activity and ensure that you condition were appropriate.

Viable cell count

The viable cell count was performed on the 15th day of treatment (Vasquez et al ., 2010), prepared according to Ruiz et al. (2013) a mineral medium were added with amounts compounds in distilled water: 0.15 g / L monobasic potassium phosphate (KH 2 PO 4); 1.156 g / L of ammonium (NH chloride 4 Cl); 0.02 g / L magnesium sulfate heptahydrate (MgSO 4 7H. 2 O); 0.04 g / L calcium chloride (CaCl 2); 0.07 g / L sodium chloride (NaCl); 1 ml / L of trace metal solution; 2 g / L peptone and 2 g / L dextrose. The compounds were added in 1 L of distilled water, the mixture was partitioned into eight 125 ml flasks each were sterilized same as in the autoclave. Each mixture was labeled in duplicate as C1, C2, C3 and C4, according to the labels of each treatment, and was added to the first four under sterile conditions, approximately 2 g of soil previously removed treatments the corresponding medium.

The inoculated medium was allowed mineral reposing for 24 h, so that bacteria will develop. After 24h the bacteria that grew on the medium were passed using a bacteriological loop to the remaining four flasks, growth steeping for 24 h. Over time the bacterial count by adding 1 .mu.l of medium inoculated on a slide using a micropipette it was performed. The slide was placed in an automated cell counter mark BIO-RAD (TC20).

The temperature was monitored every two days during the three months of treatment (Garcia et al., 2012). The schedule of temperature taking was not defined, sought to be held in the afternoons. The humidity was monitored with the cuff method every two days and lab every fortnight to keep humidity from 60 to 70% (Vasquez et al ., 2010). PH analysis were performed in the laboratory every fortnight. Manually it aired each treatment every two days to facilitate oxygen transfer (Garcia et al ., 2012).

Isolation of soil bacteria at end of treatment

Isolation of bacteria was held on 80 day of treatment to identify existing organisms at the end of the process. For this a gram of soil of each treatment was added to 5 ml of distilled water in test tubes labeled according to treatment and pre-sterilized. Subsequently the tubes were shaken on a vortex for 10 sec and left to stand for 30 minutes. dilutions were made by

taking a 5 ml aliquot of the original mixture to be poured later in a test tube with 4.5 ml of distilled water, thus obtaining a 1/10 dilution, this step was repeated until achieving dilutions 1 / 10,000,000. dilutions 1 / 1,000 were taken; 1 / 100,000; 1 / 10,000,000 to add 5 ml of distilled water supernatant of each of the tubes and placed in Petri dishes with agar culture medium brain heart infusion. The inoculated media were incubated at 27 ° C for 24 hrs. Bacterial colonies were identified that were placed in Eppendorf tubes 1.5 ml for subsequent genetic analysis. Samples of the treatments were labeled with a total of 10 samples for analysis C1 (C101), C2 (C201, C202, C203), C3 (C301, C302, C303) and C4 (C401, C402, C403).

Results

According to the Unified System of Soil Classification (USCS), the soil belongs to soils of coarse particles located within the clean sands with wide range in intermediate sizes, being located in the SW group called well graded sands, sands with gravel, permeable in compacted states, excellent shear strength and negligible compressibility.

Soil pH is 7.6 units, to be in the range of 7.4 to 8.5 is considered a moderately alkaline soil pH. Moisture presenting half saturated soil is 30.67%. For every 3.5321 g 1.5629 g would be necessary to add water to achieve saturation. 75% of field capacity necessary to achieve good bioremediation amount to add for each 3.5321 g soil, 1.1721 g of water.

The organic matter was 7.45%, according to Mexican Official Standard NOM-021-RECNAT-2000, it is considered very high (greater than 6.0). The total nitrogen was 0.46%, according to Mexican Official Standard NOM-021-RECNAT-2000 and is considered a very high nitrogen content (greater than 0.26). A match available 19.5 mg / kg was obtained, according to Mexican Official Standard NOM-021-RECNAT-2000 content is high (greater than 11.1 mg / kg).

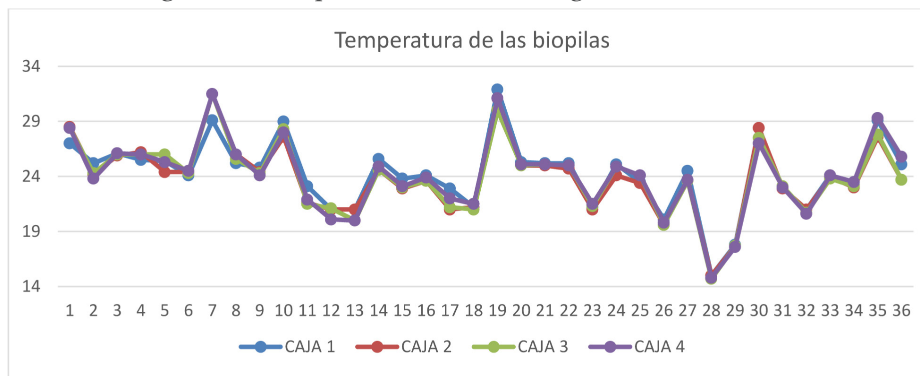
During monitoring, the biopiles moisture percentages were recorded every 15 days. The moisture content relative to field capacity is expressed in Table 1.

Table 1. Percentage of moisture during the bioremediation process.

Muestreos Tratamientos	H1	H2	H3	H4	H5	H6	H7
C1	63.06	51.45	73.39	80.99	89.63	74.50	88.52
C2	56.18	50.93	83.76	86.01	80.93	75.38	94.59
C3	64.43	42.06	87.35	84.02	88.52	75.51	86.86
C4	60.45	51.71	76.72	79.49	73.85	70.59	81.48

The humidity oscillated between 50 and 90%, and only in the second sampling where the humidity was low with an average of 49.04%, while the highest recorded was presented in the box 2 with 94.59%. On average, moisture percentage with respect to field capacity was 74.01% is (Figure 1).

Figure 1. Temperature monitoring of the treatment



Registration of pH is shown in Table 2.

Table 2. pH values obtained at each sampling during bioremediation.

Fechas de muestreos		Caja 1	Caja 2	Caja 3	Caja 4
M1	14/10/13	6.98	6.65	7.4	7.37
M2	20/11/13	7.01	7.08	7.08	7.2
M3	05/12/13	7.8	7.93	7.89	7.95
M4	18/12/13	7.84	7.71	7.9	7.88
M5	08/01/14	7.72	7.85	7.65	7.66
M6	17/01/14	7.39	7.42	7.58	7.61
M7	31/01/14	7.03	7.35	7.56	7.63

The bacteria counter showed that the amount of cells for biocell control (C1) was 5.98 x 105 cells / ml, treatment 1 (C2) 5.93 x 105 cells / ml, treatment 2 (C3) 2.29 x 105 cells / ml and treatment 3 (C4) 4.93 x 105 cells / ml. In Table 3 the initial and final concentrations of hydrocarbon fractions are shown.

Table 3. Initial and final hydrocarbon concentrations in the bioremediation process..

Tratamientos	Fracción aromática		Fracción alifática	
	Inicial ppm	Final ppm	Inicial ppm	Final ppm
C1	15387.2	35476.3	22221.8	1417.98

C2	9985.5	519.3	15471.9	1481
C3	21049.1	12885.2	17922.2	1997.85
C4	48092.3	8642	19135.2	2469

Similarly we can see the final percentage of removal of hydrocarbons obtained during treatment in aliphatic and aromatic fractions (Table 4).

Table 4. Percentages of removal in fractions of hydrocarbons in the bioremediation process..

Tratamientos	Fracción aromática	Fracción alifática
	% Remoción	% Remoción
C1	0	93.62
C2	94.8	90.43
C3	38.79	88.86
C4	82.04	87.1

The extraction and amplification of genomic DNA of 13 samples in total was achieved. Three are inoculated bacteria at baseline and 10 remaining bacteria were isolated at the end. Purification and sequencing of genomic DNA sequences of 13 with a total of 400 base pairs each was achieved.

Phylogenetic trees generated two were obtained by algorithm and nearest neighbor UPGMA respectively with the MEGA program. Within the algorithm UPGMA four clades called Group I, II, III and IV (Figures 2 and 3) are shown. The first group is composed of C302 unidentified C403 Burkholderiay C401 Mycobacterium . The second is made up of individuals initially inoculated, corresponding to OTEC 01, 02 and 03 called Acinetobacter Sp, Sphingobacterium Sp and Stenotrophomona Sp. The third group is made up of C203 Pseudomonas, C201, C301, C101 and C202 unidentified and finally the fourth group is composed of C303 Streptomyces and C402 Bacteroides. The tree generated by the nearest neighbor algorithm is composed of three clades called group I, II and III. The first group consists of C101, C202, C301, C201, C203, C303 and C404. The second group is composed of C302, C403 and C401 and the group III by OTEC 01, 02 and 03 as seen in Figure 2.

Figure 2. UPGMA algorithm Cladogram MEGA program via p-distance. The number on nodes corresponds to bootstrap support values with 100 repetitions.

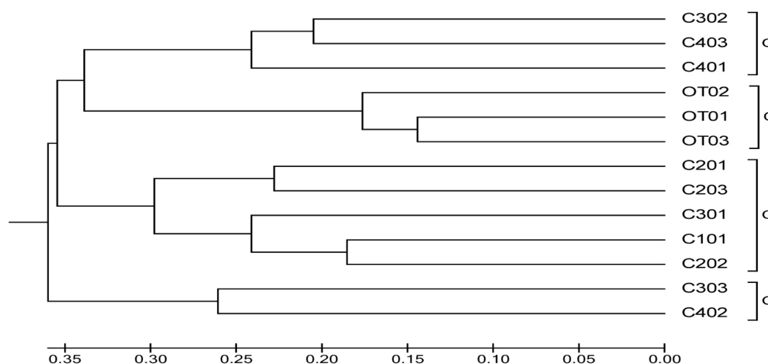
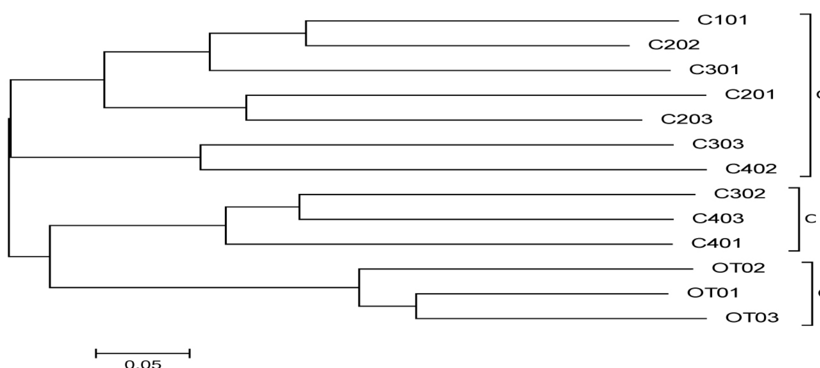


Figure 3. Cladograma of the nearest neighbor algorithm MEGA program via p-distance. The number of nodes corresponds to bootstrap support with 100 repetitions.



Discussion of Results

The soil from the UMA La Huella has a high sand content, SW group of SUCS, porous soil and has silts and clays and the presence of nutrients, which resulted: 7.45% carbon, 0.46% nitrogen and 19.5 mg / kg of phosphorus, with very high and the first two high phosphorus. It was not possible to know the bioavailability of nutrients in the soil, however, the results of hydrocarbon degradation assume that the microorganisms develop during treatment achieved in the environment, a process for which the presence is necessary nutrients bioavailable. Acuna et al . (2012) mention that the

low concentration of bioavailable nutrients (nitrate, nitrite, ammonium and phosphate) is unfavorable for the bioremediation process that could be solved with biostimulation.

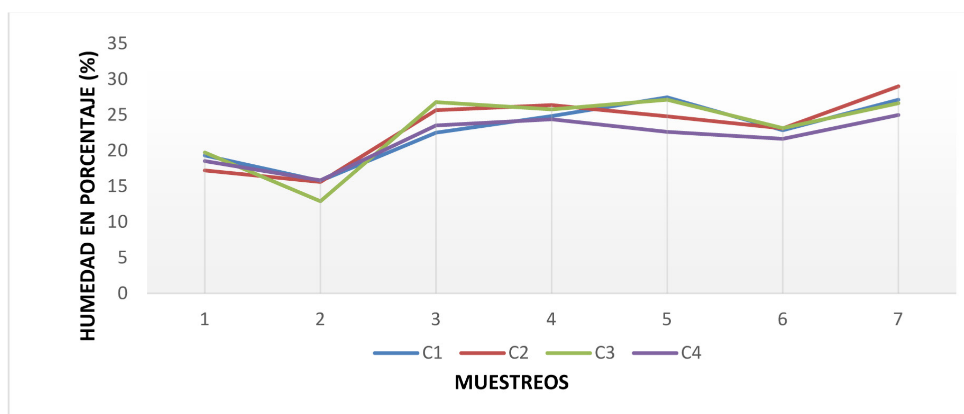
The sand prevailed in this soil, allowing oxygen to penetrate the pores thus achieving adequate oxygenation. Fine particles that were present favor the retention of water incorporated into the soil, conditions necessary for proper microbial growth (Acuña et al ., 2012). About Garcia et al . (2012) highlight that the availability and the microorganism hydrocarbon contact is key for bioremediation, soil SW group may be a viable option for the application of this technique. In addition to the above features, a neutral pH is essential for the proper development of the macro and micro in a treatment biopiles (Acuña et al ., 2012).

At the time that an oil spills in soils, as seen in the experiment, there results a series of processes that negatively impact the bacteria found to modify the environment that were acclimated, grow slowly or die (Manacorda and Cuadros, 2005) and some manage to adapt. Daubaras and Chakrabarty (1992) mention that if contaminants are synthetic and in a new environment, microorganisms have the ability to degrade pollutants as appropriate biodegradative genes could not have evolved even if the genes are present; the expression of functional genes is essential to produce appropriate degradation enzymes. The amount of oxygen decreases because the floor spaces are filled with hydrocarbon (Restrepo, 2002) and nutrients phosphorus and nitrogen that microorganisms used for development are generally limited in the middle by excess carbon generated in the oil spill (Tyagi et al ., 2011).

The bacteria used in treatments, Acinetobacter Sp , Sphingobacterium Sp and Stenotrophomona Sp , having been removed from a chronically oil contaminated soil, a place where substances such as lubricating oil are used, are considered suitable bacteria for the degradation of contaminants since they have been present at the affected site for a long period they managed to adapt to this and develop the system that allows them to degrade organic pollutants resulting from human activities (Ruiz et al., 2013). In this regard Manacorda and Cuadros (2005) highlight the importance of indigenous microorganisms, since they are the major decomposers in the ecosystem since this activity existed naturally in the environment prior to the disposition of xenobiotics (Benavides et al . , 2006; Martinez et al ., 2011).

After the spill, suitable ground conditions existed where aerobic bacteria could undertake their duties and reproduce at a faster rate and degrade the contaminant. The factors controlled during treatment were: humidity, temperature, pH and oxygen same as monitored throughout the process. Figure 4 shows the humidity recorded every two weeks during treatment.

Figure 4. Percentage of humidity recorded during treatment.



According to the results expressed we can see that the biopiles had the same moisture, although it was not constant during treatment. In Table 5 the moisture content expressed in relation to field capacity is presented. We can see that sometimes is raised up reaching 94.59%, corresponding to sampling in July of treatment 1 (C2). According Roldan and Iturbe (2005), excessive moisture is undesirable for bioremediation with biopiles, since the spaces in soil are filled by water causing a deficiency in aeration. Excess water leachate generation increases both hydrocarbon and nutrients. Due to the size and amount of soil, leaching was handled almost imperceptible in the biopiles.

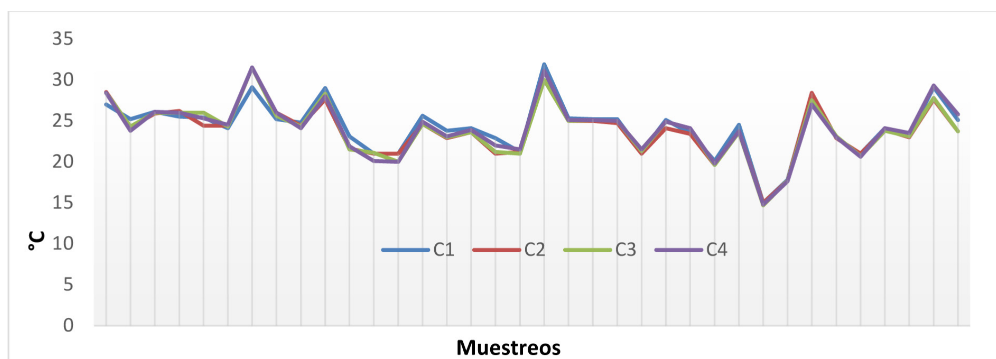
Table 5. Percentage of humidity recorded in relation to field capacity..

Muestreos tratamientos	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)	7 (%)
C1	63.06	51.45	73.39	80.99	89.63	74.50	88.52
C2	56.18	50.93	83.76	86.01	80.93	75.38	94.59
C3	64.43	42.06	87.35	84.02	88.52	75.51	86.86
C4	60.45	51.71	76.72	79.49	73.85	70.59	81.48

Optimal humidity ranges from 40 to 85% (Volke and Velasco, 2012), 70 to 95% of field capacity (Roldan and Iturbe, 2005); high humidities may be used in the process without generating problems for hydrocarbon degradation. At times the moisture was lower than that established in the experimental design (60 to 70%) during second sampling with 51.45%, the biopile control (C1); 50.93%, treatment 1 (C2); 42.06%, treatment 2 (C3)

and 51.71%, treatment 3 (C4) and treatment one (C2) of the first sampling 56.18%. Moisture is needed for the transport of nutrients, metabolic processes and maintaining cell structure (Roldan and Iturbe, 2005). Garcia et al . (2012) adjusted the moisture value between 40 and 60% of field capacity, indicating that even with low moisture conditions were achieved registered acceleration of the degradation process. Figure 5 shows the behavior of temperature in the soil recorded every other day during treatment.

Figure 5. Temperatures recorded during treatment.



Temperatures varied over treatment with upper peaks at 30 ° C and less than 20 ° C. Biopiling had similar temperatures during the procedure, indicating that the experiment was properly controlled. Volke and Velasco (2012) mention the ideal temperature between 25 and 35 ° C. Acuna et al . (2012) highlight the importance of temperature for the growth of microorganisms.

Figure 6 shows the pH recorded every 15 days throughout the procedure. The pH according Roldan and Iturbe (2005) and Volke and Velasco (2012) must be in a range of 6 to 9 and 6 to 8, respectively, with optimal pH of 7. The results held in neutral range during treatment, which favored bioremediation (Acuña et al ., 2012).

Roldan and Iturbe (2005) propose lower levels of hydrocarbons to 50,000 ppm. Vasquez et al . (2010) removed 70 to 90% at different times with concentrations between 10,000 and 50,000 ppm. The concentrations used in this study (10,000, 30,000 and 50,000 ppm) and removal levels achieved 63.82% to 92.61% are similar to those reported in the literature. Table 6 shows the bacterial species isolated from soil at the end of treatment was observed.

Figure 6. pH registered bioremediation process.

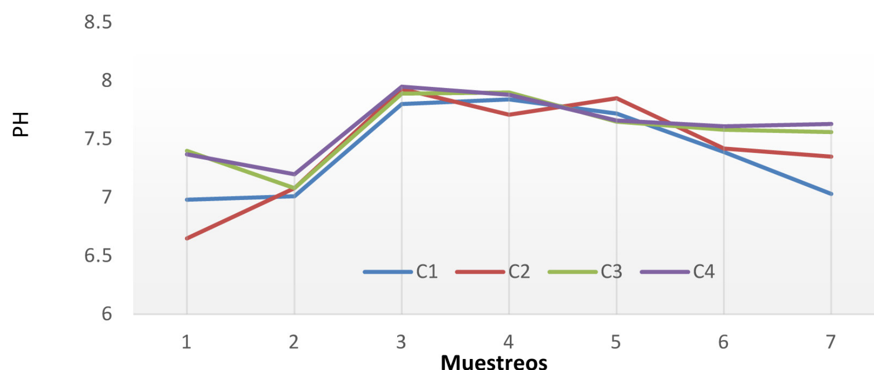


Table 6. Species isolated from contaminated soil at the end of treatment.

Biopila	Muestra	Bacteria o especie	Porcentaje Identifi-cación	Plata-forma	Observaciones
CI	C101	*No identificado*	--------	Todas	No se encontró ninguna especie de bacteria en la base de datos del genbank
C2	C201	*No identificado*	--------	Todas	No se encontró ninguna especie de bacteria en la base de datos del genbank
	C202	*No identificado*	--------	Todas	No se encontró ninguna especie de bacteria en la base de datos del genbank
	C203	*Pseudomonas*	33%	blast-blastx[1]	Productoras de biosurfactantes invo-lucrados en la remoción de aceite
C3	C301	*No identificado*	--------	Todas	No se encontró ninguna especie de bacteria en la base de datos del genbank
	C302	*No identificado*	--------	Todas	No se encontró ninguna especie de bacteria en la base de datos del genbank
	C303	*Streptomyces*	41%	blast-blastx[1]	Se encuentran en suelos y en la vegetación descompuesta y produce numerosos antibióticos de uso clínico
C4	C401	*Mycobacterium*	84%	blast-blastx[1]	Degradan hidrocarburos aromáticos policíclicos en especial pyreno, benzo (a) pyreno es altamente cancerígeno
	C402	*Bacteroides*	37%	blast-blastx[1]	Son candidatos para el tratamiento de terrenos contaminados con HTP
	C403	*Burkholderia*	75%	blast-tblastx[2]	Es excelente degradadora de hidro-carburos aromáticos

1) Search protein using a nucleotide base.

2) Search nucleotide database using a translated nucleotide query.

Roldan and Iturbe (2005) propose lower levels of hydrocarbons to 50,000 ppm. Vasquez et al . (2010) removed 70 to 90% at different times with concentrations between 10,000 and 50,000 ppm. The concentrations used in this study (10,000, 30,000 and 50,000 ppm) and removal levels achieved 63.82% to 92.61% are similar to those reported in the literature. Table 6 shows the bacterial species isolated from soil at the end of treatment was observed.

One can see that the bacteria inoculated initially were not successfully isolated from the samples obtained, regarding this Tyagi et al . (2011) mention that the indigenous microorganisms are more likely to survive and develop when reintroduced into the site compared to the transitional or strains outside the habitat. After treatment, degrading bacterial hydrocarbon strains, which survived the newly polluted environment, were obtained. The released strains may have transferred catabolic plasmids to indigenous microorganisms, the same as the environment in which they are modulates for the recruitment and evolution of degradation genes pathway in bacteria (Cisneros, 2011; Daubaras and Chakrabarty, 1992), which explains their survival and appearance at the end of treatment. The average percentages of pollutant removal at 90 days for the biopile are expressed in Table 7.

Table 7. Percentage of final removal of total hydrocarbons per treatment

Tratamiento	Porcentajes de remoción (%)
C1	46.81
C2	92.61
C3	63.82
C4	84.56

The biopile that achieved the highest amount of removal was the one that had the lowest concentration of hydrocarbon at baseline (10,000 ppm), which influenced conditions for bacteria to succeed in degrading the contaminant. In this biopile the genus Pseudomonas was identified, a bacteria recognized for their bioremediation ability , being capable of degrading aromatic hydrocarbons: benzene, toluene, ethylbenzene, xylene, naphthalene and phenanthrene; it also produces extracellular biosurfactants. Treatment with the mean concentration (30,000 ppm), achieved a lower removal; the presence of identified as not degrading hydrocarbons bacterial species involves competition with the inoculated bacteria and may cause increase in the period prior to degradation of the compounds (Steffensen and Alexander, 1995; cited by and chefs, 2011) .

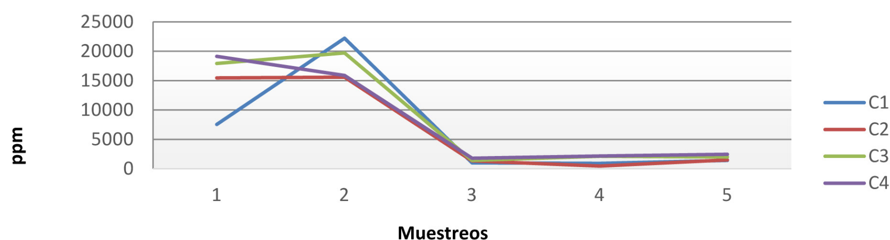
For treatments with higher pollutant concentrations (50,000 ppm), removals were achieved above average concentrations for treatment. In

Table 8, it is noted that apart from the inoculated bioremediation strains, there existed the presence of other bioremediation microorganisms such as of Mycobacterium , which is able to degrade PAHs especially pyrene, benzo (a) pyrene which is highly carcinogenic, Bacteroides genus candidate in treating contaminated with PAH and *Burkholderia* , excellent for degrading aromatic hydrocarbons (Benavides et al . , 2006; Tyagi et al ., 2011), that biopile presented more bacterial species for bioremediation, which explains the high removal percentage.

The control had the lowest biopile removal, which proves the importance of adding bacteria that are appropriate to a contaminated environment and have developed the ability to degrade the pollutants. Daubaras and Chakrabarty (1992) mention that to degrade synthetic and new compound in the environment, a microorganism must evolve genes encoding enzymes that have an affinity for the chemical, which is why it reduces or stops bacterial growth and the absence of microorganisms at the end of treatment.

In Figures 7 and 8 the reduction of hydrocarbons in the analyzed fractions is observed. In the case of biopiles control, the reduction is only observed for the aliphatic fraction (Ifeanyichukwu, 2011), it is common to find similar levels of degradation in both treatments as in biopiles controls. The PAHs, which are the most recalcitrant and dangerous engine oil fraction, are more difficult to degrade (Cerniglia, 1992) which explains a lower percentage of removal.

Figure 7. Concentration of aliphatic hydrocarbons during the bioremediation process.(Samples).



Removal percentages for the aliphatic fraction (Table 8), with stabilization of the pollutant were recorded in the environment on day 60. The aromatic hydrocarbons fraction had a different behavior to the aliphatic fraction (Table 8). A 30.56% increase for C1 was observed, indicating that the degradation process in biopile control was slow. During treatment there were observed in C3 and C4 high peaks around day 30, representing an

increase in the concentration of hydrocarbons in this fraction which was also present in the control biopile on day 60 and the end of treatment. This is due to cleavage of molecules caused by the activity of the degrading bacteria, as Cerniglia (1992) explains, bacteria use enzymes to break down the hydrocarbon chains and thus be metabolized. On the other hand the same microorganism may have different degradation rates from different backgrounds to raw or chemical compositions in the same experimental conditions (Ortiz et al ., 2005).

Figure 8. Concentration of aromatic hydrocarbons oil during treatment.

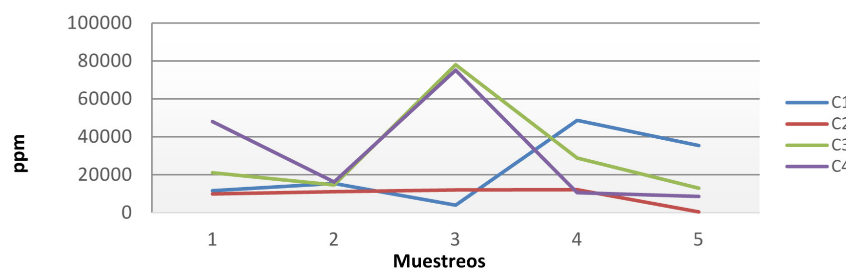


Table 8. Percentages oil removal during treatment.

Trata-miento	Día 1		Día 15		Día 30		Día 60		Día 90	
	Aromáti-cos	Alifáticos	Aromáti-cos	Alifáticos	Aromáti-cos	Alifáticos	Aromáti-cos	Alifáticos	Aromáti-cos	Alifáticos
C1	0.00	0.00	0.00	0.00	-73.93	-95.50	216.96	-95.79	130.56	-93.62
C2	0.00	0.00	10.85	0.74	-99.98	-91.43	-21.33	-97.14	-94.80	-90.43
C3	0.00	0.00	-30.08	9.97	270.64	-92.03	37.27	-88.16	-38.79	-88.85
C4	0.00	0.00	237.49	-17.04	56.26	-90.66	-77.89	-88.63	-82.03	-87.10

CONCLUSIONS

According to the physio-chemical parameters analyzed, the soil proved to be suitable for treatment with biopiles. In C1, for the aliphatic fraction there was a 93.7% removal, 90.4% for C2, for C3 88.8% and C4 87.1% was achieved. Regarding hydrocarbon removal, there were 0 for C1, 94.8 for C2, 38.8 for C3 and 82.0 for C4 in 90 days of treatment with application of bioremediation bacteria and identification of native bacterial strains able to resist change in their environment.

It was found that bioremediation can be used for treating oily residues characteristic of heavy hydrocarbons as well as reduced concentrations of hydrocarbons, aliphatic and aromatic fractions. The degradation of the

aromatic fraction was notorious in the treatments that were inoculated with bacteria.

We recommend using native microorganisms and external bacteria to allow adaptation of native bacteria and achieve lower levels of hydrocarbons. Together native and external bacteria were able to use used motor oil as a source of carbon and energy.

With the results of this investigation, it is based that the presence of external and native bacteria, the addition of water and oxygenation, has positive effects in reducing concentrations of total hydrocarbons in soil contaminated by waste oil. As part of an initial characterization of the contaminant, it is important to determine the concentration of metals, since an amount above 2,500 ppm may be detrimental to the bioremediation process (Roldán and Iturbe, 2005).

REFERENCES

Benavides, J., Quintero, G., Guevara, A. L., Jaimes, D. C., Gutiérrez, S. M., Miranda, J. 2006. Biorremediación de suelos contaminados con hidrocarburos derivados del petróleo. Nova, 4 (5), 82-90.

Cisneros Pérez, Crhistian. 2011. Identificación y caracterización de cepas degradadoras de fenol. Tesis de maestría. Instituto Tecnológico de Tuxtla Gutiérrez, Chiapas.

Daubaras, D. y Chakrabarty, A.M. 1992. The environment, microbes and bioremediation: microbial activities modulated by the environment. *Biodegradation*, 3, 125-135.

Espinoza M.E. y García D.M. 2003. Manual de laboratorio de genética. ECOSUR, San Cristóbal de las Casas, Chiapas. El Colegio de la Frontera Sur.

García, M. G., Infante, C., López, L. 2012. Biodegradación de un crudo mediano en suelos de diferente textura con y sin agente estructurante. *Bioagro* 24 (2), 93-102.

Ifeanyichukwu A. H. 2011. The Potential of ChromolaenaOdorata(L) to Decontaminate Used Engine Oil Impacted Soil Under Greenhouse Conditions.*International Journal of Phytoremediation*, 13, 627-641.

Manacorda, A. M., Cuadros, D. 2005. Técnicas de Remediación Biológicas. Escuela Superior de Salud; Microbiología Ambiental.

Maroto, M. E. y Rogel, J. M. 2013. Aplicación de sistemas de biorremediación de suelos y aguas contaminadas por hidrocarburos. GEOCISA. Div. Protección Ambiental de Suelos, 297-305. Consultada en agosto, http://aguas.igme.es/igme/publica/pdflib15/028.pdf.

Martínez Prado, A., Pérez López, M. E., Pinto Espinoza, J., Gurrola Nevárez, B. A. y Osorio Rodríguez, A.L. 2011. Biorremediación de suelo contaminado con hidrocarburos empleando lodos residuales como fuente alterna de nutrientes. Revista Internacional de Contaminación Ambiental, 27(3) 241-252.

NOM-052-SEMARNAT-2005. Norma Oficial Mexicana NOM-052-SEMARNAT-2005, Que establece las características, el procedimiento de identificación, clasificación y los listados de los residuos peligrosos. México: Diario Oficial de la Federación.

Ortiz, E., Núñez, R., Fonseca, E., Oramas, J., Almazán, V., Cabranes, Y., Miranda, A., Barbán, O., Martínez, C., Díaz, Y. y Borges, G. (2005). Biorremediación de suelos contaminados con hidrocarburos. Revista Contribución a la Educación y la Protección del Medio Ambiente. 6, 51-60.

Pérez Silva, R. M., Camacho Pozo M. I., Gómez Montes de Oca J. M., Ábalos Rodríguez A., Viñas M. y Cantero Moreno D. 2007. Aislamiento y

selección de una cepa bacteriana degradadora de hidrocarburos a partir de suelos contaminados con petróleo. Revista CENIC Ciencias Biológicas. 39(1), 44- 51.

Randy Schroeder, H., Domínguez Rodríguez V. I, García Hernández L. 1999. Potencial de la biorremediación de suelo y agua impactados por petróleo en el trópico mexicano. Red de Revistas Científicas de América Latina y el Caribe. 17 (002), 159-174.

Restrepo, R. 2002. Derrame de hidrocarburos. Impacto en los ecosistemas tropicales. ECOPETROL Instituto Colombiano de Petróleo, Colombia.

Roldán M. A. e Iturbe Arguelles R. 2005. Saneamiento de suelos contaminados con hidrocarburos mediante biopilas. Instituto de Ingeniería, UNAM. Cd. Universitaria.

Ruíz Marín A., Zavala Loria J. C., Canedo López Y. y Cordova Quiroz A. V. 2013. Aislamiento de bacterias tropicales en suelo de mangle contaminado por hidrocarburos: biorremediación por atenuación natural y bioaumentación. Revista Mexicana de Ingeniería Química. 12 (03), 1-8.

SEMARNAT. 2013. Acciones y Medidas de Descontaminación. Consultada en agosto, http://www.semarnat.gob.mx/temas/gestionambiental/Materiales%20y%20Actividades%20Riesgosas/sitioscontaminados/GTZ/B-Acciones%20y%20medidas%20de%20descontaminacion.pdf.T

Torres Delgado, K. Zuluaga Montoya, T. 2009. Biorremediación de suelos contaminados por hidrocarburos. Trabajo de grado. Universidad Nacional de Colombia. Colombia.

Tyagi, Meenu, da Fonseca, Manuela R., de Carvalho, Carla. 2011. Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. Springer. (2011). 22:231–241.

Vásquez M. C., Guerrero Figueroa, J. T., del Pilar Quintero, A. 2010. Biorremediación de lodos contaminados con aceites lubricantes usados. Revista Colombiana Biotecnológica. 12 (1), 141- 157.

Valadez, M. H. y Kahl G. 2000. Huellas del ADN en el genoma de las plantas. Editorial Mundi Prensa. México D.F. 19 pp.

Volke Sepúlveda, T. L. y Velazco, J. A. 2002. El composteo: una alternativa tecnológica para la biorremediación de suelos en México. *Gaceta Ecológica*, 66, 41- 53.