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Chapter 8

Hydrocarbon Biodegradation Potential of Native and Exogenous Microbial Inocula in Mexican Tropical Soils

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Additional information is available at the end of the chapter

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1. Introduction

Soil and water contamination by oil is one of the central environmental problems in Mexico. In the southeastern part of the country, especially in Tabasco and Veracruz states there are several oil facilities involved in extraction, transportation, processing and storage of oil and oil products. Historically in this region there have been spills that affect large areas of soil and eventually rivers, streams and lagoons. Pollutants often persist for long periods in the soil, sediments and water due to different factors like the environmental conditions prevailing in the region (heavy rains and extensive wetlands), the nature of the soils (mostly clayed soils adjacent to water bodies), and the recalcitrance of oil components (Adams et al. 2011).

The application of bioremediation methods based on degradation of hydrocarbons is a widely used alternative for the recovery of such contaminated soils (Boonchan et al. 2000, Sayara et al. 2012). However, the success of this strategy depends on the degrading capacity of the native or exogenous microorganisms applied as part of the treatment (Liu and Suflita, 1993, Van Hamme, 2003, Liu et al. 2011). In this sense, a key issue for the restoration of soils is to assess their potential for bioremediation under different treatment strategies and culture conditions. This analysis must consider first the biodegrading capacity of native microbial populations (natural attenuation). Alternatively, the use of inoculants composed by exogenous microorganisms, previously characterized as degraders (bioaugmentation), can be considered as an active bioremediation treatment.

Several authors have reported that specific microorganisms are required for the removal of various petroleum fractions, in particular those having enzymatic capabilities for biotransfor-

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mation of these compounds (Van der Meer et al. 1992; Prince, 2003). Although the bacteria can be grown in the laboratory, progress on knowledge of microbial ecology of complex communities requires more extensive studies, regarding the activity of microorganisms in environmental samples that suffered minimal modifications (Amann and Kühl, 1998). Moreover, knowledge of the mechanisms of degradation of contaminants in the environment has been generated in laboratory studies using degraders. As a result the role of microorganisms in regulating processes such as mineralization and biodegradation of polluting compounds is not fully understood (Watanabe and Hamamura, 2003).

1.1. Adaptation and presence of microorganisms in contaminated sites

Exposure of native microbial soil populations to hydrocarbon contamination is a key factor that determines the biodegradation rates for overall removal of contaminant. This phenomenon allows the increase in oxidation potential of the microbial community and is called adaptation. According to Leahy and Colwell (1990), this term applies to mixed microbial communities and single microbial species. Van der Meer et al. (1992) proposed some mechanisms on the molecular events that allow adaptation of microbial communities: i) induction and/or de-repression of specific enzymes, ii) new metabolic capabilities as a result of genetic changes, and iii) enrichment of microbial populations capable of transforming the compound of interest.

Generally, degrading-microorganisms are more frequently isolated from hydrocarbon contaminated soils, where they can reach levels between 1 to 10% of the soil microbial population. While in non polluted environments degraders normally represent 1% of the microbial community (Torsvik and Ovreas, 2002). In some cases, predominant genera of degraders are reported, while in others there is a high microbial diversity, which may be due to the composition of the native microbial community, and the environmental conditions at the affected sites. Microbial ability to grow in the presence of oil-derived compounds as a sole carbon source has been addressed by selective microbial enrichment studies (Leahy and Colwell, 1990, Viñas et al. 2002, Roldán-Carrillo et al. 2012).

1.2. Hydrocarbons biodegradation

The biodegradation process is defined as the change in the chemical structure of a compound carried out by microorganisms, and is the basis of conventional engineering techniques for the treatment of sewage and contaminated soil bioremediation (Madsen, 1998). The petroleum compounds can selectively inhibit certain microorganisms, causing changes in the number and diversity of microbial species in soil. Thus the rate of biodegradation of hydrocarbon is determined by the presence of such native microorganisms, their physiological capabilities and the environmental conditions (Prince, 2003, Zhang et al. 2012).

1.2.1. Applying degraders

In some cases, hydrocarbon biodegradation levels of native inocula had been higher than commercial or exogenous inocula (Thouand et al. 1999, Mohammed et al. 2007). It is

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considered that exogenous inocula must possess two essential properties: i) to degrade oil faster than native populations, and ii) to degrade a broader spectrum of oil compounds than native populations (Van Hamme et al. 2003, Madueño et al. 2011). Obtaining microorganisms that meet these conditions may be more feasible from sites where the microbial population is exposed for long periods to hydrocarbons pollutants (Liu and Suflita, 1993, Greenwood et al. 2009).

1.2.2. Biodegradation of hydrocarbons by mixed cultures

It is generally considered that mixed cultures have a higher metabolic versatility than pure cultures (axenic). Mixed cultures have been used as inocula for treating waste oil in various systems, applying preadaptation strategies of consortia to different types of hydrocarbons and field application conditions (Van Hamme et al. 2000). Some reports have studied the biodegrading abilities of defined mixed cultures where the composition of the consortia is previously known. Ko and Lebeault (1999), reported the co-oxidation of decalin and pristane in the presence of hexadecane, by a defined mixed culture of two bacterial strains. Finding that decalin was degraded to a greater extent by an axenic strain than the mixed culture, while pristane was largely degraded by the mixed culture. Richard and Vogel, (1999) compared the biodegradation of diesel by three bacterial strains and a mixed culture composed of the same, degradation of aliphatics was greater with the mixed culture than with the single strains, these authors suggest that degradation of hydrocarbon mixtures by mixed cultures may involve different strains that possess complementary metabolic capabilities.

Often the success of the addition of exogenous microorganisms depends on their adaptation to the environmental conditions prevailing in the soil and their ability to compete with native microorganisms. However some cases have been documented where native populations are better degraders than exogenous inoculants (Thouand et al. 1999). An alternative is the application of microorganisms native of sites contaminated with the compounds of interest, they can pre-selected based on their metabolic capabilities and subsequently be characterized in the laboratory for possible use (Medina-Moreno et al. 2005; Diaz -Ramirez et al. 2008). The application of such microorganisms is useful when the number of native microorganisms is low and they do not have a proper biodegrading capacity (Van Hamme et al. 2003). In general, bacterial cocktails used as inoculants are undefined mixed cultures, where the composition and metabolic capacities of the microbial members is not known precisely; therefore the role of each strain during biodegradation is not clear.

Another useful alternative for the treatment of oil contaminated soils is the use of native plants together with degrading microorganisms associated in their rhizosphere, known as phytoremediation or phytomitigation strategies. The use of plants for remediation of contaminated soil is well accepted, particularly when they are part of a sequence of treatments. The use of pastures allows overall improvement of soil fertility conditions and biological activity (enzymatic functions and respiratory activity), to reduce toxicity, improve, mineralization and / or integration of polluting compounds via humification (Escalante-Espinosa, 2005; Adams et al. 2011).

Diaz-Ramirez et al. (2003) reported a strategy involving bacterial strains obtained from the rhizosphere of a plant native to a contaminated site, that were used to prepare defined mixed cultures for hydrocarbon biodegradation and mineralization in liquid cultures. Ten bacterial strains were isolated and identified as members of the genera: *Bacillus, Gordonia, Kocuria, Pseudomonas, Arthrobacter* and *Micrococcus*. Then assessed for biodegradation of specific hydrocarbon fractions in liquid medium, as both; single strains and defined mixed cultures (CMD) prepared with standardized mixtures. Additionally predominant strains were selected and their population dynamics during biodegradation was analyzed, in order to design specific inocula for different types of hydrocarbons (Diaz-Ramirez et al. 2008). Under this approach consumption levels of 40% of total hydrocarbons were reached in 11 days of culture, degradation value for aromatics was 31%, and 20% for polar hydrocarbons. The results pointed that co-oxidation of aromatic and polar compounds was possible in the presence of aliphatic fraction, when using a defined mixed culture consisting of 3 *Bacillus* strains, 2 *Pseudomonas* strains and one *Gordonia* isolate (Diaz-Ramirez et al. 2008).

1.3. Monitored natural attenuation

Conventional bioremediation techniques generally allow the recovery of soils affected by organic pollutants. Natural attenuation has been considered an important remediation strategy mainly when soil, groundwater or surface water is involved, in places where biogeochemical conditions favor natural processes that degrade or immobilize harmful contaminants. Natural attenuation includes processes such as biodegradation, dispersion, sorption and volatilization of contaminants (Boettcher and Nyer, 2001). Monitored Natural Attenuation (MNA) is a viable alternative for reduction of toxicity, mobility or concentration of the contaminant compounds in soil and/or water (EPA, 1999). Some authors have noted that natural attenuation processes can be selected alone or in conjunction with more active alternatives, when achieving the objectives of remediation, in reasonable time periods, compared with other alternatives that can be costly or with high impact to ecosystems. Among the parameters that should be determined in order to assess the biodegradation potential of the microbial community are: the number and type of predominant microorganisms, degradation kinetics (measured in field or lab), enzyme activities (dehydrogenases, lipases, oxygenases), as well as the half-life of contaminants, calculated from experimental data based on zero- or first order kinetics (Boettcher and Nyer 2001, Margesin et al. 2005, Chang et al. 2010).

In this chapter, we present two experimental approaches developed to assess the ability of native and exogenous microbial populations to biodegrade different types of hydrocarbons, under lab and field conditions.

In the first experimental approach, the degrading activity of native and exogenous microorganisms on diesel and Olmeca crude oil was assessed, under different culture conditions at laboratory scale. All exogenous microorganisms were previously isolated from the rhizosphere of *Cyperus laxus* Lam., a plant native of a highly contaminated tropical swamp (Diaz-Ramirez et al., 2003, Escalante-Espinosa, et al. 2005). The biodegradation ability of these microorganisms was tested in soil artificially contaminated with diesel and biostimulated.

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Further biodegradation experiments in soil contaminated with crude Olmeca oil, were carried out in the presence and absence of co-culture.

The second experimental approach involved the monitoring of natural attenuation (MNA) as a result of a gasoil spill occurred in an area of ecological value; a riparian environment consisting of two streams and two major rivers located in the southeast of Mexico. We identified five points affected by gasoil on areas where the stream presented meanders and points of plant material accumulation. The initial TPH concentrations ranged from 8 500 to 60 000 mg kg^{-1} soil at different points, total hydrocarbon content was determined periodically in order to follow the natural attenuation process.

2. Material and methods

Experiments were carried out with tropical soils, artificially contaminated with either diesel or Olmeca crude oil. In both experimental designs total hydrocarbon content was determined at different sampling times (biodegradation kinetics), the overall biodegradation rate, the total number of microorganisms and maximum number of degraders were calculated. The composition of the residual diesel was assessed by gas chromatography and for Olmeca crude oil by column chromatography. The half-life of hydrocarbons in soil and the biodegradation constant using a first order model were calculated for both biodegradation assays.

In addition, another study was conducted in order to assess the natural attenuation potential of soil and sediments of a stream system affected by a gasoil spill. Periodic sampling was carried out and the analysis of kinetic parameters suggested alternatives that would allow restoration of different affected areas.

2.1. Soil samples

Soil was collected close but outside of an area highly impacted by human activities (truck parking lot), with high deposition of plant material and therefore high microbial activity potential. The soil samples showed dark color with fine texture (silt-clay) and pH 7. Soil humidity at the time of collection was 30% with a water retention capacity of 36% (w / w). Proportions of clay, silt and sand were 40%, 45% and 15%, respectively. Soil organic matter content was 2.88%, corresponding to a soil with medium organic matter content (NOM-021-SEMARNAT-2000); these characteristics are shared by many soils of the study area, considered representative of tropical soils of southeastern Mexico.

2.2. Diesel biodegradation

Biodegradation experiments of diesel (10,000 ppm) were performed in shaken flasks with liquid medium (adjusting the ratio C/ N ~ 20). In such assays, we tested the degrading activity of two defined mixed cultures, the first one (B-DMC) consisted of six bacterial strains; *Bacillus cereus, Gordonia rubripertincta, Kocuria rosea, Bacillus subtilis* strains 7A and 9A, and a non-identified strain UAM10AP. The second defined mixed culture was formed by two fungal

strains (F-DMC); *Aspergillus terreus* and *Aspergillus carneus.* Finally, a co-culture (CC) composed of all of the above strains was also tested. The degrading microorganisms were isolated and characterized as previously described (Diaz-Ramirez et al, 2003). In parallel, non-inoculated controls were prepared to quantify the hydrocarbons abiotic loss.

In order to evaluate the biodegradability of diesel by exogenous and native hydrocarbon-degrading microorganisms, an experimental trial was performed using artificially contaminated soil (~ 120,000 ppm of diesel), which has been biostimulated by addition of a commercial fertilizer (Triple 17), in order to get an initial C/N ratio of 20. Two treatments were established for this assay; a) bioaugmented soil (inoculated with exogenous microorganisms), and b) non-bioaugmented soil (only with the native microbiota).

2.2.1. Gas chromatography analysis

Analytical procedure to determine residual hydrocarbons was based on the gas chromatography standard method EPA8015B. A gas chromatograph (Agilent Technologies Mod 6850 – II) equipped with FID and a column Agilent HP-1. Running conditions were Initial Temp: 70°C, Initial Hold 4.00 min, Ramp 1: 10°C/min to 70°C. Ramp 2: 40°C/min to 310°C, hold for 9.00 min. Injector temp: 250°C. Detector temp: 300°C. Helium was the carrying gas at: 10.00 psi. For calibration, standards were prepared with hydrocarbons known to be present in the used diesel, obtaining a standard curve with 8 quantification points.

2.3. Olmeca crude oil biodegradation

Assays were performed in trays containing 400 g of artificially contaminated soil (10,000 mg TPH kg^{-1} soil), amended (biostimulated) with 2% w/w sugar cane bagasse used as organic conditioner, and the commercial fertilizer Gro-green 20:30:10 (Campbell, Mexico) to obtain a C / N initial ratio of 6. Soil humidity was maintained at 40% of the water retention capacity of soil. Four treatments were established as follow: a) Bioaugmented - sterile soil (B-SS), b) Non bioaugmented - sterile soil (NB-SS), c) Bioaugmented - non-sterile soil (B-NSS), and d) Non bioaugmented - non-sterile soil (NB-NSS).

The following variables were determined: TPH content at different sampling times (EPA 3540 method), the number of hydrocarbon-degrading bacteria was assessed by the most probable number assay (Wrenn and Venosa, 1996), while total heterothophic microorganisms were determined after Lorch et al (1995). With the data regarding residual TPH content, the biodegradation extent was calculated, as well as the global and maximum consumption rates. Additionally, kinetic parameters were calculated as the adjustment of the consumption data to the first order model (as described above in the section 3.2). Biodegradation kinetics of diesel in soil), the biodegradation constants and half-life under different treatments were also obtained.

2.4. Monitoring of natural attenuation

A periodic sampling protocol was designed to monitoring the natural attenuation of a soil highly contaminated with gasoil (medium fraction petroleum). Based on criteria as site

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geomorphology and direction of the stream meanders; several points with presence of hydrocarbons were identified and selected for monitoring. The residual gasoil content (medium fraction C10-C28) was used as a measure of passive biodegradation or Monitored Natural Attenuation.

The residual gasoil content (medium fraction C10-C28) was used as a measure of passive biodegradation or Monitored Natural Attenuation. Soil hydrocarbon analyses were performed by Gas Chromatography accordingly to the Mexican regulation (NOM-138-SEMARNAT/SS-2003), which is based on the EPA 8015b standard procedure. From these values, biodegradation extent and half life of the contaminant in soil were calculated. In addition, the amounts of oil-degrading and heterotrophic microorganisms were determined by

3. Results and discussion

To assess the natural attenuation potential of contaminated soils, as well as to develop inocula for bioremediation of such sites, it is necessary to know the degrading abilities of microbial native populations. Where applicable, the persistence and activity of the introduced microorganisms, without bioavailability limitations attributable to the soil, have to be addressed. Contrast the biodegradation process under this scheme and in field is one of the main challenges for the definition of the auto recovery potential of contaminated soils, as well as for the establishment of bioremediation strategies that are economic and technically feasible.

3.1. Biodegradation of diesel in liquid cultures

Higher levels of biodegradation were obtained with the CC and F -DMC that behave very similar (Table 1). Statistical analysis did not show significant differences at 95% confidence among these two treatments. While the consumption rate data also showed similar values for these treatments. Similar results were obtained with the previously reported CMD (six bacteria) using aliphatics as carbon source (10 000 mg L^{-1}) obtained from Maya crude oil, consumption rates of 282 mg L^{-1} d^{-1} in eleven days of culture were reported (Diaz-Ramirez et al. 2008).

The number of viable microorganisms was higher in cultures in liquid medium with the standardized mixture of bacterial strains and co-culture (0.36 - 8.4 ± 1.1 $X10^8$ UFC ml^{-1}) compared to the initial values (<1x10^7 CFU ml^{-1}).

These results indicated that the application of the standardized mixture of microorganisms (bacteria - fungal strains) was favorable in terms of overall growth. Considering the high biodegradation results, probably there was a synergistic effect between the populations present in the CC and F-DMC, as it has been previously reported with similarly defined mixed culture in liquid medium (Diaz-Ramirez et al. 2003).

Microbial inocula	Biodegradation extent (%)	Consumption rate (mg $L^{-1}d^{-1}$)
B-DMC	36.5 ± 6.8 a	156.63 ± 48.6
F-DMC	49.1 ± 2.8 b	204.88 ± 12.0
CC	48.3 ± 5.2 b	201.33 ± 22.0

Table 1. Diesel biodegradation by the different microbial inocula assayed during 16 days of incubation. Biodegradation extent was calculated considering an initial TPH concentration of 0.5 g/flask and corrected considering the diesel abiotic loss in the control. Values followed by the same letter did not show significant differences.

3.2. Biodegradation kinetics of diesel in soil

In order to determine the biodegradation level of diesel in soil and the activity of the exogenous and native microbial populations, the residual oil content was determined during the biodegradation assays, allowing to perform a kinetic study. Additionally, we quantified the number of heterotrophic microorganisms and soil toxicity at the end of treatment.

Figure 1 shows the degradation kinetics of diesel in artificially contaminated soil (120,000 mg kg^{-1} soil), determined as diesel residual hydrocarbons.

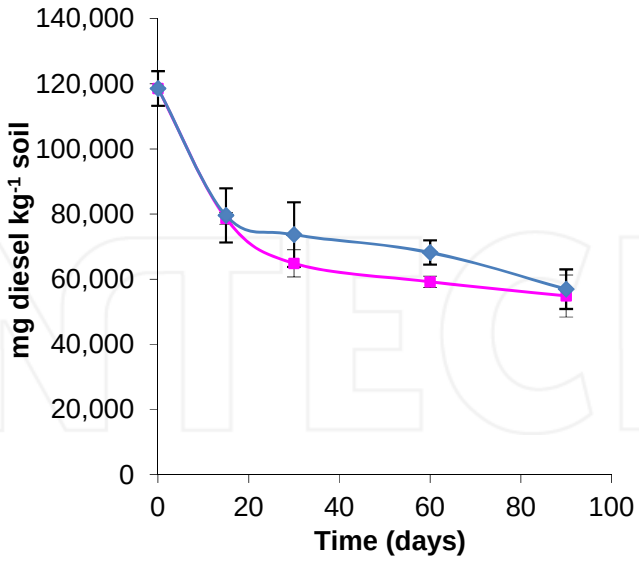

Figure 1. Soil hydrocarbon content throughout 90 day-assay, initial concentration was 120,000 mg Kg^{-1} for the bioaugmented (■) and non-bioaugmented soil (◆).

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The higher degradation rates were observed during the first 30 days of culture, being 37.8% and 45.3% for the bioaugmented and non-bioaugmented soils, respectively. This rapid initial degradation is likely due to the consumption of the easily degradable compounds, for example C10 - C18 aliphatic hydrocarbons, and some low molecular weight aromatic compounds (Vallejo et al. 2005). Subsequently, between 30 and 90 days of culture, diesel biodegradation in bioaugmented and non- bioaugmented treatments was constant until the end of the culture (Figure 1), reaching ultimate biodegradation of 52% and 54% for bioaugmented and non-bioaugmented soil, respectively. No significant differences were found between the means of the two treatments for biodegradation of diesel oil (t test, $P > 0.05$).

Further analysis of these results showed that in the first 30 days of treatment there was a rapid decline in the concentration of hydrocarbons (diesel) in both; the bioaugmented and the non-bioaugmented soils, thereafter the biodegradation rate decreased. High activity of soil microbial populations has been frequently reported for biostimulated soils (Sabaté et al, 2004; Margesin, 2005, Chemlal et al. 2012). Based on colony-morphology observations, the microorganisms inoculated in the treated soil were prevailing at the end of the assay. The predominance of these hydrocarbon-degrading strains in liquid culture and solid media has been previously reported using molecular tools (Escalante-Espinosa, 2005; Diaz-Ramirez et al, 2008), confirming that inoculated strains have good capacity to remain in soils under the assayed conditions.

Based on a first order model degradation constant (-k) and half-life ($t_{1/2}$) were determined (Eweis et al, 1998). Table 2 presents the kinetic parameters calculated for the removal of diesel hydrocarbons from the assayed treatments.

Treatment	Biodegradation (%)	GCR[a] (mg kg^{-1} d^{-1})	MCR [b] (mg kg^{-1} d^{-1})	-k [c] (d^{-1})	Half life time (d)
Bioaugmented	52.0 ± 5.1	684.4 ± 67.6	2 598 ± 555	-0.0093	74.5
Non-bioaugmented	53.7 ± 5.4	707.7 ± 71.6	2 657 ±119	-0.0106	65.4

[a] Global Consumption Rate (GCR) was calculated considering the initial and residual hydrocarbon content after 90 days.

[b] Maximum Consumption Rate (MCR) was calculated as described in the kinetic parameter section below.

[c] Biodegradation constant was calculated as:

$$C = C_0 e^{-kt} \quad (1)$$

Where:

C = Total petroleum hydrocarbon concentration at "t" time

C_0 = Initial concentration of petroleum hydrocarbon

k = Biodegradation constant represented as the slope of the *Ln* C/C_0 *vs time* (days).

Table 2. Kinetic parameters for the Diesel biodegradation assay in soil.

According to the analysis the estimated biodegradation constant was similar for both treatments. These values are comparable to those reported by Cardona and Iturbe (2003) for the biodegradation of diesel in an agricultural soil, under biostimulated conditions, adding alternate sources of oxygen and nitrogen to the soil. The values for the biodegradation constants were 0.044 d^{-1} – 0.011 d^{-1}, with concentrations ranging from 28 000 to 40 000 mg diesel kg^{-1} soil. The half-life time of spilled diesel in this soil was calculated, indicating that from 4.4 to 5 months were required to achieve a complete removal of hydrocarbons.

In similar biodegradation assays, Hye Hong et al (2005), reported 60% degradation of diesel in 13 days using a *Pseudomonas aeruginosa* (IU5 strain) isolated from an oil-contaminated soil. Marquez-Rocha et al (2001), reported rates of biodegradation (GCR) of diesel (118 g kg^{-1} dry soil) of about 2 130 to 2 780 mg kg^{-1} diesel dry soil d^{-1}, with a bacterial consortium previously acclimated to diesel. Diaz-Ramirez (2000) evaluated the biodegradation of hydrocarbons (medium fraction) using a bacterial consortium obtained from the rhizosphere of *Cyperus laxus* Lam., finding a 62% biodegradation within 30 days, the level of biodegradation was similar to the one achieved in this work; 37.8% after 30 days of treatment in the bioaugmented soil.

3.2.1. Analysis of residual diesel in soil after 90 days

Although the level of biodegradation of diesel in the bioaugmented and non-bioaugmented treatments was similar at the end of culture (Table 2), the composition profiles of the residual hydrocarbons were different in relation to the initial composition of diesel, as shown in the comparative chromatographic analysis (Figure 2).

The commercial diesel fuel used in the biodegradation assay is constituted by a mixture of paraffinic and olefinic hydrocarbons ranging from C10 to C28, plus 30% aromatics. The maximum total sulfur content is <0.05% (PEMEX, 2008). Under Mexican standards this type of diesel fraction is regarded as medium-fraction hydrocarbons, whose presence is limited to 1 200 ppm for agricultural soil, and 5 000 ppm in industrial soil (NOM-138-SEMARNAT/SS-2003).

Figure 2a shows the initial composition of diesel fuel used in both treatments; the most prominent peaks corresponded to the alkanes present in larger proportion in the mixture. In bioaugmented soil (Figure 2b), there was a marked reduction in the number of peaks and the area under the curve after 90 days, whereas in the bioaugmented soil (Figure 2c) an increase in the area under the curve was observed. This later change indicated that the residual hydrocarbons were composed of a more complex mixture (unresolved complex mixture), characteristic of a soil with residual compounds of higher molecular weight and chemical complexity. In the bioaugmented treatment the main biotransformation of hydrocarbons was probably due to degradation of short chain compounds (low molecular weight) and medium-sized alkanes (Figure 2b).

This chromatographic analysis is consistent with the results obtained by Hye et al. (2005), which shows a comparison of diesel before and after culturing with a *P. aeruginosa* strain IU5, suggesting that such a decrease is mainly due to the degradation of short to medium chain

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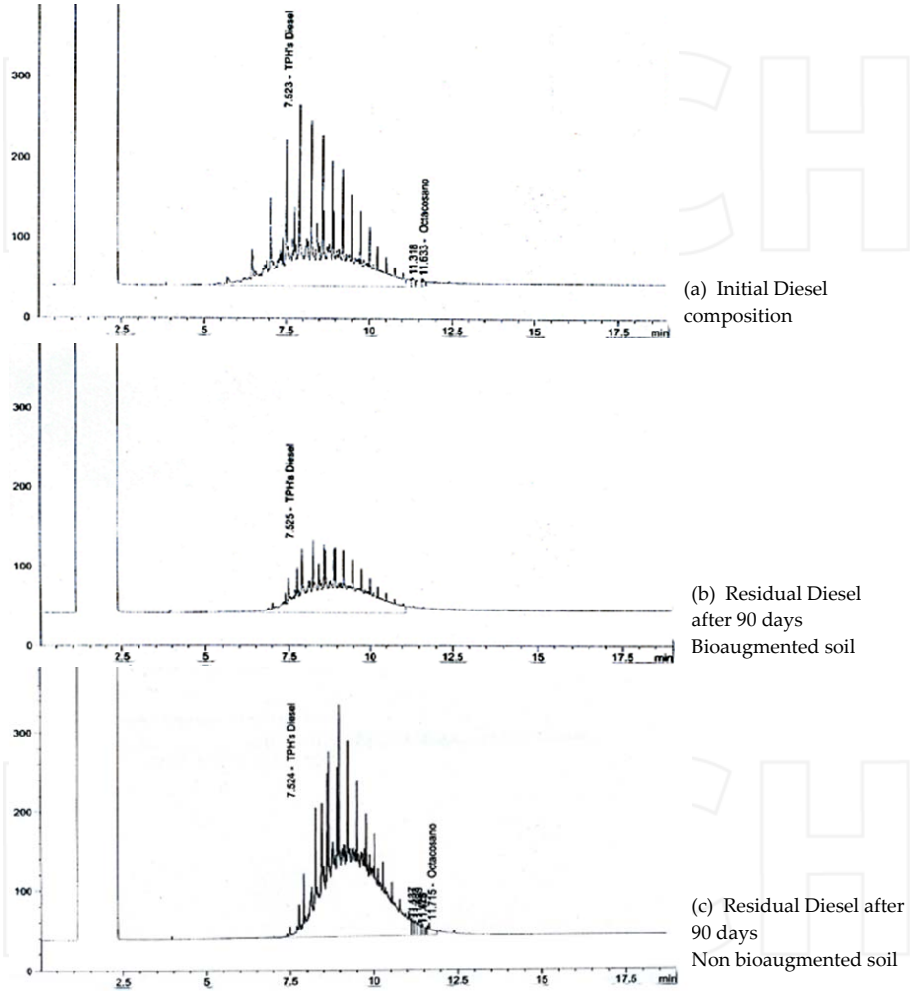
aliphatics (C10 - C24). Moreover, Marquez-Rocha et al. (2001), reported the degradation of medium sized hydrocarbons (> C12) contained in the diesel, together with the short-chain compounds.



(a) Initial Diesel composition

(b) Residual Diesel after 90 days Bioaugmented soil

(c) Residual Diesel after 90 days Non bioaugmented soil

Figure 2. Gas chromatography of diesel used as carbon source after 90 days of culture on bioaugmented and non-bioaugmented artificially contaminated soil. Chromatography analysis conditions were made based on Mexican normativity (NOM-138-SEMARNAT/SS-2003) and the EPA method (EPA 8015b). Initial and final composition for both treatments is shown for comparative analysis.

3.2.2. Assessment of degrading microorganisms

The number of hydrocarbon-degrading bacteria was assessed by the most probable number assay (Wrenn and Venosa, 1996). In bioaugmented soil values were between 11.0 to 110 X 10^5 degrading microorganisms g^{-1} dry soil, and for the non bioaugmented treatment values ranged from 0.21 to 2.1 x 10^5 degraders g^{-1} dry soil. Considering that the used soil has no history of contamination, the number of diesel degraders in both treatments is high. Similar level of degraders (2.1x10^5 g^{-1} dry soil) was reported by Vallejo et al (2005) in their evaluation of biostimulation on PAH biodegradation of petroleum contaminated soils.

3.2.3. Analysis of toxicity

In order to evaluate the acute toxicity of soil due to the presence of diesel and thus the effect of microbial activity (biodegradation) during the assay, the final acute toxicity of soil samples under both treatments was addressed. The measurements were made using the Microtox ® bioassay under the Mexican regulation procedure (NMX-AA 112-SECOFI, 1995). Soil samples were prepared following the Toxicity Characteristic Leaching Procedure (TCLP) as described in NOM-053-ECOL-1993. Toxicity was calculated based on the Relative Index of Toxicity (RIT) for contaminated soils located in Mexican southeastern. RIT is a relation between the half effective concentration (EC_{50}) and the toxicity level for contaminated soils (Adams and Guzman-Osorio 2008). In tropical soils, hydrocarbons identified as medium fraction (C10 - C28) have a low toxicity in low concentrations (<10 000 ppm), mainly due to the low molecular weight compounds, with short half-life (2 - 4 months) under natural conditions. In contrast, at higher concentrations (> 10,000 ppm), toxic effects are seen together with changes in the physicochemical properties and functions of soil, like; a decrease in nutrient retention capacity and fertility, with further increases in compaction, repellency and salinization (Adams et al. 2008). Many contaminated sites in the southeast of the country have very high hydrocarbon concentrations (> 25,000 ppm) and a high degree of weathering.

The index provides a null level of toxicity for leachates of soils with more than 95 000 ppm; slightly toxic for soil leachates ranging from 84 700 to 58 900 ppm, while levels ranging between 58 900 and 36 000 ppm are regarded as a toxic. This toxicity levels can be transformed into Toxicity Units (TU= ($1/EC_{50}$) x10^6). The EC_{50} is the concentration causing a 50% decrease in the bioluminescence level of test organism.

Toxicity results are presented in Table 3, soil samples of both treatments showed a toxicity of about 15 TU. Regarding the EC_{50} calculated by comparing the results with those described in Table 3, both values lie within the range indicated as slightly toxic for soils in this region.

Results of low or null toxicity level as determined by the Microtox bioassay have been previously reported for hydrocarbon contaminated clayed soils from the same region (Adams et al. 1999), that may be due to a strong degree of sorption of diesel into the soil, in addition to some degree of degradation of the residual compounds. In our case, biodegradation and weathering processes occurring in both treatments probably generated compounds that increased the toxicity of soil leachates, as compared to the initial TU=2.0 value, equivalent to an EC_{50} of 179,317 ppm.

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Treatment	Toxicity (TU)*	EC_{50} (ppm)
Bioaugmented	13.7 ± 2.8	75 218 ± 17 679
Non-bioaugmented	17.0 ± 0.2	59 010 ± 762.2

*Toxicity unit = $(1/EC_{50}) \times 10^6$.

Table 3. Toxicity analysis results for the artificially diesel-contaminated soil, under bioaugmented and non-bioaugmented treatments, after 90 days of culture.

3.3. Biodegradation assay of Olmeca crude oil in soil

Among the different types of oil produced in Mexico are the Maya heavy oil with gravity of 22° API (3.3% sulfur by weight); the Istmo light oil with 33.6° API (1.3% of sulfur by weight) and the Olmeca super light oil with API gravity of 39.3 ° (0.8% sulfur by weight). Olmeca and Maya oils are produced in oil fields located in the southeastern region of the country, in the states of Veracruz, Tabasco and Chiapas. There are several sites affected by the accidental discharge of either Maya and Olmeca oils or their refined products. The removal efficiency of the co-culture (CC) of degrading bacteria and fungi was evaluated by addressing residual total petroleum hydrocarbons (TPH) from Olmeca crude oil. The CC was the same used in the diesel biodegradation experiments.

3.3.1. Biodegradation kinetics of Olmeca crude oil in soil

The results corresponding to biodegradation and concentrations of residual and consumed hydrocarbons during the 90 days assay are shown in Figure 3. Among the four treatments tested, both bioaugmented treatments recorded the highest levels of biodegradation (panels a and c), being 58.3 ± 3% and 55.8 ± 2%, respectively. While in the non-bioaugmented treatments (panels b and d) the final biodegradation was about 48 ± 2% and 37.6 ± 2%, respectively.

The period of increased oil consumption corresponded to days 1 to 45 of assay, for the bioaugmented treatments. This consumption was less accelerated for NB-SS, where after 22 days of treatment, oil removal decreased considerably (Figure 3b). Furthermore, in the B-NSS treatment (Figure 3c), there is a marked decrease of the residual hydrocarbon concentration, between days 1 to 60, which is less pronounced in the non bioaugmented treatments (Figure 3b and 3d). These two treatments also showed a marked decrease in the oil removal trend from day 60 to the end of the assay (90 days). In the case of bioaugmented treatments, in this same period (60 to 90 days) was still detected a slight consumption of oil.

Values for residual hydrocarbon content at the end of the assay were statistically analyzed (ANOVA, F-test, $p<0.05$), bioaugmented treatments showed significant differences compared to the non- bioaugmented. However, no differences were found between both bioaugmented treatments that presented similar behavior.

Regarding the number of microorganisms, all treatments remained at levels higher than $1x10^6$ CFU g^{-1} dry soil. Maximum values were obtained at 60 days in the bioaugmented trays (ca $1x10^8$ CFU g^{-1} dry soil). Fertilizer was added to all trays at the start of assay, this fact probably limited the efficiency of biodegradation, as after 60 days of assay there was a decrease in the trend of oil removal (Fig. 3a – 3c).

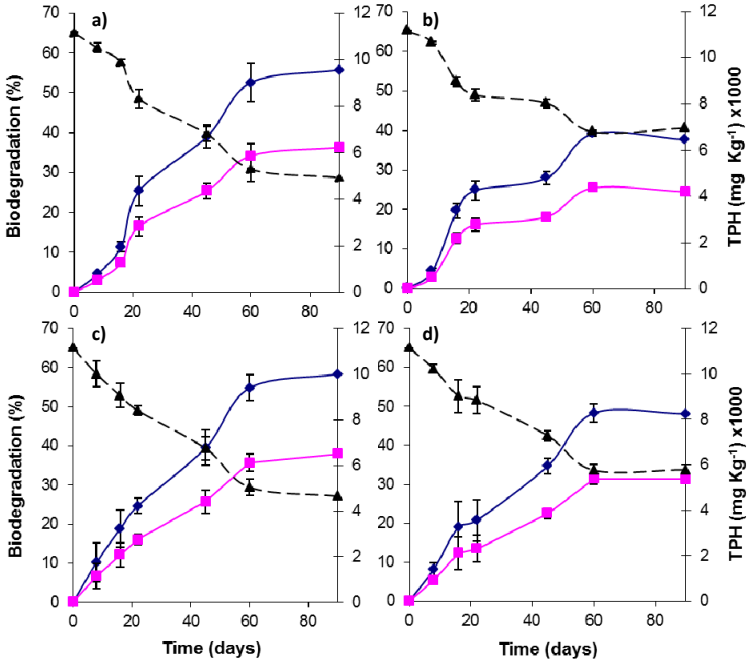

Figure 3. Kinetics of hydrocarbons consumption in soil under different treatments: a) bioaugmented - sterile soil (B-SS), b) non bioaugmented - sterile soil (NB-SS), c) bioaugmented – non sterile soil (B-NSS), d) non bioaugmented – non sterile soil (NB-NSS). Curves represent values of biodegradation (◆), consumed hydrocarbons (■) and residual hydrocarbons (▲). The values are the average of three replicates at each sampling time. The data were corrected considering extractable fat content of the soil.

3.3.2. Kinetic parameters for biodegradation of Olmeca crude oil

With the hydrocarbons consumption data we calculated the following consumption rates: a) global consumption rate; b) maximum consumption rate. Both parameters are volumetric values representing the biodegradation rate allowing a more representative comparison (Diaz-Ramirez et al. 2008). Data are presented in Table 4.

Samples of the B-NSS treatment reached maximum consumption rate after 22 days of experiment (data not shown), while for samples B-SS and NB-SS (sterile soils) the maximum rate was obtained later (between 22 and 45 days).

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Treatment	Biodegradation (%)	GCR [a] (mg kg^{-1} d^{-1})	MCR [b] (mg kg^{-1} d^{-1})
B-SS	55.8 ± 2.0	69.1	259.4
NB-SS	37.6 ± 2.3	46.6	210.6
B-NSS	58.3 ± 3.2	72.3	142.6
NB-NSS	48.1 ± 2.3	59.6	150.5

[a] Global Consumption Rate (GCR) calculated as:

$$GCR = \frac{\left[TPH_{final}\right] - [TPH_{inital}]}{t_0 - t_{final}} \quad (2)$$

Where:

GCR: Global consumption rate

TPH_{final}= Total petroleum hydrocarbon concentration at the end of the assay

TPH_{inital}= Initial total petroleum hydrocarbon concentration

t_0= initial time

t_{final}= final experimental time

[b] Maximum Consumption Rate (MCR) calculated as:

$$MCR = \frac{\left[TPH_r\right]_n - [TPH_r]_{n-1}}{t_{n-1} - t_n} \quad (3)$$

Where:

MCR: Maximum consumption rate determined between each sampling interval

$[TPH_r]_n$= Residual total petroleum hydrocarbon concentration at t_n

$[TPH_r]_{n-1}$= = Residual total petroleum hydrocarbon concentration at t_{n-1}

t_n= Sampling at "t" time

t_{n-1}= Sampling time previous to t_n

Table 4. Olmeca oil biodegradation, global and maximum consumption rates, calculated for the different soil treatments after 90 days of assay.

In order to calculate quantitative kinetic parameters, the obtained quantitative data were adjusted to a first order model equation (see section 2.2.2 Biodegradation kinetics of diesel in soil). Table 5 presents the biodegradation constant and half life time obtained from the first-order linear fit performed for the different treatments.

The better fit to first order model was achieved with the data obtained from bioaugmented treatments (B-SS, B-NSS) and even for NB-NSS (r^2> 0.94), in contrast, residual hydrocarbon concentration in NB-SS samples showed a non-linear behavior (r^2 ~ 0.7).

Similarly to the biodegradation experiments of diesel in soil, it was determined a degradation constant (-k) and the half life time average data of three replicate trays are presented in Table 5.

Treatment	-k (d^{-1})	Half life time (d)
B-SS	-0.0103	67.5 ± 5.0
NB-SS	-0.0067	103.6 ± 5.2
B-NSS	-0.0110	63.0 ± 1.0
NB-NSS	-0.0088	79.1 ± 1.4

Table 5. Biodegradation constant and half-life time for Olmeca crude oil in soil, after a 90-day assay.

The biodegradation constant values were consistent with the results described, being higher (1.2 to 1.5 times) in the bioaugmented treatments (B-SS, B-NSS). The half life time was over 100 days (3.4 months) to the NB-NSS, while this value was slightly above 60 days (2 months) in the bioaugmented treatments. This indicates that it would take about 4 months to achieve complete biodegradation of the carbon source by applying the tested inoculum, regardless the soil is previously sterilized. For TPH (1,010 – 2,141 mg kg^{-1}), first-order biodegradation rate constants were obtained by Chang et al. (2010) in similar range (0.011 - 0.024 d^{-1}).

It is noteworthy that as in the case of diesel biodegradation (Figure 1), with Olmeca crude oil also was observed a decrease in the consumption rate after 60 days of assay (Figure 3). It is likely due to the reduction of the easily assimilable compounds after this period, staying in the soil compounds more resistant to biodegradation. Another possibility is a reduction in the availability of secondary nutrients and minerals, provided by the sugar cane bagasse and commercial fertilizer, respectively, restricting the removal of oil compounds still present.

The results obtained are comparable to those obtained in similar studies that used Diesel as carbon source and added inocula to promote biodegradation. Margesin et al, (2007) reported similar results, finding that with concentrations of 10,000 mg TPH (diesel) Kg^{-1} soil, the biodegradation was 31% to 53% in 38 days, assayed soil was biostimulated using inorganic fertilization with NPK nutrients (C/N ratio of 20:1) or oleophilic fertilization with Inipol EAP22. Mishra et al, (2001), tested the inorganic nutrient addition and the bioaugmentation using a defined mixed culture composed by five oily-sludge-degrading indigenous bacterial strains, these strains belong to genus *Acinetobacter (two strains), Burkholderia and Pseudomonas*, one of them was not identified. It inoculum was used for *in situ* bioremediation of hydrocarbons, finding a 48.5% TPH decrease in presence of the defined mixed culture and 17% in the biostimulated (non-inoculated) treatment after 120 days.

3.4. Monitoring of Natural Attenuation (MNA) of contaminated soil and sediments in a riparian environment

A severe gasoil spill (TPH-medium fraction) affected soils and rivers in a mountainous area southeast Mexico. In this tropical region most soils have silt-clayed texture characteristics, being the main human activities livestock and rain fed agriculture. Another distinctive feature

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of this area is its diverse basins containing most of the country's water resources. The oil spill occurred on a site adjacent to a river system consisting of two streams and a river that flows into the upper Coatzacoalcos River basin. The first of the streams was the most affected due to the increased flow of hydrocarbons. The stream surrounding area has certain degree of conservation and ecological characteristics of a healthy forest area, with silty riverbed and sediments. Based on current Mexican regulations for soils contaminated with hydrocarbons, characterization and site diagnosis was required to propose alternatives for restoration of the affected areas. Particular interest was put in determining gasoil passive biodegradation potential under field conditions, in a series of points located along the stream and close to the spill site. Table 6 shows the results of three points selected for MNA of the gasoil spill occurred in this area.

Sampling point	Sampling time (d)	Gasoil concentration (mg Kg^{-1})[a]	Biodegradation extent (%)[b]	Half life time (d)[c]
P 2	0	59 626	0	
P 2	36	44 092	26.0	
P 2	87	9 670	83.8	
P 2	117	ND	ND	
P 2	383	1 985	96.6	73.0
P 9	0	9 627		
P 9	36	12 682		
P 9	87	3 846	60.5	
P 9	117	3 583	62.8	
P 9	383	3 351	65.2	192.5
P 10	0	34 678	0	
P 10	36	14 817	57.2	
P 10	87	12 033	65.3	
P 10	117	7 486	78.4	
P 10	383	6 715	80.6	51.7

[a] Residual gasoil determined as medium fraction (C10 – C28) EPA 8015b.

[b] Biodegradation calculated considering the initial content of gasoil.

[c] Half life time obtained from first order adjustment of the soil residual concentration data.

Table 6. MNA parameters of gasoil measured at three sampling points of the spill-impacted area.

Sediment samples and superficial soil (0-10 cm) were collected from sampling points 9 and 10 (P9 and P10). Soil samples from 30 - 45 cm depth were taken from sampling point 2 (P2). Residual hydrocarbons data were well described by the first order model having a high lineal

correlation (r^2 = 0.926 to 0.937). Additionally, in P2 hydrocarbons were detected in the streambed sediments (17 100 mg kg^{-1}), with a later 84% reduction (2 662 mg kg^{-1}) in 81 days. For some of the samples we found heterogeneous results, even finding increases from baseline (P9, times 0 to 36 d), this is explained by considering the inherent field variability on the presence of hydrocarbons and the stream dynamics in the site. However, the general behavior of the residual hydrocarbons concentration was towards an accelerated biodegradation, with reductions between 60 to 80% compared to the initial concentrations. According to the half-life calculated from gasoil biodegradation data, the residual concentrations will reach levels below the permissible limit established in current Mexican regulations (<1 200 mg kg^{-1}) after 6 – 9 months.

The calculated global consumption rates (GCR) ranged between 51 mg kg^{-1} soil d^{-1} for a low contaminated site to 232 mg kg^{-1} soil d^{-1}, for the most contaminated (P2). While average maximum consumption rates for P10 and P2 scored 551 and 675 mg kg^{-1} d^{-1}, respectively, these values were achieved between 36 and 87 days of monitoring. Regarding the presence of PAHs, in most of the studied sampling points the levels were below the quantification threshold for CG analysis, except for benzo [a] pyrene that reached 0.884 - 2.76 mg kg^{-1}.

In general, the numbers of diesel degraders and heterotrophic bacteria and fungi were high in the sampled soils. Values for degraders ranged from 10^4 to 10^7, corresponding to 0.1 to 30% of the total microbial population. This range of degraders is even larger than previous reports. Prince, (2003) found that the number of oil-degrading bacteria in a contaminated soil was around 0.1 to 10% of the total microbial population. Our results can be attributed to the favorable environmental conditions detected on the walls of the stream, with abundant vegetation, high soil moisture, rich in organic matter that has been previously deposited by water flow.

Regarding sediment samples, moderate numbers of oil-degrading microorganisms (10^3 to 10^6) were found, however, a high removal of hydrocarbons was scored. This may be explained by desorption of the gasoil present in sediments, due to washing effect of water flow. Subsequently, biodegradation could take place in the liquid phase.

Accelerated biodegradation rates were observed for most of the monitored points resulting in significant reduction of gasoil concentration after two to three months (short half-life), even at the points of highest content. On view of these results, monitored natural attenuation can be considered as an option for site restoration. Although a site characterization strategy is needed, in order to determine environmental conditions that may influence hydrocarbons disappearance, in addition to the sole presence of an oil-degrading community. MNA strategies could be applied to other sites of comparable ecological value or similar tropical regions.

4. Concluding remarks

Diesel biodegradation in liquid media was higher in presence of the co-culture (bacterial and fungal standardized mixture) and the F-DMC. These previously characterized microorgan-

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isms showed high capacity for diesel biodegradation in liquid medium and in soil (120,000 mg kg^{-1} soil). Limitations in biodegradation extent probably were due to depletion of nutrients necessary for the growth or activity of the oil-degrading microorganism. Another possible cause was the low bioavailability of diesel (soil weathering), which affected the activity of both native and exogenous degrading microorganisms. Based on the conspicuous colony morphology of the introduced bacterial strains *B. subtilis* 7A and *G. rubripertincta* during biodegradation in soil assays, they prevailed at the end of the experiments, showing a good degrading capacity and ability to compete with native soil bacteria.

The high diesel and Olmeca crude oil degrading activities registered in both liquid medium and soil assays are promising alternatives for the application of this type of co-culture as a part of active remediation strategies for contaminated soils based, together with biostimulation and bioaugmentation approaches.

Finally, MNA schemes like the one here assayed, could be useful for contaminated sites with a good ecological conservation degree, where an active remediation technique may result in more damage and increased costs. Special attention should be put in the physicochemical effects of hydrocarbons on the soil properties (i e. field capacity, water repellency, salinization, lixiviate residual toxicity). These approaches may favour re-establishment of vegetation and fertility of the soil matrix.

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