Bioremediation of urban soils contaminated with oil by-products - case study Cuenca, Ecuador

Augusto Polibio Martínez Vega[1*], *Mónica* Eulalia Abad Terán[1], *Sergio* Fernando Iglesias Abad[1] and *Manuel* Salvador Álvarez Vera[1]

[1]Catholic University of Cuenca; Av. Américas y Humbolt, CP. 010101, Cuenca, Ecuador

Abstract. In Ecuador and several Latin American countries, the degree of contamination of urban soils by hazardous waste from petroleum derivatives is a matter of great concern, because according to the Environmental Protection Agency of the United States explains that a gallon of used lubricating oil contaminates a million gallons of water, the same that meets the needs of fifty people per year [1]. When oil is spilled on the land, it causes infertility in the soil because the used oil contains hydrocarbons that cause the death of the soil and transforms the vegetation into inert. Despite the existence of legislation regulating the use, storage, processing and treatment of waste, there are very few efficient methods that guarantee adequate environmental management of urban soil, either because they are technically complex or economically unfeasible. The objective of this research is to technically and economically evaluate the bioremediation of urban soils contaminated with petroleum derivatives in the city of Cuenca, Ecuador, using *Pseudomonas* bacteria. There are different methodologies and methods for soil remediation, the technique used for the recovery of soils contaminated with petroleum hydrocarbons in this research was bioremediation, through the application of an association of *Pseudomonas* bacteria obtained from the same soil, a technique called bio augmentation, and applied in three different concentrations and on the four soil samples obtained from the mechanics of the city of Cuenca. The *Pseudomonas* bacteria obtained, especially *aeruginosa* and *fluorescence*, demonstrated in the experimentation that they have the property of degrading hydrocarbons derived from petroleum, by feeding on carbon compounds in an exponential manner. For the calculation of the remediation cost-benefit, the value of the benefit acquired is divided by the remediation cost found. If the value is higher than the unit, the relation presents benefits; the relation obtained is 5.077, allowing to establish that the remediation process studied is economically viable. It is concluded that the method used is adequate and does not alter the soil with the introduction of foreign bacteria to it, in addition, the method studied serves for the remediation of soils contaminated with non-volatile petroleum hydrocarbons. The cost of its implementation is economically viable.

* Corresponding author: amartinez@ucacue.edu.ec

1 Introduction

"Hydrocarbons are fossil fuels widely used around the world as fundamental generators of various forms of energy, they are in nature thanks to the accumulation of biomass over millions of years. However, it is possible that their extraction generates contamination in water and soil due to constant accidental spills, which are very common in producing countries" [2].

Awareness for the environment is becoming more and more notable [3], and society in general demands that a more rigorous control be carried out on possible pollution sources. Oil hydrocarbon spills are one of the main sources of soil and water contamination as they cause disturbances in ecosystems by affecting their structure and bioprocesses, directly affecting plants, animals and humans, and especially to populations of microorganisms, which represent an important part of ecosystems and are key to biogeochemical processes [4].

Bioremediation is a spontaneous or controlled process in which biological methods, mainly microbiological, are used to degrade or transform pollutants into non-toxic or less toxic products, reducing environmental pollution [5].

According to Pacheco [6], "Bacteria that survive in areas contaminated with oil become allies in soil remediation processes... These bacteria, which cannot be observed with the naked eye, are found in areas where there was a spill or contamination. They are native organisms that, despite the presence of hydrocarbons, have survived in this environment".

Among the different techniques, biodegradation is currently considered the least expensive alternative to transform contaminants present in the soil and in different ecosystems [7], taking into account that a great variety of bacteria have the enzymatic machinery to transform persistent xenobiotic compounds and that they can be isolated from places where there has been previous exposure to the contaminant [8].

The present experimental research work establishes the relationship between the dosage of an association of bacteria and its effect on the recovery of soils contaminated with hydrocarbon derivatives such as gasoline, oil, and grease, which are used by artisans who provide services in automotive mechanics in the city of Cuenca.

The experimentation was based on obtaining colonies of bacteria that are naturally present in the soil contaminated by these hydrocarbons, reproducing them and applying them again to the contaminated soil, as proposed by Belloso, et al. [9].

Finally, the results are presented and analyzed to determine the degree of soil recovery obtained through the application of each of the treatments used, and an evaluation is made of the cost-benefit of using this type of biotechnology for soil recovery, establishing its feasibility for large-scale use.

2 Materials and methods

The present research has the quantitative approach of experimental type, as stated by Hernandez et al. [10], on the types of research, and studies the effect of the variation of *Pseudomonas* concentration (as independent variable) on the concentration of total petroleum hydrocarbons TPH (as dependent variable) applying to soils contaminated with non-volatile petroleum derivatives.

2.1 Soil sampling

Study Universe: The study universe consisted of 98 artisan mechanics of the city of Cuenca, data provided by the Association of Mechanics of Azuay.

Sample size: From the application of the universal formula to determine the sample size proposed by Morillas [11], where n=((k^2)*p*q*N)/((((e^2)*(N-1))+(k^2)*p*q) and in

which the sample size is considered to be the soils of 24 artisan mechanics, maintaining a significance of 95%.

Experimental design: The experimental design used was 4x3 randomized complete blocks with three replications, whose factors under study were:

- Four types of soil,
- Three concentrations of *Pseudomonas,*
- Three replicates of application for each concentration.

2.2 Sample treatment

The soil samples collected in 24 mechanically, were taken according to a division in four terraces of the canton Cuenca with the objective of obtaining samples of four different types of soil.

Six mechanical samples were taken from each terrace, these were taken from the inner part of the site that showed evident contamination due to oil, grease and petroleum fuel spills, by excavating to the level recommended by the TULAS standard (Book VI Annex 2 Soil Resource). Fig. 1 shows the excavation process carried out by the researcher.

It should be noted that in the lower sectors of the city (Chaullabamba) the sampling depth was shallower because of the thinner soil layer and the superficial excavation of large rocks.

These samples were taken to the laboratory of the Faculty of Environmental Engineering of the Catholic University of Cuenca and were transferred in appropriate containers for later handling and treatment.

Fig. 1. Excavation at the automotive shop located at Abelardo J. Andrade and Driver Av.

2.3 Characterization of contaminated soil samples

In the laboratory, the samples were unified according to the terrace from which they came to generate a single sample representing the terrace under study.

At the end of the experiment, a soil sample was taken from each terrace to proceed to the characterization according to its texture (TULAS Book VI Annex 2 Soil Resource), obtaining the data shown in Table 4.

2.4 Initial total petroleum hydrocarbons determination

At the beginning of the investigation, total petroleum hydrocarbons (TPH) were determined in the soil samples as a starting point for the bioremediation process, according to the procedure established in the Environmental Regulations for Hydrocarbon Activities in Ecuador [12].

Table 1 shows the results of the initial determination of total petroleum hydrocarbons using the EPA-3540 procedure, called gravimetric method of TPH determination, with Soxhlet equipment, and compares these data with the values established in the Environmental Regulations for Hydrocarbon Operations in Ecuador [12], in its annex 2 table, which talks about the soil remediation criteria used in all phases of hydrocarbon commercialization.

Table 1. HTP concentration and criteria for compliance with the standard

Sample	Concentration HTP mg/kg	Experimentation Day	RAOHE Annex 2 Table 6	Compliance criterion Yes/No
Terrace 1	44690	0	<4000 mg/kg	Does not comply
Terrace 2	42020	0	<4000 mg/kg	Does not comply
Terrace 3	37040	0	<4000 mg/kg	Does not comply
Terrace 4	51020	0	<4000 mg/kg	Does not comply

2.5 Culture of bacteria present in each of the four contaminated soil samples.

For bacterial culture, we proceeded as recommended by Sanz [13], using Nutrient agar, and the sowing was carried out by preparing five Petri boxes for each terrace, giving a total of twenty sowing boxes. The agar was prepared by weighing 11.5 g of agar and dissolving them in 500 cm^3 of distilled water (according to the manufacturer's recommendations), the solution was kept boiling for 5 minutes, in order to dissolve and cook the agar well, and finally, it was kept at 121° C for one hour, for sterilization, and then in an oven at 60° C until use.

After sowing, after 48 hours in the incubator at 23 ºC, the bacteria present were identified by means of three tests, the first one called Gram test, to which they were negative (typical of *Pseudomonas*), Then they were observed under the microscope, identifying that they had stick forms (typical of *Pseudomonas*) and finally the fluorescence test was performed by exposing them to ultraviolet light, to which they reacted giving a greenish-yellow reflection, which is a reaction typical of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens, without ruling out the possibility* of other microorganisms that could be present.

Then, to prepare the bacterial association, the largest colonies from the first seeding were separated and reseeded on new agar, thus reproducing the best strains from the soil samples of the four terraces.

2.6 Identification and taxonomy of bacteria

The taxonomy of the bacteria was based on the identification of some of their physical properties such as having a characteristic greenish-yellow colour in the presence of ultraviolet light, in the Gram test to which they were negative, and by their shape for which they were identified under the microscope as bacilli (rods), and a property of the bacteria to develop in round colonies, thus confirming that they were *Pseudomonas and especially Pseudomona aeruginosa and Pseudomona fluorescens*. Fig. 2 shows the characteristic way in which *Pseudomonas* develop, that is, in rounded colonies.

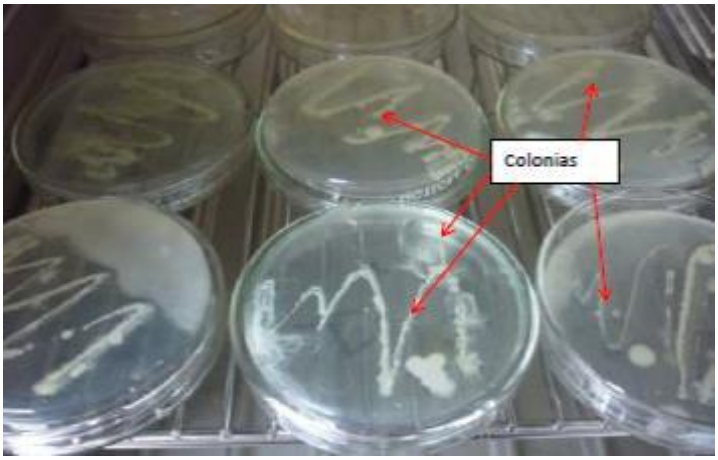

Fig. 2. Bacteria in round-shaped colonies.

2.7 Preparation of a solution of *Pseudomonas* obtained from contaminated soil samples

The *Pseudomonas* association was prepared by obtaining 100 colonies from twenty Petri dishes prepared in a number of five per terrace.

The largest colonies were selected and reseeded, thus obtaining a better quality of *Pseudomonas* strains. After seeding, a solution of distilled water was prepared in which 100 colonies obtained from the twenty reseeding boxes were placed and 20 cm^3 of prepared nutrient agar was added and diluted in 1000 cm^3 of distilled water in a volumetric balloon. This first preparation was conformed with a concentration of 1/10, that is to say, one colony of *Pseudomonas* per 10 cm^3 of solution, becoming the first concentration to be used for the experimentation and denominated concentration one (C1).

2.8 Preparation of *Pseudomonas* solutions with different concentrations

The technique used for the preparation of the different concentrations of the association of *Pseudomonas* was the technique of serial decimal dilutions [14], obtaining the first concentration with the dissolution of one hundred (100) colonies in 1 000 cm^3 of distilled water giving a concentration of 1/10 (1 in 10) called C1. With this solution C1, homogenized by means of constant rotational movements during one hour, the second concentration of 1/100 (1 in 100) was prepared, and it was called C2, that is: 100 cm^3 of the initial preparation was taken and diluted in 1000 cm^3 of distilled water in a volumetric balloon, then homogenized during one hour. The third concentration of the *Pseudomonas* association was prepared with 100 cm^3 of the second concentration and diluted in 1000 cm^3 of distilled water,

thus obtaining a concentration of 1/1000 (1 in 1000), denominated as C3. Fig. 3 shows the three solutions of a consortium of bacteria, prepared with three different concentrations.

Fig. 3. Balls with the three concentrations 1/10, 1/100 and 1/1000 The 1/10 concentration was named C1, the 1/100 concentration C2 and 1/1000 C3.

2.9 Inoculation of strains in contaminated soil samples

The technique used for the recovery of soils contaminated with petroleum hydrocarbons was bioremediation, through the application of an association of *Pseudomonas* obtained from the same soil, a technique called bioaugmentation [15], and applied in three different concentrations and on the four soil samples obtained from the mechanics of the city of Cuenca.

Pseudomonas bacteria, especially *aeruginosa* and *fluorescens,* have the property of degrading petroleum hydrocarbons by feeding on carbon compounds.

2.10 Inoculation of strains in contaminated soil samples

Three concentrations of bacteria were prepared for inoculation: the first 1/10, called C1, the second 1/100, called C2, and the third 1/1000, called C3. After the solutions were prepared, they were inoculated into the soil by spraying. The amount of solution used in each application was 300 cm^3. Fig. 4 shows the researcher in the process of inoculating the consortium of bacteria with different concentrations to the soil, by spraying.

Fig. 4. Application of bacterial consortium concentrations.

3 Results and discussion

3.1 Concentration of HTP

The soil samples, which were obtained from artisanal mechanics from the four terraces into which the Cuenca canton was divided, contained concentrations of petroleum derivatives that exceeded the established RAOHE standard (<4 g/kg), which establishes that the concentration of petroleum hydrocarbons must be below 4 grams per kilogram of soil, data shown in Table 2.

Table 2. Initial HTP data and its % over the norm.

Sample	Sample weight g	HTP g	HTP %	RAOHE Annex 2 Table 6 <4 g/kg	HTP g/kg	% about the standard	Compliance criterion Yes/No
Terrace 1	11,5	0,48	4,17	<4	41,74	1043.48	NO
Terrace 2	8,88	0,35	3,94	<5	39,41	985.36	NO
Terrace 3	13,99	0,49	3,5	<6	35,03	875.63	NO
Terrace 4	5,23	0,25	4,78	<7	47,80	1195.03	NO

According to table 2, it is found that in terrace one (S1) the concentration of HTP in the soil exceeds the norm by 1043.48%, in terrace two (S2) 985.36% higher, in terrace three (S3) 875.63% higher and in terrace four (S4) 1195.03% higher than the norm, the latter being the most contaminated with HTP.

Table 3 shows the percentage reduction of HTP according to the soil and the concentration of bacteria used at the end of the 45 days of experimentation.

The degrading action of the bacteria could be evidenced by the tendency towards a decrease in the concentration of hydrocarbons obtained in the analysis of the samples after fifteen days of experimentation.

The results show that the application of the concentration of bacteria C1, with a higher concentration of bacteria (1/10) degrades the hydrocarbons present faster and that as the concentration of bacteria decreases, the degradation behaviour decreases and resembles the natural degradation behaviour.

Table 3. Percentage of HTP degradation by soil type and type of bacteria concentration, arithmetic average.

Soil sample	Bacteria concentration	HTP initial g/kg	HTP at 45 days, g/kg	HTP downgraded at 45 days g/kg	% of reduction	Arithmetic average of the % of degraded HTP
S1	C1	41.74	7.01	34.73	83.21	
S2	C1	39.41	12.91	26.5	67.24	70.92
S3	C1	35.03	9.62	25.41	72.54	
S4	C1	47.8	18.79	29.01	60.69	
S1	C2	41.74	29.33	12.41	29.74	
S2	C2	39.41	27.39	12.02	30.49	27.88
S3	C2	35.03	28.61	6.42	18.33	
S4	C2	47.8	32.05	15.75	32.95	
S1	C3	41.74	36.16	5.58	13.37	
S2	C3	39.41	34.96	4.45	11.3	14.55
S3	C3	35.03	30.66	4.37	12.47	
S4	C3	47.8	37.73	10.07	21.07	

3.2 Evaluation of Results

The statistical method of analysis of variance called ANOVA (ADEVA) and Duncan's test at 5%, applied to soils, concentrations and treatments, were used to evaluate the results.

4 Conclusions

The best treatment applied is S1C1, corresponding to the soils of terrace 1, to which a bacterial concentration of 1/10 was applied, which is the highest concentration.

- The concentration of C1 bacteria equivalent to 1/10 is the one that degraded the petroleum hydrocarbons present in the contaminated soil samples to a greater extent, giving according to Duncan's test at 5% for the HTP behaviour with the application of the bacteria concentrations, a range "a" obtained at 45 days of the experiment.
- At 45 days of experimentation, three ranges of bacteria concentrations for TPH performance were observed when applying Duncan's test at 5%.
- According to the 5% Duncan test for the behaviour of the TPH concentration in % for soils at 45 days of experimentation, it shows that soils S3, S1 and S2, have the same range of significance "a".
- The method used is adequate and does not alter the soil environment with the introduction of foreign bacteria.

References

1. Torres P. Diseño de un plan de recolección y el re-refinamiento de los aceites lubricantes usados en la ciudad de Loja. [Tesis de grado]. Quito: Universidad Internacional del Ecuador, 2014.
2. Velásquez J. Contaminación de suelos y aguas por hidrocarburos en Colombia. Análisis de la fitorremediación como estrategia biotecnológica de recuperación. Revista De Investigación Agraria Y Ambiental, 2017; 8(1): 151–167
3. Alonso R. Proyecto de recuperación de suelos contaminados por hidrocarburos [Proyecto final de carrera]. Barcelona: Universidad Autónoma de Barcelona; 2012.
4. Pelaez J, Vacaro G, Yanez E, Saqalli M. Un Sistemas para Detección de Contaminación por Hidrocarburos: Aplicación al Oriente Ecuatoriano. RISCI: Revista Iberoamericana de Sistemas, Cibernética e Informática, 2016; 180-185.
5. Moreno C, González A, Blanco M. Tratamientos biológicos de suelos contaminados: Contaminación por hidrocarburos. Aplicaciones de hongos en tratamientos de biorrecuperación. Revista Iberoam Micol. 2004; 21: 103-120.
6. Pacheco, M. Bacterias nativas comen el petróleo y remedian suelos contaminados en Ecuador. El Comercio. 29 de junio de 2019.
7. Shah P. Una aplicación de la estrategia de bioaumento para decolorar y degradar el tinte negro reactivo por Pseudomonas SPP. Revista internacional de biorremediación ambiental y biodegradación, 2014; 2.2; 50-54.
8. Marquez F, Hernández V, Lamela T. Biodegradation of Diesel Oil in Soil by a Microbial Consortium. Water Air and Soil Pollution 128. En: Centro de investigación científica y de educación superior de Ensenada. Ensenada: México; 2021. p. 313-320.
9. Belloso C, Carrario J, Viduzzi D. Biodegradación de hidrocarburos en suelos contenidos en terrarios. XXVI Congreso interamericano de Ingeniería Sanitaria y Ambiental; 1998 noviembre 1-5. San Lorenzo: Argentina; 1998.
10. Hernández R, Fernández C, Baptista P. Metodología de la Investigación. México: Mc Graw Hill; 2014, p. 4- 129.
11. Morillas, A. Muestreo en poblaciones finitas. [Trabajo de grado]. Málaga: Universidad de Málaga; 2007.
12. Decreto Ejecutivo-Reglamento Sustitutivo del Reglamento Ambiental para las Operaciones Hidrocarburíferas en el Ecuador, Anexo 2. (Presidencia de la República, Registro Oficial 265, Quito, 2001, p. 55).
13. Sanz S. Prácticas de Microbiología. [Servicio de publicaciones]. La Rioja: Universidad de La Rioja; 2011.
14. Arana I, Orruño M, Barcina I. Como abordar y resolver aspectos prácticos de microbiología. España: Universidad del País Vasco; 2010.
15. Valenzuela B, Galindo L, Mantilla D, Moncada D, Orjuela E, Romano K, Rincón J. Técnicas de biorremediación de suelos contaminados por hidrocarburos con fines de uso en el municipio de Tibú, norte de Santander. Suelos Ecuatoriales, 2021; 51: 108-118.