GROWTH OF THE CALIFORNIAN RED WORM (*Eisenia foetida*) IN VERMICOMPOSTING PROCESS OF DIESEL CONTAMINATED SOIL

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SUMMARY

*The goal of this work was to determine the growth and adaptability of the Californian red worm (*Eisenia foetida*) in a medium contaminated with diesel and to know the efficiency of degradation of the hydrocarbon in the vermicomposting process. Six different diesel concentrations were tested: 1, 5, 10, 15, 20 and 25%. The physicochemical properties of the soil were analyzed in three stages: soil mixed with organic matter (previous to diesel addition), in the soil/organic matter/diesel mixture once the vermicomposting process had started, and at the end of the vermicomposting process. Evolution in the population of the red worm and the removal of contaminant during the process was measured. The highest diesel removal achieved was 97%, reached in the contaminated soil with a 20% pollutant concentration. The results show that the vermicomposting process with Californian red worm represents an efficient alternative for bioremediation of soils contaminated with hydrocarbons.*

Introduction

In recent years, the oil industry in Mexico has ranked 11th internationally in oil production (EIA, 2013); the products and by-products derived from this activity, such as gasoline and diesel, are among the main pollutants of the environment. When inappropriately discharged, hydrocarbons cause deterioration in water, soil, and vegetation, and lead to a decreased biodiversity of microorganisms and micro fauna. In the soil, they produce changes in its structure and physical and chemical characteristics, causing soil infertility (Madigan *et al*., 2003). According to PEMEX (2018), in 2018 there were more than 223 accidents due to spills caused by illegal drilling and connections in pipelines, adding to the accidents that the company has, mainly during the operation of drilling, extraction and transportation of hydrocarbons, as well as for the corrosion of the infrastructure. PROFEPA (2018) refers that in the last 10 years, about 427ha of soil have been contaminated by hydrocarbon spills throughout the country, of which only 107.97ha were remediated, in roughly 693 sites (SEMARNAT, 2006). During 2017, a total of 100.16ha were contaminated.

In Mexico, as in other countries, oil spills cost billions of pesos invested in the use of physicochemical, thermal, and biological remediation techniques, with somewhat favorable results. Currently, cost effective hydrocarbon degradation is sought through bioremediation techniques. The most common of such techniques is biostimulation, consisting of putting nutrients into the contaminated environment and thus stimulating the growth of bacteria and other microorganisms (Varjani *et al*., 2016). A biological treatment method using bacteria, fungi and algae has recently gained a lot of attention due to its efficiency and lower cost (Sayed *et al*., 2021). The application of micro-organisms for the bioremediation of petroleum hydrocarbon pollutants in this day and age is a priority in the effort to establish green technologies (Cai, 2021).

Recently, studies have been carried out with earthworms for soil remediation, especially *Eisenia* species. Earthworms are degrading species that together with other organisms, such as bacteria and fungi, form a symbiosis in the degradation of organic and inorganic materials during the vermicomposting process. According to Dabke (2013) earthworms stimulate and accelerate microbial activity creating favorable conditions for bacteria and improving soil aeration, and Hickman and Reid (2008) indicate that earthworms can be used directly within bioremediation strategies to promote biodegradation of organic pollutants. *Eisenia foetida* is easily cultivated and is a strong macro-organism with a high tolerance to temperature, humidity and acidity potential (Callaghan *et al*., 2012). In the past, the earthworm has been used as a bioaugmentation agent in the degradation of crude oil, obtaining excellent results (Schaefer and Filser, 2007).

There is a wide variety of worms that could be used for bioaugmentation in minimizing

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CRECIMIENTO DE LA LOMBRIZ ROJA CALIFORNIANA (*Eisenia foetida*) EN EL PROCESO DE VERMICOMPOSTAJE DE SUELO CONTAMINADO CON DIESEL

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RESUMEN

*El objetivo de este trabajo fue determinar el crecimiento y adaptabilidad de la lombriz roja californiana (*Eisenia foetida*) en un medio contaminado con diésel y estudiar la eficiencia de degradación del hidrocarburo en el proceso de vermicompostaje. Se probaron seis diferentes concentraciones de diésel: 1, 5, 10, 15, 20 y 25%. Las propiedades fisicoquímicas del suelo se analizaron en tres etapas: al mezclar el suelo con materia orgánica (previo a la adición de diésel), en la mezcla diésel/materia orgánica/suelo al iniciar el proceso de vermicompostaje y al final del proceso. Durante el proceso se midieron la evolución de la población de lombriz y la remoción del contaminante. La mayor remoción alcanzada fue de 97%, en el bioensayo con una concentración de 20% de hidrocarburo. Los resultados muestran que el proceso de vermicompostaje con lombriz roja californiana representa una alternativa eficiente para la bioremediación de suelo contaminado con hidrocarburos.*

CRESCIMENTO DA MINHOCA VERMELHA-DA-CALIFÓRNIA (*Eisenia foetida*) NO PROCESSO DE VERMICOMPOSTAGEM DE SOLO CONTAMINADO COM DIESEL

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RESUMO

*O objetivo deste trabalho foi determinar o crescimento e adaptabilidade da minhoca vermelha-da-califórnia (*Eisenia foetida*) em meio contaminado com diesel e estudar a eficiência de degradação do hidrocarboneto no processo de vermicompostagem. Foram testadas seis diferentes concentrações de diesel: 1, 5, 10, 15, 20 e 25%. As propriedades físico-químicas do solo foram analisadas em três etapas: no momento de misturar o solo com matéria orgânica (antes da adição do diesel), durante a mistura diesel/matéria orgânica/solo ao iniciar o processo de vermicompostagem e, ao final do processo. Durante o processo foi medida a evolução da população de minhocas e a remoção do contaminante. A maior taxa de remoção alcançada foi de 97%, no bioensaio com concentração de hidrocarboneto de 20%. Os resultados mostram que o processo de vermicompostagem com minhoca vermelha-da-califórnia representa uma alternativa eficiente para a bioremediação de solo contaminado com hidrocarbonetos.*

soil pollutants, not only hydrocarbons. They adapt quickly to the environment, have a short reproductive cycle (they reach sexual maturity in approximately two months) and are extremely prolific (they lay eggs every 7 to 10 days). Use of earthworms for bioaugmentation is a viable alternative that could be less expensive than other methods. Contreras-Ramos *et al*. (2008) affirm that the application of earthworms to a soil contaminated with oil is a safe, friendly and economical detoxification alternative to remove polycyclic aromatic hydrocarbons from the soil.

In this investigation, the growth and adaptation of the Californian red worm (*E. foetida*) in five substrate media with hydrocarbon (diesel) in concentrations of 1, 5, 10, 15, 20, and 25% was analyzed. Subsequently, the decrease of hydrocarbon in the contaminated soil was measured and the feasibility of the vermicomposting process in the removal of contaminated soil hydrocarbons was verified.

Experimental

Earthworms used in the bioassays

Californian red worm *E. foetida* specimens used for the study were obtained from the vermicomposting area of the Universidad Politécnica de Durango, previously donated by Tecnológico de Veracruz. A Hanna digital scale was used to weight every specimen. Only adult worms with an average weight of 0.6 ±0.15g were chosen for the bioassays.

Soil preparation

The soil was prepared in plastic containers with a capacity of 3kg. On each container, 1kg of soil and 1kg of organic matter from fruits and vegetables were mixed. A volume of diesel, indicated by a completely randomized experimental design with six treatments and three repetitions (to complete a total number of 18 bioassays), was then added to each container (average data from the three repetitions are shown). The diesel/soil concentrations tested were 1, 5, 10, 15, 20 and 25%. To demonstrate that red worms grow better in healthy environments without contaminants, the results of Rodriguez *et al*. (2018) were considered, with the same amounts of substrate and organic matter that in such study. Diesel used was purchased at a Pemex service station. After thoroughly mixing the mixture, it was allowed to stand for 15 days to achieve stabilization.

Vermicomposting process

After the stabilization period, 10 adult worms were added to each container and the evolution of the population in each bioassay was constantly monitored. After earthworm addition, the containers were left at room temperature for a period of four months; no extra nutrients were incorporated to the bioassays, but water was added due to high ambient temperatures during the process (25-30°C). Temperature and humidity control was carried out with a Hanna brand hygrometer.

Soil samples were analysed and only three measurements were considered: prior to the addition of hydrocarbon and earthworms, during the vermicomposting process and at the end of the process. At each point the physicochemical characteristics of the soil were determined to measure the effect of vermicomposting on the contaminated soil.

Soil sample analysis

The pH and conductivity parameters were determined by the methodology indicated by NOM-021-RECNAT-2000. Macro and micronutrient contents were measured: phosphorus, manganese, magnesium, calcium, sulfate, ferric iron, nitrite nitrogen, ammonia nitrogen and copper, using a Model AST-15 Kit (5412-01) from La Motte Co., Chestertown, MD, USA.

The determination of total petroleum hydrocarbons (TPH) was performed by the reflux method using Soxhlet equipment, according to the method reported by Fernández *et al*. (2006) and taking as reference the methods D5369-93 of ASTM (2003), and 3540 C of US EPA (1996). This analysis was performed with samples taken during the vermicomposting process (two months after the process had started) and at the end of it (after four months of vermicomposting).

In the hydrocarbon extraction process, dried samples of 5g were ground to a fine powder and placed in a filter paper circle that was transferred to the Soxhlet extraction equipment. Reflux extraction was carried out with a 400ml volume of hexane for 8h, performing 6 to 8 refluxes per hour. Upon completion of the extraction process, the flask containing the sample was placed in the oven at 30° for 24h and then placed in a desiccator until it reached room temperature; finally, the weight of the flask containing the organic extract was recorded. TPH quantification (in mg TPH by kg^{-1} of dry soil) was obtained as

where $Weight_2$: weight (mg) of the flask with the concentrated organic extract, $Weight_1$: weight (mg) of the empty flask, Soil: amount (g) of soil in the sample, and HCF: humidity correction factor equivalent to 1-(humidity %/100).

Statistical analyses were performed for the growth and productivity of the earthworm and soil nutrients data with the Infostat (2019) program and the Sigma Plot (2019). A Tukey test was performed for the hydrocarbon removal results.

Results and Discussion

Soil characteristics

The results obtained on the 20% diesel concentration bioassay from the physical and chemical analyses of the soil of the samples previous to diesel addition (March 20, 2018), during vermicomposting (May 22, 2018) and on vermicomposted soil (July 20, 2018), are shown in Table I.

The results showed variations in pH and electrical conductivity, which are important characteristics in soil composition. According to Abed *et al.* (2002), Vallejo *et al*. (2016), and García *et al*. (2011), a pH between 6 and 9 favors the growth of microorganisms and also influences the balance of macro and micronutrients. In the present study, an increase in pH was observed in the middle of the process with respect to the initial pH of the sample, due to the addition of the hydrocarbon; however, by the end of the vermicomposting process the pH had again fallen to a value of 6.5, presumably due to earthworm action. In the manual for remediation of contaminated soils of SEMARNAT (2006) it is mentioned that the pH also influences cation exchange process, and an optimal pH range of 6-8 units for microbial activity is indicated. The values of pH observed in the present experiments (Table I), although it varies between an acid to neutral values, fall within these ranges and meet the maximum permissible limit set by the NOM-021-RECNAT-2000 (DOF, 2002), referring to the productive capacity of the soil.

The increase in the content of NO^-_3 (1 to 3.5mg·kg^{-1}) and NH^+_4 (10 to 32.73mg·kg^{-1}) during bioassays, also had an effect on the growth of the earthworm. Havlin *et al*. (1999) and García *et al*. (2011) mention that a high concentration of ammonia is associated with the observed pH, generating an inhibitory effect on the microbial population. This can be observed in the present experiment, due to the low earthworm density (see next section) and characteristic rotten smell (ammonia) at the end of the process. However, other authors mention that the action of worms increases both the rate of N mineralization and the conversion rates of NH^+_4 to NO^-_3 (Atiyeh *et al*., 2002).

Margesin *et al*. (2000) point also to phosphorus as an important factor for microbial growth; nevertheless, in this research the element remained constant. The values of Ca and Mn concentrations at the end of the process were high enough to consider the resulting vermicomposted soil as appropriate for plant production.

Growth of E. foetida

Figure 1 shows the evolution of earthworm population in the bioassays from the start of the experiment (March) through the end (July). During the first weeks, there was a decrease in the number of individuals in all the percentages of diesel tested according to the experimental design (1, 5, 10, 15, 20, and 25%). This effect is attributed to the pH and electrical conductivity presented at the beginning by the contaminated soil. However, the species showed adaptation to the conditions and at the third week, eggs were found inside the containers. The growth rates of worms in the substrates studied are higher than those observed by other authors in similar laboratory

TABLE I
PHYSICAL AND CHEMICAL PROPERTIES OF THE SOIL

Measured variable	Soil sample previous to diesel addition	Sample during vermicomposting	Sample at the end of vermicomposting
pH	5.5	7.6	6.5
Electric conductivity (mS·cm^{-1})	2.4	4.5	3.2
Organic material (%)	6.1		9.1
Humidity (%)	75	85	70
Average temperature (°C)	25	21.5	24
Total P (mg·kg^{-1} dry soil)	40	32.74	43.64
Sulfates (mg·kg^{-1} dry soil)	11	125	218.2
Fe (mg·kg^{-1} dry soil)	239	265	216
Mn (mg·kg^{-1} dry soil)	28	46.19	43
Mg (mg·kg^{-1} dry soil)	4.55	5	5
Ca (mg·kg^{-1} dry soil)	5	14	28
Cu (mg·kg^{-1} dry soil)	5	7	6
NO^-_3 (mg·kg^{-1} dry soil)	1.53	1	3.5
NH^+_4 (mg·kg^{-1} dry soil)	7	10	32.73

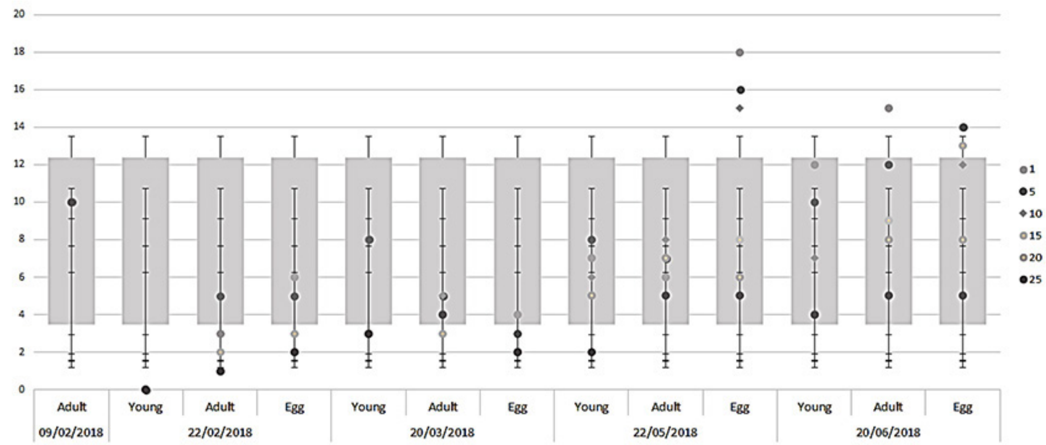

Figure 1. Evolution of *E. foetida* population in the different bioassays.

experiences (Haimi and Hultha, 1990; Elvira *et al*., 1998).

The largest egg production occurred in the final stage of biostimulation with Californian redworm, when the organism was fully adapted to the environment. It is believed that electric conductivity above $8mS\cdot cm^{-1}$ during the composting and vermicomposting processes, would negatively affect worm and microbial populations as well as the biotransformation of organic compounds (Santamaría *et al*., 2001). In this study, the variation of electric conductivity was between 2.4 and $4.5mS\cdot cm^{-1}$, so conductivity was appropriate for earthworm growth.

Diesel degradation in soil

The results presented in Figure 2 show that the bioassay with a concentration of 20% (200ml diesel added) had the highest removal of TPH (97%), followed by the bioassay with concentration of 15% (150ml) with 95% removal, and the bioassay with 1% (10ml). A lower removal level (59%) was reached by the 5% concentration bioassay (50ml added), which lead the authors to believe that there was sufficient oxygen transfer to the soil matrix, allowing the development and growth of the Californian red worm. Accodring to Del Aguila *et al*. (2011) during vermicomposting pollutants are sequestered reducing its toxicity. However, more studies are desirable to determine the permanence of diesel in the soil.

Researchers such as Arrieta *et al*. (2012) obtained removal rates of 36.86 and 50.99%, respectively, during four months of treatment; they mention that the efficiency of hydrocarbon removal in the soil is affected by the physicochemical characteristics of the nutrients added to the environment (source of N and P). The results of this investigation confirm these claims. It is believed that in the vermicomposting process the percentage of hydrocarbon is reduced because during its growth and development, red worms (*E. foetida*) can improve the chemical, physical and biological properties of the soil (Sinha *et al*., 2010). Azaripa *et al*. (2013) describe that the matter that makes up the vermicompost, when mixed with contaminated soil, causes earthworm to adapt quickly to the conditions of the environment, and report a remarkable reduction (50-80%) in trace elements and soluble salts in sheep manure and garden soil with *Eudrillus eugeniae*.

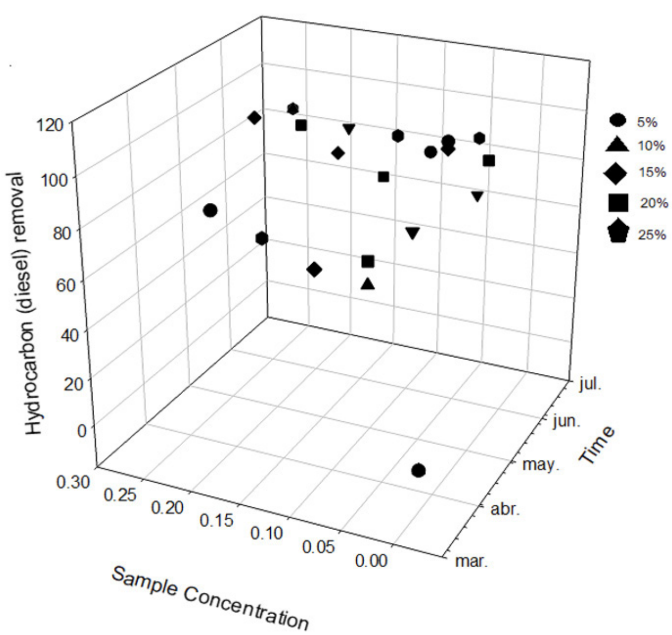

Figure 2. Removal percentages from vermicomposting bioassays with different diesel concentrations.

Table II shows the statistical analysis performed through the Tukey test, which shows that there are significant differences between the variables, with $p<0.001$.

Conclusions

At the beginning of the vermicomposting process, there was a low population density of *E. foetida*, and in the final phase there was a great variation in the number of adults, young worms and eggs within the different bioassays.

The bioassay with a concentration of 20% (200ml diesel added) had the highest removal of TPH (97%) followed by the bioassay with concentration of 15% (150ml) with 95% removal and the bioassay with 1% (10ml). A lower removal level (59%) was reached by the 5% concentration bioassay (50ml added).

TABLE II
TUKEY TEST RESULTS FOR DIESEL REMOVAL PERCENTAGES

Variable comparison	Differences between ranges	q	p	p<0.050
Time *vs* diesel concentration (%)	800	10.243	<0.001	Yes
Time *vs* Removal (%)	400	5.121	<0.001	Yes
Removal % *vs* diesel concentration (%)	400	5.121	<0.001	Yes

This study demonstrates that the use of vermicomposting process with *E. foetida* represents an inexpensive and viable option in the removal of TPH from contaminated soil.

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