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Treatment of Diesel-Contaminated Soil by Bioaugmented Composting with Bacteria from the Larva *Tenebrio Molitor*

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ABSTRACT: In this study, soil composting and bioaugmentation processes were combined to remediate diesel-contaminated soil. Deciduous corn flour, wheat bran, and sawdust were used as co-substrates for soil composting. In addition, consortia of bacteria from the inner tract of the *Tenebrio molitor* larva were isolated and selected for the bioaugmentation of the composting reactors. It was observed that the isolated inoculum enhanced the treatment and combined with deciduous corn flour, a higher efficiency (87%) and a better removal rate (9.10% diesel removed/week) were registered. In soils with a concentration of 11,796 mg diesel/kg, this combined treatment reduced, in 10 weeks, the pollutant to values below the maximum permissible limits in soils stated by Mexican regulations.

KEY WORDS: diesel-contaminated soil, bioaugmented composting, hydrocarbon bacteria, *Tenebrio molitor*

INTRODUCTION

Soil contamination by hydrocarbons from fossil fuel spills, a global problem, is the source of several adverse effects on the environment [1]. This kind of pollution originates serious risks to human health due to its toxicity, its persistence owed to its low biodegradability and the possibility of entering food chains [2]. Such is the case of spilled diesel, a fuel characterized by its high content of medium-fraction hydrocarbons (MFH), a mixture of compounds containing between 10 and 28 carbon atoms [4].

Bioremediation processes, including soil composting, can accelerate the degradation of hydrocarbons [1]. Composting entails the addition of biodegradable co-substrates, macronutrients, aeration, and moisture to the soil, which stimulates the proliferation of mixed consortia. Noteworthy, the hydrocarbonclastic bacteria present in polluted soils are of interest, because they can simultaneously metabolize hydrocarbons and cosubstrates [3]. In addition, cosubstrates improve nutrient availability for microbial activity and increase porosity and water retention capacity of soil [5].

The bioaugmentation or addition of microorganisms previously adapted to biodegrade hydrocarbons is a process that has also been used for the remediation of soils contaminated with total petroleum hydrocarbons (TPH). It is common to apply cultures from bacterial consortia that were isolated and cultured from soils contaminated with hydrocarbons [6, 7]. For example, Chen *et al.* [8] applied a consortium of hydrocarbonclastic bacteria and reported a 75% reduction in diesel pollution (20,300 mg/kg) over a 27-week period. Also Koshlaf *et al.* [2] used a consortium of hydrocarbonclast strains to bioaugment a soil with 18,966 mg TPH/kg and after 12 weeks of the concentration of the pollutant was reduced in 80%.

The larva of *Tenebrio molitor* is the larval stage of black beetles, from the family *Tenebrionidae*, of the order *Coleoptera*. It is also known as mealworm and is native to Europe, but is currently a cosmopolitan species, as it was introduced to all continents because it is easy to raise and feed and has a high protein value that makes it attractive to the animal feed industry. [9]. The life cycle of *Tenebrio molitor* ranges from approximately 280 to 630 days. Its mature larval stage is reached from 3 to 18 months. The mature larva is light brown, measuring between 20 and 32 mm; and weighs between 130 and 160 mg. These larvae can degrade polymers such as polystyrene, polyethylene, and even polyvinyl chloride, which are petroleum derivatives [10, 11]. In addition, they can increase their degradation capacity when wheat bran and maize flour are added as cosubstrates, since these are natural foods for the larvae [9, 12]

They have also been adapted to hydrocarbon environments through selective breeding [13].

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This ability to degrade is due to the fact that the digestive tract of the larva contains consortia of bacteria that can use polymers as carbon source. Specifically, the genera of bacterial species that predominate in the larval tract are *Exiguobacterium sp*., *Klebsiella sp*., *Alcaligenes sp*., *Citrobacter sp*., *Bacillus sp*., and *Kosakonia sp*. [12, 14, 15, 16]. This ability suggested us the idea of using bioaugmentation with hydrocarbonclastic bacteria isolated from the digestive tract of *T. molitor* larvae in the composting process.
In this work we studied the composting process of two soil samples originally contaminated with different concentrations of diesel, applying diverse cosubstrates: deciduous corn flour, wheat bran, and sawdust, and we added bioaugmentation with hydrocarbonclastic bacteria isolated from the digestive tract of the *Tenebrio molitor* larva.

METHODOLOGY

Two contaminated soil samples, named A and B, were obtained from a gas station in the vicinity of the Azcapotzalco district, in Mexico City. An initial characterization was made using the methods listed in Table 1.

Table 1. Parameters determined in the initial characterization of soil samples.

Parameter	Method
Field capacity	Determination of humidity at field capacity [17]
Apparent density	Test tube method
Real density	Metod AS-04, pycnometer method [18]
Porosity	Indirect calculation
Soil moisture	Method AS-05, determination by gravimetry [18]
Soil textura	Method AS-09 or Bouyoucos' procedure [18]
Usable phosphorus	Method AS-11, procedure of Olsen and collaborators [18]
Organic material	Method AS-07, Walkley and Black procedure [18]
Total Kjeldahl nitrogen (NTK)	Method AS-25, procedure by digestate[18]
pH	Method AS-06 [18]
Bacterial count	Plate dilution [19]
HFM hydrocarbon concentration	Method 8015 C EPA [20].

Cosubstrates

The cosubstrates used were deciduous corn flour, wheat bran and sawdust.

Inoculum of hydrocarbonclastic bacteria

Larvae of *Tenebrio molitor* were used to obtain an inoculum of hydrocarbon bacteria. The larvae, in sets of 10 individuals, were subjected for 36 days to four different concentrations (1,300, 6,000, 13,000 and 23,000 mg/kg) of MFH, using a sample of commercial diesel, and 10 g of deciduous corn flour as a matrix. Dead larvae or those that evolved into pupae were constantly removed and the survivors were reserved until they were transformed into beetles. The second generation of larvae was obtained from these beetles and three larvae were randomly chosen and dissected. The consortium from the digestive tract of the larva were seeded in a selective culture medium (Noble Agar, DIFCO brand) to isolate colonies of hydrocarbonclast bacteria [11].

Selection and adaptation of hydrocarbonclast bacteria

The inoculum used in the bioaugmentation of composting was obtained with pre-enriched bacteria broth (MERCK brand), seeded with the hydrocarbon bacteria previously isolated. With 600 µL of this broth, tubes containing the selective medium of liquid combined carbon (9.8 mL) and diesel as a carbon source (90 µL) were inoculated [23]. Using McFarland's standard turbidity method [21]. the growth of hydrocarbon bacteria was monitored for 280 hours. With 990 mL of selective carbon medium and 10 mL of the previous culture, one liter of inoculum was prepared and incubated at 28 C for 24 hours, before bioaugmenting the composting cells. The isolated strain of hydrocarbonclastic bacteria was subjected to biochemical tests for identification [22]

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Bioaugmented composting process.

Eight composting experiments were carried out, in triplicate. Control reactors were also set up for each type of soil, A and B, where no bacteria or cosubstrates were added, but they were aerated and moistened in the same way as the others.

In each reactor, 453 g of soil were used, 34 g of cosubstrate and 200 mL of hydrocarbonclastic bacteria inoculum (19.1 x 106 CFU/L) were added. The bioaugmented composting process lasted 18 weeks, during which aeration by mechanical mixing once a week and humidity (at field capacity) were maintained.

RESULTS AND DISCUSSION

Soil characterization

The results of the characterization of the soil samples are presented in Table 2. The porosity results of both soils were around 30%, so they are classified as light soils [24] The sand content was close to 50% and the silt content was 35%, a composition that produces greater flow of air and water between its structure and the contaminant would be more available, which could benefit composting.

Both soils had a high phosphorus content, around 58 mg/kg. In the case of organic matter, the values found were 7.62 and 8.68 %, and the total nitrogen values in both soils were 0.2 %, which gives a classification of soil with high nutrient content. Finally, with the pH results (7.75 and /.69 units, respectively), both soils were classified as moderately alkaline.

Soil A presented 45,866 mg/kg of diesel and soil B, 11,796 mg/kg. Both exceeded the maximum permissible limit, MPL, (5,000 mg/kg dry basis) dictated by the Official Mexican Standard NOM-138-SEMARNAT/SSA1-2012, for pollution due to hydrocarbons in soils and guidelines for sampling, characterization and specifications for remediation [4].

Table 2. Characterization of soil samples

Parameter	Unit	B soil	SD	A soil	SD[1]
Field capacity	%	51.15	2.318	42.57	0.612
Apparent density	g/cm^3	1.70	0.351	1.57	0.228
Real density	g/cm^3	2.37	0.003	2.38	0.011
Porosity	%	28.27	0.100	34.03	0.304
Soil moisture	%	14.21	0.2509	12.76	0.0332
Soil texture		Loamy	-	Loamy[2]	-
Usable phosphorus	mg/kg	59.97	3.5408	57.42	2.5951
Organic material	%	8.68	0.2453	7.62	0.2453
Organic carbon	%	5.04	0.3890	4.42	0.2003
Total Kjeldahl nitrogen (TKN)	%	0.256	0.0195	0.207	0.0005
pH	-	7.75	0.012	7.69	0.008
Bacterial count	CFU/g DS[3]	2.74x10^8	7.5x10^7	4.36x10^7	7.1 10^6
Hydrocarbonoclastic bacteria count	CFU/g DS	1.07x10^6	6.7x10^4	2.38x10^6	3.2x10^5
HFM hydrocarbon concentration	mg/kg DS	11796.71	2215	45866.76	4257

[1]SD: Standard deviation
[2]Both soils are loamy (%50% sand, 15% clay and 35% silt)
[3]DS: Dry Soil

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Total bacterial counts indicated that there is a difference of 2.3 x 10^8 CFU/g between the soils, the highest concentration was in soil B and could be due to the fact that the environment of this soil is less toxic. In the same way, the count of hydrocarbonclastic bacteria resulted in a difference of 1.31 x 106 CFU/g between both soils; the increased number of bacteria in soil A could be due to excess diesel.

Inoculum of hydrocarbonclastic bacteria

Most of the larvae used to develop the inoculum of hydrocarbonclast bacteria were not affected by the elevated concentrations of diesel (Figure 1). Of the 40 larvae used in the test, only two perished. The survivors were transformed into beetles.

Figure 1. Live larvae on day 15 of the test

The graphs in Figure 2 show the number of larvae and pupae progressively during the test. The lines representing each quantity are symmetrical, i.e. the number of larvae that decreases is equal to the number of pupae that increases. This confirms that the diesel did not affect the specimens.

Selective reproduction of larvae

The results of the count of hydrocarbonclastic bacteria in the larval tract are reported in Table 3, it can be observed that there is a higher concentration in the second generation compared to the first.

Table 3. Colonies of hydrocarbonclastic bacteria in the digestive tract of the larva *Tenebrio molitor*

Larvae	CFU/mL	SD
First generation	3.58 x 10^3	5.54 x 10^2
Second generation	6.19 x 10^3	7.8 x 10^2

Selection and adaptation of hydrocarbonclast bacteria

In the first adaptation process, hydrocarbon bacteria reached a population of 2.29 x 10^9 CFU/mL in 24 hours, using a medium with 10,893 mg/L of diesel. In the second process, in a medium with 1,210 mg/L of diesel, the bacteria reached a population of 2.83 x 108 CFU/mL in the same period. This suggests that the amount of diesel promotes their growth, *i.e.*, if there is greater availability of diesel then the population of hydrocarbon bacteria will be more abundant.

For the second adaptation process, a growth curve of the bacteria was generated (Figure 3) and double exponential smoothing was applied. It has an 8% average relative error and 9.94 x 10^7 mean square error (RMSE). An accelerated growth was observed between 100 and 150 hours, represented by the steepest slope; after 150 hours the slope was almost constant corresponding to a constant growth.

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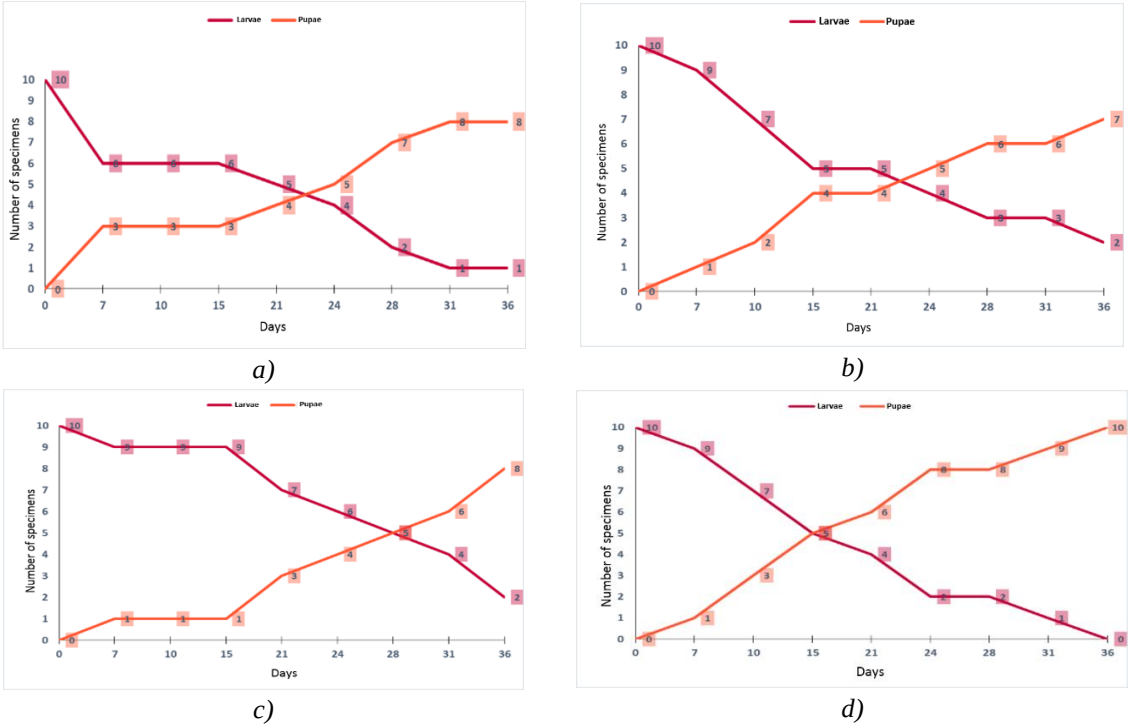

Figure 2. Larvae-pupae in the diesel-contaminated matrix
(a) 20000 mg/kg b) 13000 mg/kg (c) 6000 mg/kg (d) 1300 mg/kg

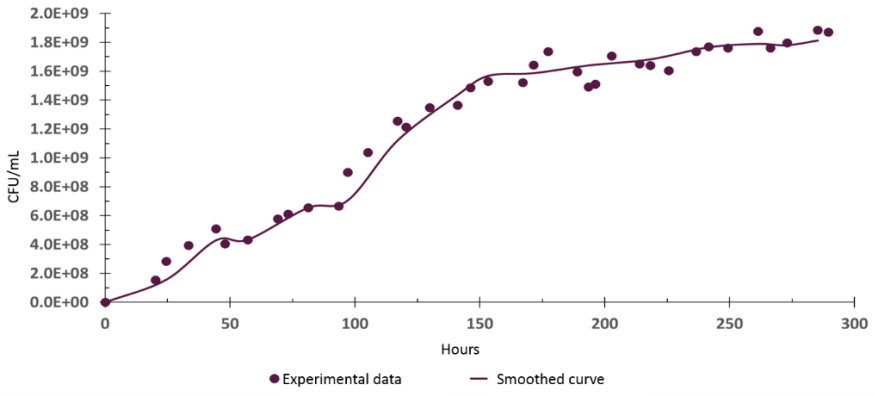

Figure 3. Growth of hydrocarbonclastic bacteria in the adaptation process

Bacterial growth

The colonies of the strain isolated and purified from the tract of the larva *Tenebrio molitor*, are shown in Figure 4a. The colonies that grew on the plate were white, circular in shape, and milky in appearance. They resulted in a Gram-positive bacillus that was distributed linearly, as can be observed in Figure 4b. The species was biochemically identified as *Bacillus coagulans*, within a 92% range of similarity. This genus has been found in the digestive tract of *T. molitor* by other researchers [15, 16].

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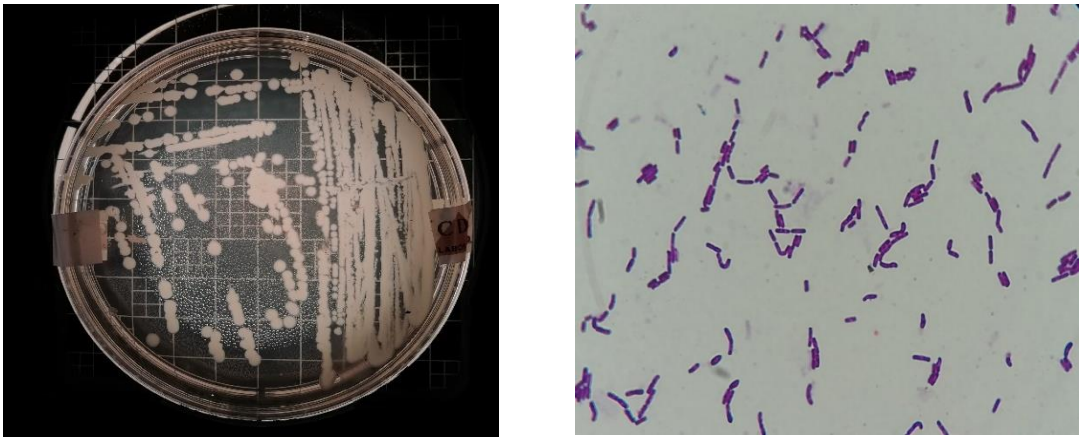

a) *b)*

Figure 4. Pure strain of hydrocarbonclastic bacteria

a) Colonies in striation seeding b) Gram-positive bacilli (violet color), (100 X)

Bioaugmented composting process.

Diesel removal efficiencies

The results corresponding to MFH degradation are presented in Table 3. Regarding soil A, the experiment with deciduous corn flour as a cosubstrate was the most efficient, the concentration of diesel was reduced to 5,833 mg/kg in a period of 18 weeks, that is, the contaminant was reduced 7.8 times its initial concentration. However, it did not reach the maximum permissible limit (5,000 mg/kg) established by the official Mexican standard NOM-138-SEMARNAT/SSA1-2012 [4].

On the topic of soil B composting, with expired maize flour, the concentration of HFM was reduced up to 3,954 mg/kg in 10 weeks, which is below the MPL.

The removal efficiencies of bioaugmented composts in soil B are represented by the cumulative graph in Figure 5. Treatment with deciduous corn flour was the most efficient in this group (66%), followed by compost with wheat bran (49%), then sawdust (32%) and finally the bioaugmented experiment without cosubstrate (24%). The efficiency of the control trial was 11%.

The removal efficiencies in soil A are presented in Figure 6. The most efficient process was the deciduous corn flour cosubstrate (87%), followed by wheat bran (75%), then sawdust (49%), the bioaugmented experiment without cosubstrate (21%) and finally the control trial (14%). In summary, the cosubstrates that worked best in conjunction with the bioaugmentation of hydrocarbonclastic bacteria were deciduous corn flour and wheat bran.

The inoculum prepared with the bacteria of the *Tenebrio molitor larva* tract gave added value to the composting, since differences were observed between the curves of the control experiments and the bioaugmented cells without cosubstrates of both soils.

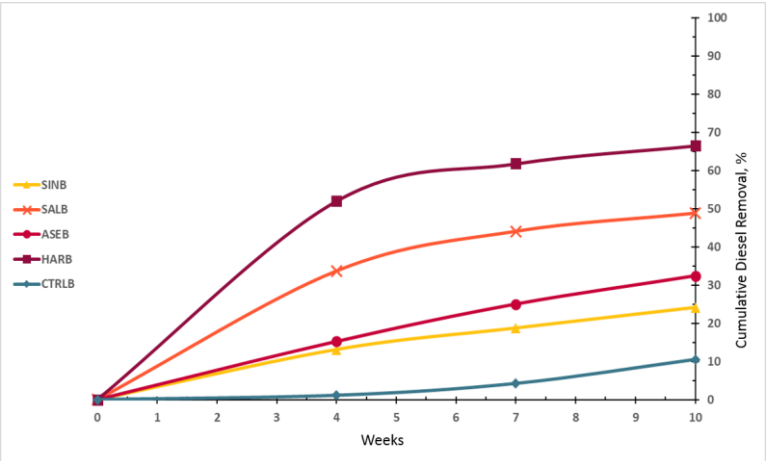

Figure 5. Cumulative Diesel Removal in B Soil Treatments.

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SINB: bioaugmented cell without cosubstrate: SALB: cell with wheat bran; ASEB: cell with sawdust; HARB: cell with deciduous cornmeal; CTRLB: Control Cell

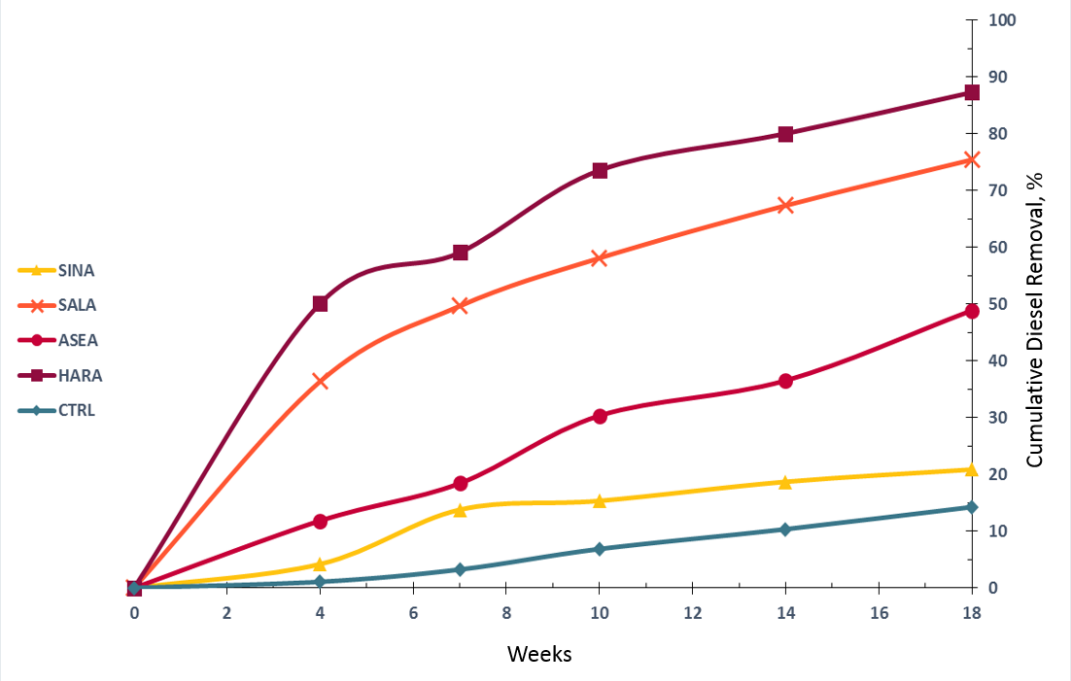

Figure 6. Cumulative Diesel Removal in Soil Treatments A.

SINA: bioaugmented cell without cosubstrate: ROOM: cell with wheat bran; ASEA: cell with sawdust; HARA: cell with expired corn flour; CTRLA: Control Cell

CONCLUSIONS

The diesel had no toxicological effect on the larvae of *Tenebrio molitor* and they were able to complete their life cycle exposed to the pollutant. From second-generation larval tracts, an inoculum of hydrocarbonclastic bacteria was isolated and selected that could have used diesel as a carbon source. A strain of *Bacillus coagulans was isolated from the inoculum.*

The bioaugmented composting process with bacteria from the *Tenebrio molitor* larval tract and using cosubstrates, such as deciduous corn flour, wheat bran or sawdust, removed diesel from contaminated soils regardless of the concentration of the contaminant.

The treatment with expired corn flour with bacterial inoculum was the most efficient compared to the other treatments carried out and 87% of diesel was removed from the soil contaminated with 45,866 mg/kg, in 18 weeks, up to a value below the limits established by the Official Mexican Standard NOM-138-SEMARNAT/SSA1-2012.

Diesel removal efficiencies by bioaugmented composting without cosubstrates were close to 20%, in 18 weeks in the soil with the highest concentration and in 10 weeks in the soil with the lowest concentration. Although the removal was greater than those obtained in the control trials of 15 and 10%, in soils with higher and lower concentrations, respectively, such removal did not reach the MPL established by the Official Mexican Standard NOM-138-SEMARNAT/SSA1-2012.

By adding cosubstrates to the composting process, the removal of diesel was enhanced. The highest average removal rate (9.10% diesel removed/week) occurred with deciduous corn flour and in the soil with the lowest concentration the contaminant was reduced in 10 weeks to 3,954 mg/kg, a value below the MPL of the respective Official Mexican Standard.

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