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Reduction of the soil environmental impact caused by the presence of total petroleum hydrocarbons (TPH) by using *Pseudomona*s sp.

Key words: bioaugmentation, biodegradation, total petroleum hydrocarbons, efficiency, bacterium

Introduction

Fossil fuels are limited resources that are used to obtain energy. Within these, we can find oil, natural gas, and liquefied petroleum gas, which have been formed from the accumulation of large quantities of organic remains from plants and animals (Abas, Kalair & Khan, 2015). The use of fossil fuels causes atmospheric pollution, generators greenhouse gases, acid rain and respiratory diseases (Singh, 2017; Herndon & Whiteside, 2019).

The hydrocarbon activity in Ecuador is one of the main engines that move the economy of the country and therefore it is impossible to stop or replace the use of this type of resource (Merchán-Rivera & Chiogna, 2017).

Thanks to biotechnology, various methodologies have been developed that allow the restoration of soil, water, and even air, according to the needs and dimensions of the problem (Kuppusamy et al., 2017). One of the techniques used today for recovery of hydrocarbon contaminated soils is bioremediation in which bacteria are used for effective biodegradation of total petroleum hydrocarbons (TPH) (Quintella, Mata & Lima, 2019). Today, due biological and technological advances, it is much easier to

resort to any of the bioremediation processes such as phytoremediation, biostimulation, bioaugmentation, among others; where they use living organisms to treat environmental pollutants (Paredes-Páliz et al., 2016a; Anza et al., 2019).

In this context, bioaugmentation arises from the need to reduce the environmental impact of an area affected or contaminated by the presence of hydrocarbons to detoxify pollutants in different environments (soils and water) through the strategic use of microorganisms, plants, or enzymes (Arora, 2015). This mechanism involves the artificial introduction of viable populations through bioaccumulation using live cells and biosorption by dead microbial biomass (Paredes-Páliz et al., 2016b).

Within the bacterial groups mostly used for bioremediation purposes, *Pseudomonas* sp. is considered as one of the most heterogeneous and ecologically important groups of hydrocarbon-degrading bacteria (Rabus et al., 2016).

The first studies about this microorganism date back to the late 50s, when Leadbetter and Foster (1959) observed that *Pseudomonas methanica* oxidized ethane but could not use it as a carbon source. This was the first of a series of articles by the Foster group, who subsequently observed that this bacterium, growing in a medium with methane, could transform different hydrocarbons. Also, currently, *Pseudomonas aeruginosa* is one of the most used and studied microorganisms in bioremediation and presents a series of natural activities on xenobiotics. Unfortunately, it is also known to be an opportunistic pathogen in humans and causes serious complications in human health (Safdari, Kariminia, Nejad & Fletcher, 2017).

To evaluate the biodegradation of the TPH in soil, we isolated selective bacteria, using optimal environmental conditions for bacterial growth (Jiang et al., 2016). These studies were made in the Laboratory of Analysis and Environmental Assessment (AqLab), located in the city of El Coca, Orellana Province, Ecuador.

The aim of this study was to evaluate the bioremediation capacity of *Pseudomonas* sp. to propose economic and efficient solutions to treat soils contaminated with hydrocarbons with the finality of promoting the interest of companies specialized in bioremediation in the use of this bacteria as a potent degrader of oil contaminants.

Material and methods

Location of the study

This research was carried out in the province of Francisco de Orellana, Ecuador, in the AqLab laboratory, with the geo-referential coordinates 279265 m E and 9948497 m S located in zone 18M, WGS-84 S - UTM, extracted from Google Earth.

Experiment design

The completely randomized design (DCA) was used, which helped us to know if one treatment is different from the other treatment about native bacteria vs. commercial bacteria, with four repetitions. Our methodol-

ogy included: first group of buckets: S1.a, S1.b, S1.c, S1.d corresponding to the sterile native soil with its respective four replicates (a, b, c, and d) were inoculated with the bacterial colonies of *Pseudomonas aeruginosa* (S1). The second group of buckets: S2.a, S2.b, S2.c, S2.d, which corresponds to the sterile native soil with its respective four aftershocks (a, b, c, and d) were inoculated with *Pseudomonas* sp. bacteria previously isolated from same soil (S2). The standard soil called S3 (native non-sterile soil) which was not carried out replicas with the purpose to finalize the total contaminated soil, was bioaugmented with the bacterium *Pseudomonas* sp. (Table 1).

Unlike the previous physicochemical results, the TPH of the soil to be treated was above the permissible limits in Ecuador established in the Ecuadorian Executive Decree 1215 (RAOHE). To execute the treatments with their respective repetitions, the division into eight cubes was carried out with 2 kg of contaminated soil each, divided into two variables: the bacteria used for inoculation and the biodegraded TPH in the 80 days of treatment. For the test it was necessary to homogenize the soil and its sterilization avoiding the presence of other microorganisms at the time of bioaugmentation with the isolated bacteria (*Pseudomonas* sp.) and the commercial bacteria (*Pseudomonas aeruginosa*).

Physicochemical analysis of soil samples

A total of 6 kg of soil was extracted for the initial physical-chemical analysis and the isolation of *Pseudomonas* sp. The sterilization of native soil was carried out utilizing an autoclave at 120°C for 15 min for three times. Physicochemical parameters such temperature (T°), pH, humidity, electrical conductivity (EC), organic matter (MO), total organic carbon (COt), nitrogen (N), phosphorus (P) was measured using the methodology based on the "Standard methods for examination of water and wastewater" (American Public Health Association, American Water Works Association, Water Environment Federation [APHA, AWWA, WEF], 2017), EPA and ASTM methods, standardized methods private laboratory AqLab, duly

TABLE 1. Design and characteristics of the treatments (own studies)

Treatment	Description	Inoculation	Repetition
Soil 1 (S1)	native sterile soil	commercial bacteria (*Pseudomonas aeruginosa*)	S1.a S1.b S1.c S1.d
Soil 2 (S2)	native sterile soil	autochthonous bacteria isolated (*Pseudomonas* sp.)	S2.a S2.b S2.c S2.d
Soil 3 (S3)	native non-sterile soil	autochthonous bacteria isolated (*Pseudomonas* sp.)	no repetitions were realized

accredited by Ecuadorian service of accreditation – Servicio de Acreditación Ecuatoriano (SAE).

Isolation and massification of *Pseudomonas* sp. and *Pseudomonas aeruginosa*

To verify the presence of *Pseudomona*s sp. in native soil, media were prepared for the isolation of colonies. Selective medium Difco™ Pseudomonas Isolation Agar (DPI) was used as a first media for subsequent incubation in broth infusion brain-heart (BHI) at 35°C for 24 h. After 24 and 48 h, the cultures in Petri dishes were examined, using always a blank to verify that the medium is not contaminated with any type of microorganisms. Also, peptone water was used to reduce the microbial concentration by dilution (10^{-1}, 10^{-3}, 10^{-5}, 10^{-8}). Those isolates that presented blue/green or yellowish-brown pigmentation (Palleroni, 2005), due to the production of pyocyanin or pyoverdine were considered positive for the biodegradation treatment (APHA, AWWA, WEF, 2017).

The procedure for the isolation of *Pseudomonas* sp. (Native Strain No AqLab-001) and activation of *Pseudomonas aeruginosa* (Commercial Strain: ATCC@ 15442™* Catalogue No 0693, Lot No 693-1323) was done according to the methodology used in the laboratory AqLab and its comprehensive provider of commercial bacteria MEDILAB, laboratories accredited by the SAE, based on the book of Standard Methods No 9213E and the Association of Official Analytical Chemists (AOCA, 2003.07.2005). The reactivation and massification of the *Pseudomonas aeruginosa* was made using the isolation method proposed by Morales-Guzmán et al. (2017) to isolate petroleum degrading and emulsifying bacteria were performed with the addition of the nutrient broth for *Pseudomonas* sp. in the procedure standardized by the AqLab laboratory, whose massification is in agitation at 180 rpm for 72 h.

Bacteria efficiency and TPH biodegradation

The efficiency of the bacteria was determined based in the levels of biodegradation of TPH after some days of treatments (80 days), with data measured in $mg \cdot kg^{-1}$ in samples every 20 days. The methodology used for extraction measurement was made according to Schwartz, Ben-Dor and Eshel (2012). Consequently, no specific identification was carried out in this study, since only the presence of total oil-pollutants hydrocarbons (TPHs), was reported.

RESULTS AND DISCUSSION

Characterization of soil samples

The optimization and control of bioremediation processes is a complex system of many factors that include: the existence of a microbial population capable of degrading the pollutants; the availability of contaminants to the microbial population; the environment factors (temperature, type of soil, the presence of oxygen or other electron acceptors, pH, and nutrients) (Hlihor, Gavrilescu, Tavares, Favier & Olivieri, 2017).

Physical chemical parameters such organic carbon (TOC), organic matter (MO), phosphorus (P), nitrogen (N), humidity, pH, electrical conductivity (EC), temperature and irrigation were daily monitoring for a total of 80 days. At the same time, TPH and the presence of *Pseudomonas* were also measured. The collection of data through the control generates statistical data that will allow us to perform the efficiency calculations and maintain the soil conditions in optimal conditions for the bacterium to work in the biodegradation of TPH.

The parameters analyzed in the investigation of environmental liabilities contaminated with TPH are presented in Table 2, whose results showed that the physicochemical conditions of the soil, are in midpoint of the permissible limits of the current legal regulations of Ecuador. Also, reference was made in the quality criterion of the minimum parameters of soil fertility in TULSMA book VI, Ecuadorian Executive Decree 3516. Studies have shown that crude oil removal was improved in the presence of sufficient nitrogen and considerably reduced by insufficient nitrogen levels, by the other hand, lack of phosphorus can significantly decrease TPH removal (Sun et al., 2021). Besides, microorganisms produce enzymes in the presence of carbon sources which are responsible for attacking the hydrocarbon molecules. Many of these enzymes have different pathways involved to degrade a hydrocarbon contained in petroleum. Lack of an appropriate enzyme will either prevents attack or will act as a barrier to

TABLE 2. Physical-chemical parameters (own studies)

Parameter	Reference	Unit	Analysis results			Permissible limits		
			initial (0 days)	medium (40 days)	final (80 days)	min.	half	max.
pH	EPA9045C	–	7.32	7.3	7.64	6	–	8
Electric conductivity	EPA9050A	$mmho \cdot cm^{-1}$	240	248	252	–	–	< 2 000
Humidity	ASTM D3976-92	%	73	67	68	50	–	70
Organic material	gravimetric	%	4.12	2.6	1.74	≤ 1.0	1.0–2.0	≥ 2
Total organic carbon*	EPA9060	%	2.5	1.5	one	≤ 0.6–1.5	1.5–3.0	3.0–≥ 6.0
Total nitrogen*	Kjeldahl, EPA351.2	%	0.216	0.13	0.09	0–0.15	0.16–0.3	≥ 0.31
Phosphorus	Booker Tropical Soil Manual (Landon, 1991)	$mg \cdot kg^{-1}$	206.7	257.3	447.2	0–10.0	11–20	≥ 21

*Percentage ratio of C / N 11.6 is within the parameters since the regulations do not determine the accuracy of the permissible limit of the amount of organic matter, nitrogen and phosphorus in terms of the C / N ratio will be < 30 maximum for agricultural land according to current legal regulations (TULSMA book VI, Ecuadorian Executive Decree 3516).

complete hydrocarbon degradation (Barboza, Guerra-Sá & Leão, 2016). For this reason, it is essential that there is an adequate carbon balance for the removal of pollutants, as we can see in our results.

Morphological characterization of *Pseudomonas* sp.

Pseudomonas sp. showed a yellow-brown pigmentation in the first 24 h of incubation at 35°C (Fig. 1), changing to fluorescent red after 48 h of observation in incubation. The presence of polar flagella was also verified, so a minimum displacement of the colonies was observed at the end of the 7 days of observation. *Pseudomonas* oxidatively degrades glucose, which is why it is considered oxidase and catalase positive (Luján, 2019). Specifically, cytochrome monoxygenase enzymes allow the catalytic oxidation of THPs, degrading up to 100% of aromatic fractions (Araujo-Blanco et al., 2016; Cevallos Paguay & García Díaz, 2018).

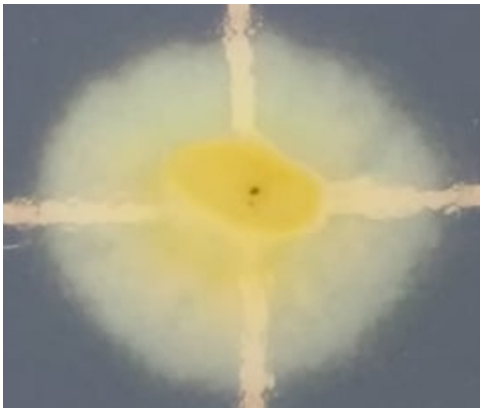

FIGURE 1. Isolation and growth of a colony from dilution 10^{-1} of *Pseudomonas* sp. after 24 h incubation at 35°C (own studies)

These characteristics helped confirm that the isolated bacterium belongs to the genus *Pseudomonas* (Powell et al., 2016). Because in the sponsoring company AqLab it does not have the accreditation of the SAE for molecular characterization, however, using API tests we were able to carry out the biochemical characterization of the native and commercial strains. Considering that only molecular tests can define the bacterial species to 100%, we prefer to describe the native strain only up to genus.

Control of the presence of *Pseudomonas sp.*

For an efficient bioremediation process, it is essential that the quantity and quality of the bacteria used are always optimal (Ojuederie & Babalola, 2017). For this reason, verification of *Pseudomonas* sp. colonies present in the contaminated soil was carried out at the beginning, during and at the end of the treatment of the biodegradation of TPH. The results were beneficial due the periodic control of the bacterial presence every 20 days during the entire treatment, making two massification: at the beginning and 60 days since the presence in colonies of *Pseudomonas* sp. and *P. aeruginosa* began to suffer a decrease in the CFU.

At 40 days of bioaugmentation in the native soil sterile using *Pseudomonas* sp. and *Pseudomonas aeruginosa*, CFUs are maintained with a high number of colonies in the dilution 10^{-3} to 10^{-8}. After 60 days, a deficit was observed in the CFUs in dilution 10^{-1} of the eight treatment cubes, so a count was made in some

cases by sectioning it into quadrants, obtaining between 89 and 373 CFU per Petri dish so that the activation of the bacteria in cryogenics was carried out and its massification in BHI broth, which is necessary a new bioaugmentation of the soil with the bacteria investigated thus without affecting the analysis of the degradation of the TPH.

Evaluation of TPH in soil samples

Biodegradation of total petroleum hydrocarbons (TPHs) is particularly limited by the low availability of contaminant compounds due to their low water solubility and strong absorption in inorganic and organic soil components. However, many studies associated with TPH biodegradation have focused on the use of single strains or an artificial microbial consortium constructed by mixing several known strains, which can grow on TPHs as the only carbon source (Harmsen & Rietra, 2018; Safdari et al., 2018). In our case, we used a native and commercial bacteria of genus *Pseudomonas* sp. Bioaugmentation carried out with *Pseudomonas* sp. is one of the most ecological and economical methods, since they convert hydrocarbons into innocuous subproducts such as CO_2 and H_2O (Prakash & Irfan, 2011; Luján, 2019). This bacterium uses and degrades N alkanes between 11 and 40 carbon atoms reaching 60% effectiveness, using C_{20} as a substrate (Brito, Flores, Howard, Cedillo & Hu, 2008).

Due the sterilization, the TPH can be significantly reduced in the samples evaluated, resulting in an average reduction of 56% at the initial TPH concentration of the standard sample; this occurs because they have different volatile organic compounds and boiling temperatures. Therefore, the initial results of the treatment show a reduction of an average from 20,640 $mg \cdot kg^{-1}$ in a sample of TPH (S3) to an initial of 9,366 and 8,977 $mg \cdot kg^{-1}$ (S1 and S2) without bacteria inoculation (Table 3).

Pseudomonas aeruginosa reduces the initial TPH to 1,996 $mg \cdot kg^{-1}$ and *Pseudomonas* sp reduces the initial TPH to 994 $mg \cdot kg^{-1}$ after 80 days of treatments (Fig. 2).

We can note that the highest concentration of TPH decreased at the first 20 days of treatment in the soil bioaugmented with *Pseudomonas* sp. (S2), unlike

TABLE 3. TPH quantity during treatment

Soil sample	TPH in the samples			Permissible limits in Ecuador			Unit
	initial (0 days)	medium (40 days)	final (80 days)	agricultural use	industrial use	sensitive ecosystems	
S1*	9 366	5 150	1 996	< 2 500	< 4 000	< 1 000	$mg \cdot kg^{-1}$
S2*	8 977	2 992	994	< 2 500	< 4 000	< 1 000	$mg \cdot kg^{-1}$
S3*	20 640	7 710	739**	< 2 500	< 4 000	< 1 000	$mg \cdot kg^{-1}$

*The representation of the acronyms S1 is the bioaugmented native sterile soil with *Pseudomonas aeruginosa*; S2: it is the bio-augmented native sterile soil with *Pseudomonas* sp.; S3: it is the bio-augmented non-sterile standard soil with *Pseudomonas* sp.

**They are the final TPH after 100 days of treatment to give final disposal to the total soil.

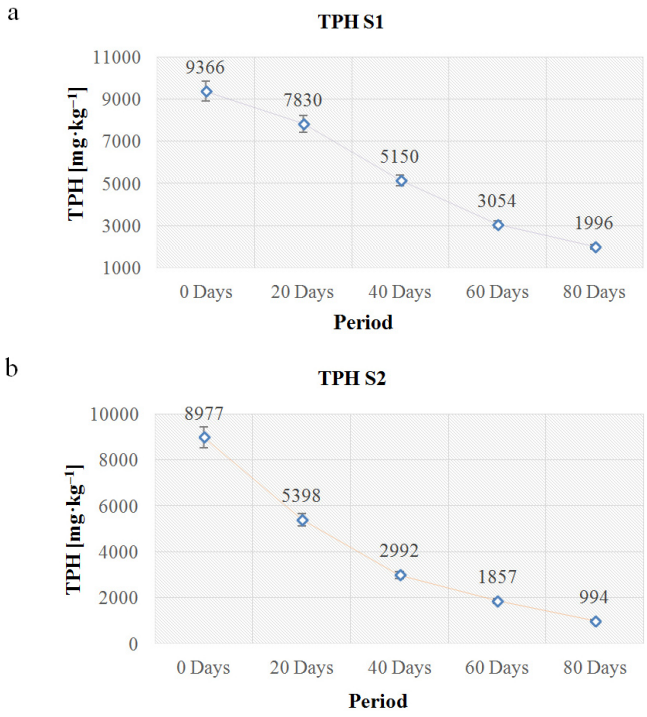

FIGURE 2. TPH biodegradation in soils inoculated with commercial and native bacteria (values are mean ±*SE* of four replicates): a – TPH biodegradation after 80 days of treatment with *Pseudomonas aeruginosa* (S1); b – TPH biodegradation after 80 days of treatment with *Pseudomonas* sp. (S2) (own studies)

the soil inoculated with *Pseudomonas aeruginosa* (S1) that decreased the TPH, but in a smaller percentage compared to the second S2 treatment. From here, the bioaugmentation proceeded only using *Pseudomonas* sp. since the purpose of the research is to reduce costs that generate high socio-economic benefits.

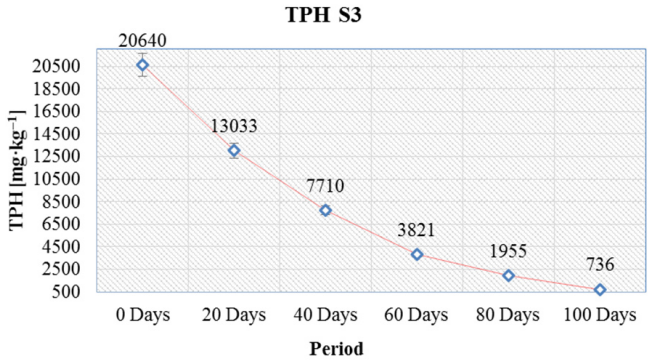

FIGURE 3. TPH biodegradation of the non-sterile standard sample in 100 days of treatment with *Pseudomonas* sp. (S3) (own studies)

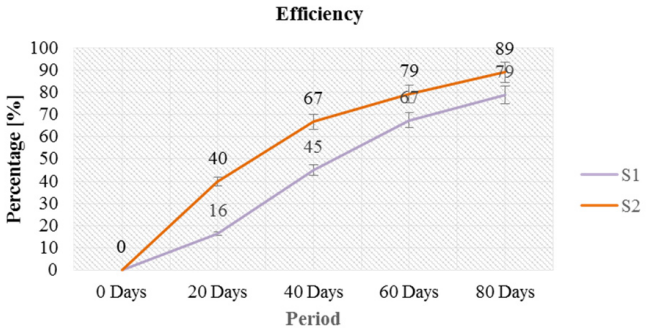

FIGURE 4. Efficiency growth by degrading TPH of *P. aeruginosa* (S1) and *Pseudomonas* sp. (S2) bacteria in sterile native soil during 80 days of treatment (own studies)

Because the laboratory permissible limit must be < 1,000 mg·kg^{-1} exclusive for sensitive ecosystems, according to Ecuadorian Executive Decree 1215, the evaluation of the biodegradation of TPH in the standard not sterilized sample was extended for 20 days over 80 days of treatment. At the end of the treatment, the soil complies with the permissible limits of current legal regulations having a result of TPH in a sample of 736 mg·kg^{-1} (Fig. 3).

Efficiency analysis

The degradation efficiency was calculated, allowing continuity to the statistical analysis and in this way to verify the hypothesis raised at the beginning of the study. As a result, the treatment with the native bacterium *Pseudomonas* sp. (S2) obtained a degradation percentage of 89% compared to commercial bacterium *Pseudomonas aeruginosa* (S1) that reached 79%, having a range of amplitude from one to the other of 10% (Fig. 4) whose average is a biodegradation of 84%. Data that could be compared with efficiency studies reported by Safdari et al. (2017) that show that the removal of hydrocarbons of up to 10 carbons reaches an efficiency of up to 90%, while for hydrocarbons of 20 carbons or more, the efficiency is of about 69%.

Conclusions

Bioaugmentation with isolated and massive native bacteria belonging to the Pseudomonadaceae family, specifically the *Pseudomonas* genus, reduced the concentration of initial total oil hydrocarbons (TPH) in contaminated soils to a final percentage of 4–12%. These results show an efficiency compared to the biodegradation rate of the commercial bacterium *Pseudomonas aeruginosa* that showed a final concentration of 20–22% of TPH in the agglomerates or environmental liabilities stored in the AqLab laboratory that was used in this research. The use of native bacteria in the area is decisive to ensure accelerated and efficient biodegradation due to the innate adaptability that these strains have compared to others that are not native to the site to be remedied.

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Summary

Reduction of the soil environmental impact caused by the presence of total petroleum hydrocarbons (TPH) by using *Pseudomonas* sp. This research focuses on the bioaugmentation with *Pseudomonas* sp. (native) and *Pseudomonas aeruginosa* (commercial) for the biodegradation of total petroleum hydrocarbons (TPH) of the environmental soil samples of the AqLab laboratory in Orellana, Ecuador. Two treatments of sterilized soil (one inoculated with the native strain and the other inoculated with the commercial strain), where used for physical--chemical analyzes as well as the degradation of TPH. They were evaluated every 20 days for a total period of 80–100 days. The native bacterium was isolated from the laboratory agglomerates in a selective culture medium specific for *Pseudomonas* sp. The biodegradation of the TPH exhibited a positive result after 80 and 100 days of treatment, with a reduction of 84 and 96% of initial TPH after the bacterial inoculation. The comparison between the two strains evaluated, commercial and native, showed a greater efficiency of biodegradation by the native strain isolated directly from the agglomerates, suggesting working with native strains of the place that have a greater adaptability to the contaminated environment that would ensure bioremediation processes faster and more efficient, low cost and environmentally friendly.

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