
Ingeniería Ambiental

Revista Mexicana de Ingeniería Química

Vol. 12, No. 3 (2013) 553-560

TROPICAL BACTERIA ISOLATED FROM OIL-CONTAMINATED MANGROVE SOIL: BIOREMEDIATION BY NATURAL ATTENUATION AND BIOAUGMENTATION

AISLAMIENTO DE BACTERIAS TROPICALES EN SUELO DE MANGLE CONTAMINADO POR HIDROCARBUROS: BIORREMEDIACIÓN POR ATENUACIÓN NATURAL Y BIOAUMENTACIÓN

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Received March 14, 2013; Accepted June 4, 2013

Abstract

The biodegradation of DRO compounds was evaluated by the processes of natural attenuation and bioaugmentation in mangrove soil. Prior to the experiments, a consortium of bacteria capable of degrading hydrocarbons was isolated and identified: *Pseudomonas aeruginosa*, *P. luteola*, *Sphingomonas paucimobilis* and *P. fluorescens*. For natural attenuation, mangrove oil contaminated soil was placed in horizontal tubular reactors with air supply and no addition of bacteria. While for bioaugmentation three initial inocula from bacterial consortium (0.02, 0.04 and 0.06 g L^{-1} biomass dry weight) were added to the mangrove soil, maintaining aeration and moisture (30%). The samples were collected every 30 days during 3 months and the oil content was analyzed with a gas chromatograph (GC). The degradation of diesel oil range (40.3%) was higher with an inocula size of 0.06 g L^{-1}, where C_{12}, C_{18} and C_{26} were the most susceptible to degradation, while in the process of natural attenuation only 4.51% was removed, suggesting that the low nutrient content in the mangrove soil and bacteria number could limit the hydrocarbon degradation. Therefore it is possible to increase the degradation through bioaugmentation in a system as the mangrove soil.

Keywords: bioremediation, bioaugmentation, natural attenuation, mangrove soil, bacterial consortium.

Resumen

La biodegradación de DRO componentes fue evaluada por procesos de atenuación natural y bioaumentación en suelo de mangle. Previo a los experimentos se realizó el aislamiento e identificación de un consorcio de bacterias capaces de degradar hidrocarburos: *Pseudomonas aeruginosa*, *P. luteola*, *Sphingomonas paucimobilis* y *P. fluorescens*. Para atenuación natural, el suelo de mangle contaminado con hidrocarburo fue colocado en reactores tubulares horizontales con suministro de aire y sin adición de bacterias. Para bioaumentación tres tamaños de inoculo inicial del consorcio de bacterias (0.02, 0.04 y 0.06 g L^{-1} biomasa peso seco) fueron adicionados al suelo de mangle contaminado, manteniendo la aireación y humedad (30%). Se colectaron muestras cada 30 días durante 3 meses y se analizaron por cromatografía de gases. La degradación en rango diesel (40.3%) fue mayor con un tamaño de inoculo de 0.06 g L^{-1}, donde los hidrocarburos C_{12}, C_{18} y C_{26} fueron más susceptibles. En el proceso de atenuación únicamente el 4.51% fue removido, sugiriendo que el bajo contenido de nutrientes y numero de bacterias en suelo de mangle podría causar una limitación en la biodegradación de hidrocarburo. Por lo tanto, es posible incrementar esta degradación a través de la bioaumentación en suelo de mangle.

Palabras clave: biorremeadiación, bioaumentación, atenuación natural, suelo de mangle, consorcio bacterial.

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1 Introduction

One of the biggest challenges at present is the restoration of contaminated sites. With the advances in biotechnology, bioremediation has developed rapidly in the field of restoration sites through the use of microorganisms, and thus reduce the concentration and toxicity of various chemical compounds, such as oil, polycyclic aromatic hydrocarbons (Baird *et al*., 2002), polychlorinated biphenyls, esters, nitro-aromatic compounds, industrial solvents, pesticides and other contaminants as heavy metals (Dua *et al*., 2002).

These compounds enter to the environment through various forms, as components of fertilizers, insecticides and herbicides, some distributed by direct application. Combustion processes release other compounds such as polycyclic aromatic hydrocarbons (PAHs), dibenzo-p-dioxins and dibenzofurans (CDD / Fs). The local concentration of a pollutant depends on its present amount and rate at which it is released, as well as its stability in the environment under aerobic and anaerobic conditions, dilution capacity in the environment, and mobility of the compound in a particular environment and its rate of biological and non biological degradation (Janssen and Anderson, 1992). Diesel fuel is classified as the middle fraction of crude hydrocarbon distillate oil and consists of a number of carbon atoms mainly in the C_9-C_{20} range. A proportion of diesel fuel may be subject to volatilization (C_2-C_{20}), however it is small (1500-6300 μg g^{-1}) and some of these volatile compounds can be toxic to human health.

All soils contain microorganisms that are capable of feeding on petroleum products (Röling and Verseveld, 2002). A large number of microorganisms able to degrade compounds that were previously considered to be non-degradable have been recently isolated (Balagurusamy, 2005). These microorganisms produce biomass by the conversion of complex organic materials into simple organic molecules and finally to carbon dioxide and water. The microorganisms have enzymes that can degrade natural organic compounds, but usually organic compounds derived from human activities can also be degraded, although with greater difficulty. Therefore the enzyme system of native bacteria must be adapted to these persistent organic compounds before their degradation and the capacity of these bacteria in developing in this type of environments increases the processes of biodegradation.

Most oil spills in the ocean converge on coastal ecosystems such as mangroves. Microorganisms are directly involved in this mangroves biogeochemical cycle as the main cause of degradation of many carbon sources, including petroleum hydrocarbons (Santos *et al*., 2010). Microorganisms provide a wide range of services to the ecosystem, such as bioremediation, and are a promising alternative for the recovery of environmental impacts. Previous studies have been conducted on the development and selection of strategies for bioremediation in mangroves, though rather at laboratory level, with little application described in the literature. Hydrocarbon biodegradation in contaminated soils is dependent on three factors: the creation of optimum environmental conditions to stimulate biodegradation activity, the predominant variety of hydrocarbons present in the contaminated matrix and the bioavailability of contaminants for organisms. Oil degradation is also affected by the molecular composition of oil, characteristics that are directly related to bioavailability, diffusion -sorption and the biodegradation rate that can be altered (Marquez-Rocha *et al*., 2001; Medina-Moreno *et al*., 2009).

A major challenge for the restoration of mangrove areas is to define the bacterial population levels and to measure the recovery of these soils. Although mangroves are rich in organic matter, there is usually a deficiency of nutrients, especially nitrogen and phosphorus (Holguin and Bashan, 2001). Despite this limitation, mangroves are highly productive. This can be explained by their efficient nutrient recycling system. When a nutrient deficiency occurs, new material is generated by the decomposition of organic matter, so microorganisms are responsible for most of the carbon flux in the sediment of mangrove, though this occurs mainly near the sediment surface where aerobic conditions enable aerobic biological processes.

The concentrations of available nitrogen and phosphorus often limit the rate of microbial degradation; some studies show that concentrations of these nutrients in seawater limit the rate of oil degradation after spills. Nitrogen demand has been found to be 4 nmoles of nitrogen per gram of oil, therefore, when the degradation of hydrocarbons in the environments is slow, it is probably due to low concentrations of bioavailable nitrogen and phosphorus (Atlas, 1997; Shin *et al*., 2001; Nwachukwu *et al*., 2001; Mishra *et al*., 2001).

Although hydrocarbons in mangrove areas can become degraded by native bacteria, this process can be slow; therefore an alternative is the use

of bioaugmentation with bacteria which has proven effective and feasible in oil removal. The present study aims to determine the population of bacteria capable of growing in the presence of hydrocarbons in mangrove soil and assess the removal of petroleum hydrocarbons using the technique of bioaugmentation. For this study a tubular bioreactor was used to scale laboratory for the treatment of approximately 2 Kg of contaminated soil with hydrocarbons under aerobic conditions.

2 Materials and methods

2.1 Study area

Terminos lagoon is located south of the Gulf of Mexico in the state of Campeche, at 18º 24' and 19º00' N and 91º 15' and 92º 00' W. It is the largest lagoons in the country with a length of 70 km and 28 km wide, with an area of 1566.5 km^2 and an average depth of 3.5 m (De la Lanza Espino and Lozano-Montes, 1999).

The lagoon receives freshwater discharges mainly from four rivers (Usumacinta, Palizada, Chumpan and Candelaria) and communicates to the sea through two entries named: Puerto Real and Ciudad del Carmen. The prevailing winds from the east cause an east-west movement of coastal waters, and a net flow of seawater into the lagoon on the Northeast area, and outside in the Southeast. This movement causes a semi-permanent salinity in the lagoon, with accumulation of fresh and brackish water mainly in the Southeast. The deposition of fine sediments (clay and silt) occurs in this area while in the Northwest area, calcium carbonate rich in marine salts prevails (Yañez-Arancibia and Day, 1988). The margins of the lagoon are covered mainly by three types of mangroves: *Rhizophora mangle* (red mangrove), Avicennia germinans (black mangrove), *Laguncularia racemosa* Gaertn (white mangrove) (Rivera-Monroy *et al*., 1998). Its climate is tropical with a rainy season from June to October, a north-wind season from November to March and a dry season from April to June. Due to its ecological importance, it is of interest to evaluate the capacity to degrade hydrocarbons in mangrove areas so as to create restoration alternatives at potentially contaminated sites.

2.2 Isolation of bacteria

Mangrove soil samples with possible contamination by hydrocarbons were collected near industrial sites at depths of 13 to 30 cm. Each soil sample weighed approximately 3 kg and stored at 4 °C until use.

Subsequently, the soil samples were placed in glass containers (20 cm in diameter x 10 cm high) in triplicate with approximately 500 g of soil. The moisture content of each sample was adjusted with distilled water to 30% and maintained at this level for 7 d to reactivate the microbiological properties of the soil. Then 35.87 mL of Maya crude oil were added, according to the method described by Xu and Obbard (2003) and it was manually homogenized every 24 hours.

After 40 days of incubation at 27 ±1 °C, soil samples of approximately 1 g (wet weight) were collected from each containers and suspended in 25 mL of sterile saline solution (0.85%). Subsequently the suspension was inoculated in Petri plates with (g L^{-1}): 1.71 K_2HPO_4; KH_2PO_4 1.32; 1.26 of NH_4Cl; $MgCl_2$ 0.011. $6H_2O$; 0.02 $CaCl_2$, 1 mL of trace metal solution and solidified with 2% agar (Marquez-Rocha *et al*., 2001). About 150 μL of hydrocarbon oil were added to each plate as the sole carbon source, distributed on the agar surface. Plates were incubated at 37 °C until detecting bacterial growth and the appearance of isolated colonies. Later, colonies were transferred to liquid mineral medium in 250 mL flasks previously sterilized. The liquid mineral medium contains the same concentrations than the medium described above but without agar at 2% (Marquez-Rocha *et al*., 2001).

The isolation and identification of oil-degrading bacteria were performed using the method developed by Merino (1998). The previously identified colonies were cultured in Palleroni broth and Mineral Acetate broth at 30 °C and 150 rpm for 72 hours. The bacteria acclimated in Palleroni broth were grown in King B agar, while the bacteria obtained from the Acetate Mineral broth were grown on Brain Heart agar (BHI). Prior to isolation, the cultures were incubated at 30 °C for 24 and 48 hours, for the identification of bacteria according to their morphology.

The taxonomic identification was performed according to the oxidase test in each isolated strains, using the technique described in API 20NE System (BioMerieux, St. Louis, MO, USA); previously grouping the types of colonies using the Gram stain technique: *bacilli*, *coccobacilli*, Gram-positive and gram negative *cocci*. For quality control we used strains of *E. coli* ATCC ® 25922 and *P. aeruginosa* ATCC® 27853. The identified bacteria were cultivated as a consortium for later experiments.

2.3 Experimental design in natural attenuation and bioaugmentation

For the experiments, horizontal tubular reactors (50 cm of length; 10 cm of diameter and 7 cm of height) were installed with operation capacity of 2 kg of soil, with air supply at the lower part through a compressor for both treatments of natural attenuation and bioaugmentation; every 12 hours the soil was mixed manually. For the treatment of natural attenuation, the mangrove soil was placed immediately after collection in the horizontal bioreactors in triplicate, without previous drying treatment and with addition of 35.87 mL of Maya oil per kg of soil.

While for the bioaugmentation trial, the mangrove soil was dried at 60 °C for 24 hours, with the intention of remove any bacteria from the soil. Once dry, the soil was artificially contaminated with 35.87 mL Maya oil per kilogram of soil according to the procedure described by Xu and Obbard (2003), with subsequent homogenization and left to adsorb for 24 hrs. Humidity was adjusted to approximately 30% with distilled water. The contaminated soil was used in subsequent laboratory experiments.

In the bioaugmentation treatment, different sizes of inocula of the bacterial consortium previously isolated of 0.02 g L^{-1} (A), 0.04 g L^{-1} (B) and 0.06 g L^{-1} (C) dry weight were added in the horizontal reactors in triplicate. Soil samples were collected every 30 days for natural attenuation and bioaugmentation during 3 months. The hydrocarbons concentration in the diesel range (DRO: C_{10}-C_{28}) was determined by extraction from the soil samples, according to the methodology described in standard methods (NOM-138-SEMARNAT/SS-average fraction hydrocarbon determination) and method 9071 - EPA.

The analysis of DRO fraction hydrocarbons was performed by gas chromatography (GC). An Agilent Technology model 7890 chromatograph was used, with a flame ionization detector (FID) and capillary column of 30 m $\times$ 0.32 mm, 0.25μm thick of layer with nitrogen as carrier gas to a flow of 1.5 mL min^{-1}. The injector and detector temperatures were 350 °C and 360 °C respectively, and an oven temperature program of 50 °C for 4 minutes at 300 °C (10 °C min^{-1}), maintained for 15 minutes.

2.4 Soil physicochemical analysis

The content of organic matter (OM), organic carbon (OC) in the mangrove soil samples were determined according to the ignition method described by Heiri *et al*., (2001), based on the weight loss of the burned sample with respect to the original sample. The OC content was determined in relation to the OM content by a 0.58 factor. To determine TN it was used the Kjeldhal method of digestion and distillation. Samples between 0.25 and 1 g were used depending on the content of MO. The soil sample digestion was performed using sulfuric acid and to distill the digested sample we adjusted the pH to 12 with a NaOH solution (10 N), recovering the distillate in boric acid solution with indicators and titrating with sulfuric acid (0.01 N). The quantification of phosphorus (P) was performed by fixing phosphorus complexes in a molybdate-vanadate solution (APHA, 1995). All the analyses were by triplicate for each treatment.

3 Results and discussion

Although mangroves soil are rich in organic matter, some studies suggest that there is usually a deficiency of nutrients, especially nitrogen and phosphorus (Holguin and Bashan, 2001). This is consistent with the percentage values of N and P in the soil of mangrove in the present study of 0.08% and 0.12%, respectively. According to the granulometric properties, the mangrove soil is classified as sandy-clay, with a low level of nutrients and organic matter content of 4.09% and 2.37% of organic carbon (Table 1). Despite this limitation of nutrients, the mangroves are highly productive. This can be explained by their efficient nutrient recycling system. When a nutrient deficiency occurs, new material is generated by the decomposition of organic matter; therefore, the microorganisms are responsible for most of the carbon flux in the sediment of mangrove, which occurs mainly near the sediment surface where aerobic conditions allow aerobic biological processes.

Table 1. Initial physico-chemical characteristics of the mangrove soil.

Parameter	Concentration
Composition (%) (sand : silt : clay)	52 :25:21
	Mean ± DE
Phosphorus (%)	0.12 ± 0.031
Total nitrogen (%)	0.08 ± 0.002
Organic matter (%)	4.09 ± 0.01
Total carbon (%)	2.37 ± 0.01

Table 2. Removal (%) of Diesel Range Organic (DRO) compounds using natural attenuation and bioaugmentation treatments.

Treatment	C_o	C_f	% Removal
Natural Attenuation	0.680±0.017	0.650±0.012	4.51
Bioaugmentation			
Treatment A	0.783±0.021	0.578±0.009	26.2
Treatment B	0.877±0.018	0.638±0.010	27.2
Treatment C	0.851±0.020	0.519±0.004	40.3

*C_o and C_f: initial and final concentration of Diesel Range Organic compounds (DRO: μ g g^{-1} soil)

Studies suggest that the organic load from mangrove areas can modify the flow of nitrogen and phosphorus in estuaries which can be sources of nutrients. The mangrove detritus amount can reach 345 g C m^{-2} yr^{-1} (Twilley, 1988; Dittmar and Lara, 2001). Thus, the amount of nutrients exported from the mangrove can be greater than that exported by rivers in the dry season (31.39 tons) and rainy season (55.14 tons) (De la Lanza-Espino and Rodríguez-Medina, 1990; Rivera-Monroy *et al*., 1988). The low content of nitrogen and phosphorus in the present study suggest features of a non-eutrophic environment. Similar results were reported by Aike *et al*., (2009) for sediments in rivers of Brazil, of 1.46 ± 0.20 and 0.86 ± 0.3 μg g^{-1}, respectively. The reason for a low level of nutrients is related to the low affinity between the chemical species and the soil matrix (Catherine and Raymond 2004); while a high content of organic matter was observed in the present study, similar to that reported in sediments (Aike *et al*., 2009), which suggests a high participation of the system as producer of organic matter.

In the present study, we were able to identify degrading bacteria of petroleum compounds in contaminated soils such as: *Pseudomonas aeruginosa*, *Sphingomonas paucimobilis, Pseudomonas luteola* and *Pseudomonas fluorescens*.

With the identified bacteria, we prepared a consortium, with which was assessed the capacity to remove petroleum compounds with the treatments of bioaugmentation corresponding to the inoculum sizes of A, B and C bacteria. Results suggest that in the mangrove soil it is possible to carry out the degradation of hydrocarbons since the bacteria present in the consortium show the ability to grow and degrade hydrocarbons, providing the opportunity to develop technologies of restoration in-situ (Table 2). Xu and Obbard, (2003) reported that approximately 72.5% of total microorganisms in the beach sediments are capable of degrading oil. This suggests a potential for restoration of sites contaminated with oil in sensitive ecosystems such as mangrove areas.

Results show a lower total hydrocarbon removal using natural attenuation (4.51%) compared to treatments with bioaugmentation (26.2 % - 40.3 %) during 3 months of treatment (Table 2). The degradation of hydrocarbons in the different treatments was increasing as the inoculum size was larger, being the largest removal registered with an inoculum size of 0.06 g L^{-1} (treatment C). The removal percentages for treatments A and B (26.2 % to 27.2 %, respectively) showed no significant differences ($P \geq 0.05$); while, a higher removed was obtained for treatment C of 40.3 % (Table 2). The effective removal using a consortium of bacteria on mangrove soil indicates that bacteria have the capacity to degrade petroleum compounds. Marquez-Rocha *et al*., (2001) reported a greater removal of 85 % to 96% of diesel in a soil using a bacterial consortium of *Pseudomona, Serratia, Acinetobacter* and *Flacobacterium*. The difference in the removal percentage with the present study could be related to the oil initial concentration and the type of hydrocarbon, as well as, soil characteristics and the treatment by biostimulation. This suggests that bioaugmentation in mangrove soil contributes to the removal of oil; however, it is possible to achieve higher efficiency if the treatment is combined with biostimulation by the addition of nitrogen and phosphorus. Aike *et al*., (2009) reported that the 74% biodegradation was reached after 30 days in a biostimulated process, while only 35.5% was removed by natural attenuation, higher than that reported in this study of 4.51% hydrocarbon removed without biostimulation. Bento *et al*., (2005) reported a degradation of hydrocarbons in the range of C_{12}-C_{23}

Table 3. Total concentration of N and P in mangrove soil with natural attenuation and bioaugmentation.

Parameter	Natural Attenuation	Bioaugmentation		
		A	B	C
Phosphorus (%)	0.130 ± 0.028[a]	0.260 ±0.08[a]	0.260±0.07[a]	0.250±0.076[a]
Total Nitrogen (%)	0.084 ± 0.004[b]	0.092±0.004[b]	0.087±0.004[b]	0.110±0.001[b]

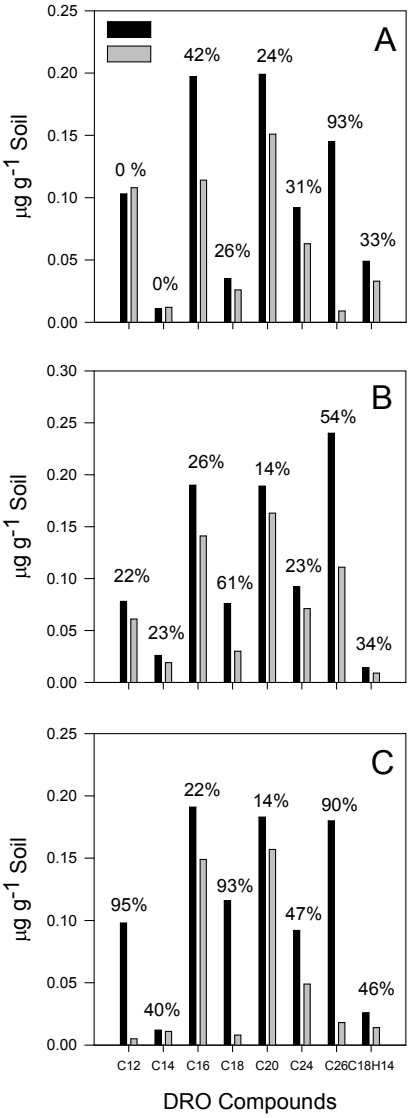

Fig. 1. Diesel Range Organic (DRO) compounds removal (%) in treatments with bioaugmentation in reactors A (0.02 g L^{-1}), B (0.04 g L^{-1}) and C (0.06 g L^{-1}). Black bars: initial concentration; Grey bars: final concentration.

of 48.7 % ± 0.33 in a process of natural attenuation with respect to a 75.2 % ± 0.17 with the bioaugmentation treatment.

Respect to the content of phosphorus and nitrogen, these showed no significant differences ($P \geq 0.845$) to the end of the period of treatment for both natural attenuation and bioaugmentation (Table 3); which probably is related to the decomposition of organic matter and nitrogen-phosphorous release under aerobic conditions, suggesting a system of effective nutrient recycling that could favors the biodegradation of petroleum compounds.

For aliphatic hydrocarbons in the diesel organic range (C_{10}-C_{28}), there was a greater degradation in treatment C compared to treatments A and B. More biodegradation was observed in the compounds C_{12}, C_{18} and C_{26} of about 95%, 96% and 90% respectively (Figure 1). The natural attenuation process is known to be generally used in highly sensitive environments, where the process and use of equipment can be aggressive to the environment. Röling and Verseveld (2002) reported that a natural bioremediation process will be efficient only if the site shows efficient microorganisms to degrade the pollutant, sufficient nutrient and contaminant bioavailability. This shows that under selective pressure of environmental pollution, microorganisms develop catabolic capacity either to degrade or convert them to innocuous products (Balagurusamy, 2005). It is important to consider that though in the present study was not possible to reach a higher oil biodegradation in mangrove soil compared to other studies, the bacteria consortium used offer an opportunity to be applied for proposed of biodegradation on mangrove soil. An alternative to increment the oil degradation is the addition of N and P, due to poor nitrogen and phosphorus content and the bioavailability of the contaminant observed in the present study.

Conclusions

The increase of bacterial population (bioaugmentation) and under aerobic conditions it is possible to increase the petroleum compounds degradation with respect to a natural attenuation process. Further study of bioremediation processes in mangrove areas in tropical regions is required, since many of the investigations have been conducted in temperate climates. The analysis of biodegradation shows that the bioaugmentation in the mangrove soil favors the removal of oil unlike the treatment with natural attenuation. The present study proposes the introduction of bacteria, which belong to the same habitat and have the ability to use oil as a carbon source, suggesting that environmental impacts would be minimal compared to chemical and physical treatments. Therefore, it is necessary to conduct further studies of mangrove soils, where bioaugmentation combined with biostimulation could contribute to achieving higher levels of degradation.

Acknowledgments

The authors wish to express their gratitude to the Consejo Nacional de Ciencia y Tecnología (CONACYT) Mexico, for the financial support provided to conduct this study.

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