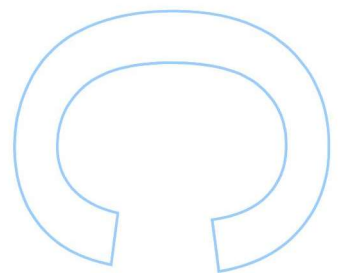
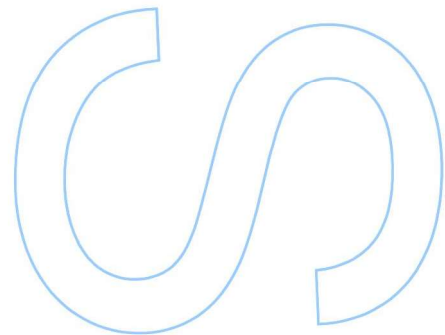
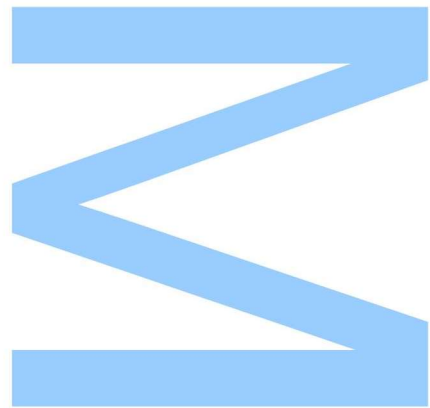


Bio and phytoremediation of soils contaminated with hydrocarbons

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Orientador

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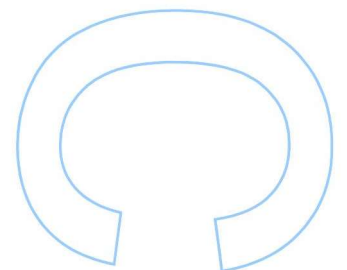
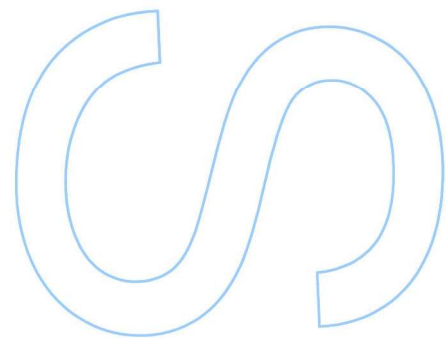
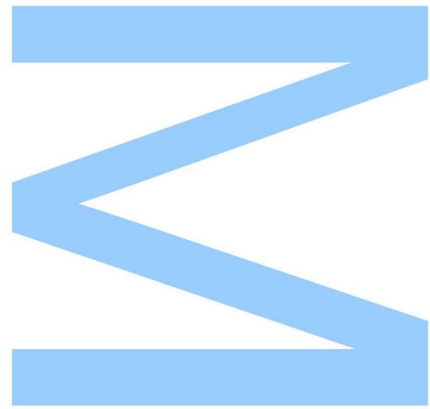




Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, ____ / ____ / ____



Acknowledgment

In this two-year master's degree, I learned to accept the help of many, and many were the ones who offered me help. First and foremost, I would like to thank my family that, even though they were on another continent, made themselves present on my everyday here, supporting me and cheering me on.

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Abstract

Recovery of degraded soils is a topic of great importance today, especially of those contaminated with hydrocarbons. Techniques such as bioremediation and phytoremediation appear as possible solutions to mitigate this problem. In this project, we put these techniques into practice, to analyse their efficiency in a soil contaminated with benzene, a component of greater toxicity present in hydrocarbons. The first part of this study consisted of collecting contaminated soils from a refinery and cultivating the bacteria present there to use them in the process of bioremediation of a soil contaminated with known amounts of benzene. The results obtained were measured by quantifying the concentration of benzene in the gaseous environment of the flasks assembled with the contaminated soil and the inoculum formed from the cultivated bacteria. The results obtained were then compared with a mathematical model of an ideal bioremediation process. These results also served to generate a mathematical model for phytoremediation.

key words: Bioremediation, phytoremediation, Benzene, contaminated soil.

Resumo

A reabilitação de solos degradados é hoje um tema de grande importância, especialmente para aqueles contaminados com hidrocarbonetos. Técnicas como a biorremediação e a fitorremediação aparecem como possíveis soluções para mitigar este problema. Neste projeto, fomos estas técnicas em prática, para analisar a sua eficiência num solo contaminado com benzeno, um componente de maior toxicidade presente nos hidrocarbonetos. A primeira parte deste estudo consistiu em recolher solos contaminados de uma refinaria e extrair e adaptar as bactérias aí presentes para as utilizar no processo de biorremediação de um solo contaminado com quantidades conhecidas de benzeno. Os resultados obtidos foram medidos através da quantificação da concentração de benzeno na fase gasosa dos frascos montados com o solo contaminado e do inóculo formado a partir das bactérias cultivadas. Os resultados obtidos foram então comparados com um modelo matemático de um processo ideal de biorremediação. Estes resultados também serviram para desenvolver um modelo matemático para a fitorremediação.

Palavras-chave: Biorremediação, fitorremediação, Benzeno, solo contaminado.

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Abbreviations and acronyms

APA - Agência Portuguesa do Ambiente

BTEX - Benzene, Toluene, Ethylbenzene and Xylenes

CFU - colony forming units

EC - enrichment culture

EDS - Energy-dispersive X-ray spectroscopy

MM - mineral medium

SEM - High Vacuum Scanning Electron Microscopy

TPH - Total petroleum hydrocarbons

U.S. EPA - United States Environmental Protection Agency

VOCs - volatile organic compounds

XRF - X-ray fluorescence

1. Introduction

1.1 Theme Importance

Soil is an extremely important natural resource for society as it serves not only as a structural base, providing mechanical support, but also as a source of water and nutrients for plants and raw materials for construction and other industries. As such, it is a resource under strong anthropogenic pressure and, as it is not renewable within the human time scale, it must be protected. The present work is based on the environmental concern of soil protection, aiming to reverse the effects of contamination that it may suffer.

Contamination of soil by hydrocarbons is a frequent problem, considering that today's technologies are heavily based on the use of fossil fuels. Such contamination is not only harmful to the environment but also to human health. Consequently, remediation technologies for this type of contamination are highly needed and relevant.

Currently, numerous researches related to the remediation of hydrocarbons contaminated areas are being carried out with the purpose of restoring the quality of these soils. Various methods may be used including bioremediation, which consists on using living organisms (bacteria and / or fungi) that employ these types of contaminants in their metabolism, transforming or degrading them into non-toxic chemicals or at least less toxic than the original molecules. This type of remediation, although recent, is considered very promising not just because it aims to minimize anthropogenic impacts and restructure natural habitats, offering greater safety, but also because it offers less disturbance to the environment and relatively low costs. Bioremediation strategies include: the use of autochthonous microorganisms, i.e. from the site itself, without any interference from active remediation technologies (intrinsic or natural bioremediation); the addition of stimulating agents such as nutrients, oxygen and biosurfactants (biostimulation); and the inoculation of enriched microbial pools (bioaugmentation) (Bento et al., 2003).

1.2 Objectives

This thesis aims to analyse the bioremediation of soils contaminated by hydrocarbons, more specifically the most common and harmful compound in its composition, the benzene, by cultures of bacteria original from soils at two different sites of an oil refinery and the efficiency of this treatments.

It was also intended to evaluate the decontamination efficiency of the same soil as the treatment by bio-phytoremediation, using the same cultures of bacteria and plants of the genus *Miscanthus*, this part of the work was done by mathematical modelling, due to the fact of the Covid-19 pandemic, that had all laboratory work postponed or stopped.

1.3 Organization

This work was organized in six chapters. The introductory chapter is where you find the importance of the chosen theme, the objectives of this study and the way the study was developed.

The second chapter is the bibliographic review of the subjects covered, which serves as a basis for the study. The third talks about the applied materials and methods, and is separated into an experimental part carried out in the laboratory, and a part of mathematical projection.

The fourth chapter is where the results obtained from both laboratory experiments and mathematical calculations are presented. And the fifth are the conclusions we reached at the end of this study. The last chapter is the bibliographic references used throughout the study.

In the Appendices the experimental data and the calculations performed are presented.

2. Literature review

2.1 Petroleum and its components

According to Coriolano and Morais Filho (2016), oil is still the most important source of energy today, being used mainly in the generation of fuels such as gasoline, liquefied petroleum gas, diesel oil, fuel oil and kerosene. It is also an important raw material used in the manufacture of products such as plastics, paints, synthetic rubbers, pharmaceuticals, cosmetics and some others from the advent of the petrochemical industry. What has been noted in the last two decades is that pollution caused by oil and its derivatives has been one of the problems with the most harmful effects on the environment (Andrade, Augusto & Jardim, 2010).

Petroleum is a mineral resource with a complex composition, a mixture that is formed from various compounds, mostly hydrocarbons. Its composition varies widely depending on where and how the petroleum was formed. Because of this complexity, treating areas contaminated with these substances can be quite problematic. Also, because there are so many different chemicals in crude oil and in other petroleum products, it is not often practical to measure each one separately. Being so, the total amount of TPH (Total petroleum hydrocarbons), term used for any mixture of hydrocarbons that are found in crude oil, is usually use. Chemicals that occur in TPH include hexane, benzene, toluene, xylenes, naphthalene, and fluorene.

In soils contaminated by petroleum and its derivatives, some contaminants stand out from the others, they are benzene, toluene, ethyl benzene and xylenes (isomers: ortho-, meta- and para-xylene). These volatile organic compounds (VOCs), also known as BTEX, are defined as monoaromatic hydrocarbons, whose molecular structures have as main characteristic the presence of the benzene ring (Andrade, Augusto & Jardim, 2010).

Among the hydrocarbons present in petroleum, aromatics are the ones that cause major problems in contamination, both because they are more abundant and because of their toxicity.

2.1.1 Benzene

Benzene is a colourless liquid with a sweet smell that evaporates rapidly in the air, is soluble in water and is highly flammable (Velasco, 2015). It's composed of six carbon atoms joined in a ring with one hydrogen atom attached to each. The following are some characteristics of benzene (as cited in Mackey, Shiu, Ma e Lee, 2006):

- Molecular formula: C₆H₆
- Molecular weight: 78.112 g/mol
- Melting point: 5.49°C
- Boiling point: 80.09°C
- Density: 0.8765g/cm³
- Water solubility: 1796g/m³ at 25°C
- Vapor pressure: 12680Pa at 25°C
- Henry's Law constant: 586Pa m³/mol at 25°C (concentration ratio)

- Octanol/Water Partition coefficient: 2.13 log KOW at 25°C
- Octanol/Air Partition coefficient: 2.80 log KOA at 25°C (head-space GC-FID both phases)
- Bioconcentration Factor: 1.48 log BCF (algae)
- Sorption Partition coefficient: 1.96 log KOC at 25°C (soil, calculated-molecular connectivity indices)
- Environmental Fate Rate constants k, or Half-Lives, t_{1/2}:

Volatilization: t_{1/2} = 4.81h from water depth of 1m (calculated)

K = 0.03 d⁻¹ with t_{1/2} = 23 d in spring at 8-16°C, k = 0.22 d⁻¹ with t_{1/2} = 31 d in summer at 20-22°C, k = 0.054 d⁻¹ with t_{1/2} = 13 d in winter at 3-7°C during the periods when volatilization appears to dominate, and k = 0.101 d⁻¹ with t_{1/2} = 6.9 d with HgCl₂ in September 9-15, 1980 in marine mesocosm experiments

t_{1/2} ~ 27h from a river of 1m depth with wind speed 3m/s and water current of 1m/s in 2.7 h at 20°C

Photolysis: atmospheric photolysis t_{1/2} = 2808-16152h, based on measured photolysis half-lives in deionized water

Aqueous photolysis t_{1/2} = 2808- 16152h, based on measured photolysis half-lives in deionized water

- Reaction rate constants, k = 8.84x10⁻⁴ h⁻¹ in air, and k = 1.8 x10⁻⁴ h⁻¹ in water
- Oxidation: KOH = 1.23x10⁻¹²cm³ molecule⁻¹s⁻¹ at 298K
- Biodegradation: K = 0.2yr⁻¹ with t_{1/2} = 110d
- Transpiration stream concentration factor: TSCF: 0.71

Benzene is a known carcinogenic compound. According to the Risk Assessment Information System (RAIS), created by the Oak Ridge Reservation Environmental Restoration Program, Benzene is absorbed via ingestion, inhalation, and dermal application. Nonlethal oral doses of benzene can impact the nervous, hematological, and immunological systems. Ingested and inhaled benzene produces symptoms of neurotoxicity. Inhalation of benzene vapor concentrations of 20,000 ppm for 5-10 minutes can be fatal to humans (Clayton and Clayton, 1981 apud RAIS, 2006). The limit values for benzene regarding the risk to human health, according to the Toxicological profile for benzene, elaborated by the Agency for Toxic Substances and Disease Registry (ATSDR), the values for regulations and guidelines applicable to benzene are:

Table 1: Regulations and Guidelines Applicable to Benzene (ATSDR, 2007).

Oral Slope Factor ((mg/kg)/day)	Inhalation Unit Risk (microg/m ³)	RfC* (mg/m ³)	RfD** ((mg/kg)/day)	Dermal Absorption Factor	EPA Class
1.5x10 ⁻²	2.2x10 ⁻⁶ - 7.8x10 ⁻⁶	0.03	4x10 ⁻³	0.01 ^{ac}	A ^a

* inhalation reference concentration.

** oral reference dose.

a - carcinogenic to humans

c - skin notation, refers to the potential significant contribution to the overall exposure by the cutaneous route.

Being so, the uses and presence of Benzene in the environment is controlled. Portuguese Environment Agency (Agência Portuguesa do Ambiente, APA) has the reference values (concentration of a contaminant in the soil above which there may be an unacceptable risk to human health and / or the environment) for benzene in the environment, that are as in the following tables (Tables 1 and 2). These values were adapted from the Ontario Standards (published in the document “Soil, Ground Water and Sediment Standards for Use Under Part XV.1 of the Environmental Protection Act”, Ministry of the Environment, April 15, 2011), when the initial state or the natural background values weren’t available.

Table 2: Technical Guide Reference Values, APA, 2019.

Situation	Reference values (mg / kg dry weight)			
	agricultural use	urban use	commercial use	industrial use
Environmentally sensitive soils	0.02	0.02	0.02	0.02

Source: Portuguese Environment Agency.

Table 3: Technical Guide Reference Values, APA, 2019.

Situation	Reference values (mg / kg dry weight)				
	Using groundwater			No use of groundwater	
	agricultural use	urban use	commercial / industrial use	urban use	commercial / industrial use
Soils less than 30m from a body of surface water	0.02	0.02	0.02	0.02	0.02
Shallow soils	(0.17) 0.21	(0.17) 0.21	(0.4) 0.32	(0.63) 0.5	(0.4) 0.32
For non-stratified soil remediation	(0.17) 0.21	(0.17) 0.21	(0.4) 0.32	(0.17) 0.21	(0.4) 0.32

() The values in parentheses refer to medium to fine textured soils. The values for coarse textured soils are without parentheses.

Source: Portuguese Environment Agency.

The reference values in these tables depend on the location of the soil, the type of remediation that is going to be applied and the intended use of the soil. Being so, to know which reference value to use is necessary to do a previous study of the area and the soil. In soils less than 30m from a body of surface water, such as reservoir, stream, river or channel, section of stream, river or channel, transition waters or coastal water, where the contamination has a higher probability of spreading in the ecosystem, the reference values are lower. On shallow soils and soils where the remediation applied will be non-stratified, the contamination is more likely to remain contained, so the values of reference vary according to the use that land will have.

2.2 Soil

What we call soil corresponds to the end product of weathering of rocks. There are several factors that can result in this weathering, among them we can highlight the climate and weather, the action of living organisms, the structure and composition of the matrix rocks - geological origin, and the geographic location. The joint action of the different types of weathering generates, for each soil, a distinct fingerprint, or an association of them.

The soil matrix is composed of five main components, which are: minerals, air, water, organic matter and living organisms. Mineral matter is the structural component of the soil, making up more than 50% of the total volume. Air and water make up the pore volume, which varies between 25% to 50% of the total volume. The volume of organic matter (humus) ranges from 0.2% to 3%. Finally, living organisms occupy less than one percent of the volume of a soil (M. Vila, 2004).

Soils can be classified according to their mineralogy, texture, evolution, and others. When analysing the particle size of the soil particles, these can be classified as: sand, silt and clay. Sand being the soil with the largest grains, silt having medium grains and clay with the smallest grains. To do the classification of the soil based on its particle size distribution we follow the criterion used by the United States Department of Agriculture, in which the clay consists of the fraction of soil that passes through a 2µm sieve, silt is constituted by the material that is retained in the 2µm sieve and passes through the 50 µm sieve, sand constituted by the coarse material fraction that is retained in the 50 µm sieve but passes through the 2mm mesh opening (Eweis et al., 1998, apud M. Vila, 2004).

Considering that this difference between grains affects its permeability, when we study soil contamination it is important to know which type we are dealing with, so as to know the possibility of the contamination stain to spreading superficially, infiltrating the deeper soil etc.

2.3 Soil Remediation Techniques

The recovery of degraded soils is not always an easy task and can be time consuming and laborious. There are several types of remediation techniques, of physical, chemical and/or biological nature. To choose which technique to use, first there is a need to identify the type of contaminant and the expected outcome.

Table 4 shows the various types of soil remediation techniques, and if there can be applied *in* or *ex situ*.

Table 4: Soil remediation techniques.

Physico-Chemical Treatment		Biological Treatment		Thermal Treatment	
Soil Flushing	In situ	Bioventing	In situ	Enhanced thermal SVE	In situ
Fracturing		Monitored Natural Attenuation		Vitrification	
Soil washing	Ex situ	Land Farming		Incineration	
Chemical extraction		Enhanced bioremediation		Plasma Arc Process	
Containment barriers	In & Ex situ	Phytoremediation		Pyrolysis	
Landfill cap and enhancements		Biopiles	Low Temperature Thermal desorption		
Soil vapor extraction		Bioreactor	Hot gas decontamination		
Chemical reduction/oxidation		Composting			
Electroremediation					

Source: (Adapted from Lombi; Hamon, 2005).

There are a lot of factors to consider when planning a soil remediation, and to choose which technique should be used, it is necessary to take into account in addition to its effectiveness, its safety, and the time and costs that we can invest in the recovery, since both factors vary greatly depending on the treatment applied. When considering *ex situ* techniques, we have to take into account transport costs, feasibility according to the size of the contamination stain and the impact it will have on the contaminated area. *In situ* techniques tend to be less invasive and cheaper but may take longer to achieve the desired result.

In addition, it is also necessary to know the soil itself and its use after recovery, since certain chemical and biological processes can release unwanted substances and some physical treatments can destabilize the soil and some of its characteristics such as texture, pH, temperature etc can make certain techniques unviable.

Being so, site characterization is often the first step in a contaminated remediation strategy. It consists of the collection and assessment of data representing contaminant type and distribution at a site under investigation. The results of a site characterization form the basis for decisions concerning the requirements of remedial action. Additionally, the results serve as a guide for design, implementation and monitoring of the remedial system (Demcak & Balintova, 2015)

Considering the organic nature of our chosen contaminant, in this work we will discuss the application of biological techniques, in particular bioremediation and phytoremediation.

2.3.1 Bioremediation

According to Andrade, Augusto and Jardim (2010), bioremediation involves the use of naturally occurring (native) or cultivated microorganisms to degrade or immobilize contaminants in groundwater and soil. In this case, generally the microorganisms used are bacteria or filamentous fungi. Bacteria being the most commonly used and are therefore considered as the main element in work involving the biodegradation of contaminants.

Bioremediation comes down to the metabolism of organic substances by microorganisms, so that they obtain nutrients and energy. For this to occur, microorganisms must be active to perform their biodegradation task (Andrade, Augusto & Jardim, 2010).

Bioremediation is the acceleration of the biodegradation process and may therefore be limited to nutrient availability, moisture, temperature, pH, mineral concentration, redox potential, nature of the contaminant and the physical and chemical characteristics of contaminated environments. (Rosa; Triguis, 2005). Generally, native subsurface microorganisms may develop the ability to degrade contaminants after a long period of exposure. Normally, these microscopic beings adapt in low concentrations of contaminants and are in the regions outside the contamination plume (Andrade, Augusto & Jardim, 2010).

Therefore, one of the first steps in considering a bioremediation treatment for a contaminated area is to analyse the contaminant itself, and what type of microorganism is able to degrade it.

Usually the different types of bioremediation are separated by *in situ* and *ex situ* techniques,

the table 5 shows the different bioremediation techniques and some information about them.

Table 5: Bioremediation techniques.

<i>Ex situ</i>		<i>In situ</i>		
Biopile	Involves above-ground piling of excavated polluted soil, followed by nutrient amendment, and sometimes aeration to enhance bioremediation by basically increasing microbial activities.	Natural attenuation	Passive remediation of polluted sites, without any external force (human intervention). The process relies on both microbial aerobic and anaerobic processes to biodegrade polluting substances including those that are recalcitrant.	
Windrow	Periodic turning of piled polluted soil to enhance bioremediation by increasing degradation activities of indigenous and/or transient bacteria present in polluted soil.	E n h a n c e d	Bioaugmentation	Addition of organic and inorganic nutrients that aim to stimulate microbial activity. Often populations of indigenous microorganisms are employed, with the aim of increasing biodegradation rates.
Bioreactor	Vessel in which raw materials are converted to specific product(s) following series of biological reactions.		Bioslurring	This technique combines vacuum-enhanced pumping, soil vapour extraction and bioventing to achieve soil and groundwater remediation by indirect provision of oxygen and stimulation of contaminant biodegradation.
Land Farming	In most cases, it is regarded as ex situ bioremediation, while in some cases, it is regarded as in situ bioremediation technique. Polluted soils are usually excavated and/or tilled, but the site of treatment apparently determines the type of bioremediation.		Bioventing	This technique involves controlled stimulation of airflow by delivering oxygen to unsaturated (vadose) zone in order to increase bioremediation, by increasing activities of indigenous microbes.
			Biosparging	This technique is very similar to bioventing in that air is injected into soil subsurface to stimulate microbial activities in order to promote pollutant removal from polluted sites. However, unlike bioventing, air is injected at the saturated zone, which can cause upward movement of volatile organic compounds to the unsaturated zone to promote biodegradation.

Source: adapted from Azubuiké, Chikere e Okpokwasili (2016).

In most cases the bioremediation is done using bacteria from the contaminated site, being

so, it is the contaminant itself that pre-selects the bacteria to be used, thus determining whether the process will be aerobic or anaerobic, and which technique will be implemented.

In this study we are focusing on contamination by hydrocarbons, and the chosen option to degrade this type of contaminant is a biostimulation using aerobic heterotrophic autochthonous bacteria. Therefore, the decontamination can be performed in situ. The possibility of in situ remediation is one of the advantages of this method, as it does not require removal and transport of contaminated soil and can cover large areas which save money and time. In addition, in situ is the only remediation option in dense constructed areas where it is not possible to excavate the contaminated soil.

2.3.2 Phytoremediation

As its name implies, phytoremediation (Phyto = plant + remediation) is the use of plants as a method for removal, reduction, containment, and isolation of contaminant concentrations in solid, liquid, or gaseous medium.

The type of activity the plant will perform depends not only on the medium to be treated and the type of plant used, but also on the physical and chemical properties of the contaminant. Thus, two different compounds in the same medium can interact with a plant in very different ways (Newman; Reynolds, 2004). This is because the plants can carry out a set of physical, chemical, and biological processes, which allow them to promote the mineralization and immobilization of toxic compounds in the root area, which may later accumulate and concentrate on its stem and sheets (Fiúza, 2016).

According to Tavares, Oliveira and Salgado (2013), plants have good adaptability to extremely diverse environments and to multiple organisms, facilitating their adaptation in soils almost all types (more or less rich in nutrients, acids, saline or contaminated) with different chemical compounds).

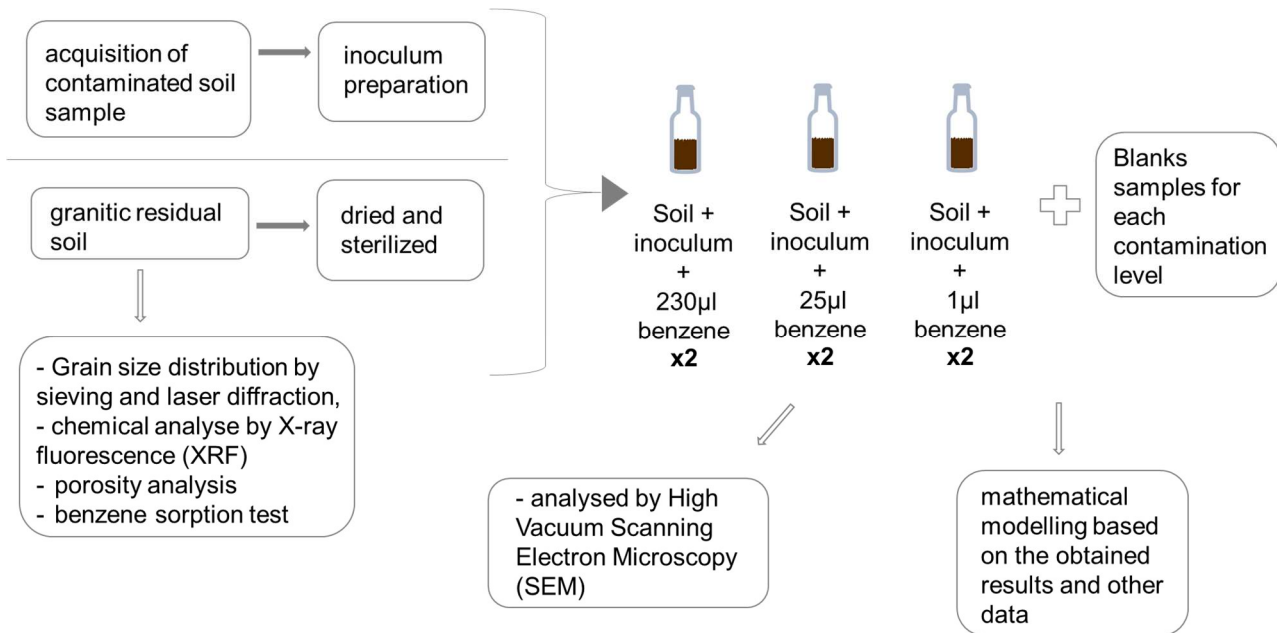
There are four different types of phytoremediation: Phytoextraction, which is the process of absorption of contaminants by the roots of plants. Phytostabilization, which consists of the incorporation of contaminants in the lignin of the plant wall or in the humus of the soil. Phytostimulation, where the growing roots of the plant promote the proliferation of degrading microorganisms in the rhizosphere. And phytodegradation, where organic contaminants are degraded or mineralized within plant cells by specific enzymes.

The phytodegradation of organic compounds can occur either inside the plant or in the rhizosphere region or even in its aerial parts. Decontamination of polluted soils through phytoremediation technique is mainly applicable to toxic metals and organic pollutants, reducing their toxicity levels (Tavares; Oliveira; Salgado, 2013).

3. Materials and methods

3.1 Experimental work

The experimental work was done following the steps shown in Figure 2.



Source: made by the author.

Figure 2: Work plan for this thesis.

Soil samples were collected from the Galp refinery in Matosinhos, from two different locations, one next to a aromatics storage tank (refined oil) and one next to a tank of crude (crude oil). From each of these samples an inoculum was developed.

Subsequently, flasks were prepared with sterilized and previously characterized soil, artificially contaminated with different concentrations of benzene, and the inoculum previously prepared. These were sealed and the level of contamination by benzene was measure in the aerial fraction by Gas chromatography, with measurements over time until the end of bacterial activity was noted.

3.1.1 Soil sampling

Samples of contaminated soil were extracted from a refinery in two different locations, samples classified as TK-6020 were taken from the side of a crude tank and samples classified as TK-802/850 were taken from the side of an aromatics tank. These samples were kept in the refrigerator until used for the inoculum preparation. The following image (Figure 3) show the location of the Galp refinery.



Source: Google Earth, image from 4/11/2015.

Figure 3: Location of Galp refinery, in Matosinhos and areas where the soil was collected.

3.1.2 Pre-characterization

3.1.2.1 Granitic residual soil

The soils have a multiphase nature. They consist of a solid phase formed by mineral particles and organic matter; a liquid phase, usually composed of water (free, pellicular and / or capillary) and a gas phase consisting of air, often thinned with oxygen, and enriched with carbon dioxide. In most soils it is also possible to find a biological phase, consisting of living microorganisms (Carvalho, 2014).

The selection of the soil that was used in this research work was done considering the representativeness of the soil and the physical, chemical, and geotechnical properties that enable the successful application of technologies under study. This soil is a granitic residual soil, collected from a fresh excavation in Leça do Balio, in the municipality of Matosinhos. The sample was taken from a depth of 2 to 3m and was air-dried and later stored at room temperature in an environment protected from light. This sample were previously used in other academic thesis.

Granite, which is a common type of felsic intrusive igneous rock that is granular and phaneritic in texture, is very abundant in Minho, Douro Coast, and Beira Alta, still being found in important patches in Beira Baixa and in the Alentejo. Residual soil is the material resulting from the alteration of the mother rock and which has never been transported from its place of origin (Blight, 1997). Therefore, these soils largely inherit the characteristics of the bedrock, especially with regard to

mineralogy, to texture and structure, factors that are determinant in its geotechnical behaviour.

In order to characterize and determine some parameters of this soil, particle size analysis by sieving and laser diffraction, analysis of chemical composition by X-ray fluorescence (XRF, a technique that offers the identification and qualification of the chemical elements present in the soil sample), and porosity analysis were carried out in the laboratory. Although these properties were previously determined when the sample was taken, since storage over a long period of time can lead to a change in some soil properties, these new determinations were made. Other data were collected and measured when the soil was initially collected.

Before being use in the test, this soil underwent a sterilization process, where it was placed in double sterilizing bags and autoclaved for 20 min at 120°C on three consecutive days, having been kept in a thermostatic camera between one sterilization and the next.

3.1.2.2 Plant species

Miscanthus is a plant that originated in eastern Asia and was, in the first instance, introduced in Europe as an ornamental plant. This perennial plant has a rapid growth rate, which combined with its long duration (20 to 25 years) makes it very valuable (Lewandowski et al., 2003 apud Lino, 2014).

It belongs to the same tribe of grasses as maize, sorghum and sugar cane, the *Andropogoneae*. Miscanthus achieves high nutrient use efficiency in three ways: (1) low entry requirements; (2) recycling of nutrients by dropping waste; and (3) translocation of nutrients back to rhizomes. Nutrient recycling is highly dependent on the efficiency of the translocation process, which in turn is mainly defined by the characteristics of the genotype and the time of harvest. The demand for nutrients for optimal growth is highly dependent on soil conditions (LEWANDOWSKI et al., 2018).

As there are already studies proving the effectiveness of this plant in the phytoremediation of contaminated soils, it was chosen for this study. Unfortunately, dual to the Covid-19 pandemic, it was not possible to perform the phytoremediation tests that were programmed, alternatively a mathematical modelling approach was adopted.

3.1.3 Inoculum preparation

The preparation of the inoculum started with the preparation of the enrichment culture (EC), from both contaminated soil samples, TK-6020 and TK-802/850, and then, in order to develop and adapt the microbial consortium, successive transfers were made (T1 and T2).

To produce the enrichment culture (EC), was previously necessary to prepare the liquid mineral medium (MM), which consists of macronutrients, micronutrients, nitrogen source ((NH₄)₂SO₄) and buffer solutions (KH₂PO₄ and Na₂HPO₄). The preparation of one litre of mineral medium (MM) is done with the following amounts:

Table 6: amounts of micro and macronutrients.

Macronutrients		Micronutrients	
Composition	Weight (g)	Composition	Weight (mg)
CaCl ₂ .H ₂ O	0.0696	FeSO ₄ .7H ₂ O	2.0000
NaCl	0.0080	ZnSO ₄ .7H ₂ O	0.1000
KNO ₃	0.1030	MnSO ₄ .5H ₂ O	0.0430
NaNO ₃	0.6980	H ₃ BO ₃	0.3000
MgSO ₄ .7H ₂ O	0.1000	CoSO ₄ .7H ₂ O	0.2400
NTA	0.1000	CuSO ₄ .5H ₂ O	0.0110
		NiSO ₄ .5H ₂ O	0.0200
		Na ₂ MoO ₄ .2H ₂ O	0.0300
		Ca(OH) ₂	0.5000
		EDTA	5.0000

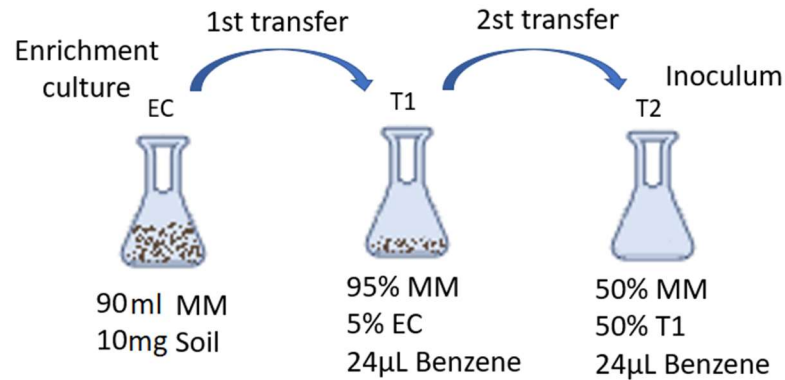
Source: (M. C. Vila, 2004).

To produce 1L of mineral medium, in a one litre Schott flask, was added 100 ml of the macronutrients solution, 10 ml of the micronutrients solution, 20 ml of potassium phosphate solution KH₂PO₄ (0.5444g), 30 ml of hydrated sodium hydrogen phosphate solution NaHPO₄.2H₂O (2.1489 g), 5 ml of ammonium sulphate solution (NH₄)₂SO₄ (0.5 g) and the volume was made up with the addition of distilled water. The pH was measured to make sure that the solution has pH 7, and if necessary, it was adjusted to that value, adding HCl or NaOH (0.1 M).

The enrichment cultures in liquid mineral medium were prepared in 250 mL Erlenmeyer flask, in which 10 g of the soil was added to 90 ml of liquid mineral medium. The cultures were then left to incubate for 24 hours, with orbital shaking (150 rpm), inside a thermostatic chamber at 28 °C.

Then, in order to develop and adapt the microbial consortium, two successive transfers were made (T1 and T2). Those were made in 500mL Erlenmeyer flasks, closed with Mininert® valves, adding inoculum (EC or T1), mineral medium (MM) and the contaminant, benzene. In first transfers (T1) enrichment cultures were used as inoculum (EC), in the EC: MM ratio of 5:95 (v: v). This transfer was kept in incubation until the level of contamination in the gas phase decrease to less than 0.5mg/L, which was about two day later. In the subsequent transfer, used as inoculants for T1 cultures, in a 50:50 T: MM ratio (v: v). T1 and T2 cultures were incubated with shaking (150 rpm), inside the chamber thermostatic at 28 ° C.

Figure 4 illustrates the transfers procedure:



Source: created by the author.

Figure 4: Enrichment culture scheme.

The quantification of the indigenous microbial population was done by the method of counting viable cells (CFU), both the MM and T1, T2 transfer cultures were spread in petri dishes, following the procedure described below:

- Sampling 1ml of the culture and adding it to a test tube with 9ml of saline solution.
- Successive dilutions in sterile saline solution (10^{-2} to 10^{-6}) by flame transfers in sterile and covered test tubes.
- Spreading 100µl of each dilution (10^{-4} to 10^{-6}) on Petri dishes containing LB medium (in duplicate).
- Incubation of Petri dishes at 28 ° C for 3 days.
- Counting of the colony forming units (CFU). They were only considered valid for counting plates with a minimum of 30 and a maximum of 300 CFU.

3.1.4 Benzene sorption tests

To analyse how much benzene is adsorbed by the soil and how much is in the gas phase, a sorption test was carried out. This test consisted of flasks of 330 mL, fill with approximately 73.75g of dry sterilize soil each and six level of contamination by benzene, in duplicates, according to the Table 7. These flasks were sealed and kept in a thermostatic camera at 25°C. After 78 and 186 days, the concentration of benzene on the gas phase was measured and calculations were made to determinate the sorption isotherm.

Table 7: Initial data for sorption test.

Benzene					
	Concentration (mg/kg)	Mass (mg)	Volume (ml)	Volume (µL)	Soil weight (kg)
1A	0.002379689	0.0001757	0.0002	0.2	0.07385
1B	0.002387123	0.0001757	0.0002	0.2	0.07362
2A	0.023829153	0.0017574	0.002	2	0.07375
2B	0.023790443	0.0017574	0.002	2	0.07387
3A	0.238226922	0.017574	0.02	20	0.07377
3B	0.238356164	0.017574	0.02	20	0.07373
4A	1.189683184	0.08787	0.1	100	0.07386
4B	1.19339943	0.08787	0.1	100	0.07363
5A	2.380978187	0.17574	0.2	200	0.07381
5B	2.38388497	0.17574	0.2	200	0.07372
6A	4.765830508	0.35148	0.4	400	0.07375
6B	4.769710951	0.35148	0.4	400	0.07369

3.1.5 Bioremediation tests

The bioremediation tests were done in 1L flasks, fill with approximately 200g of dry sterilized soil, inoculum and know concentrations of benzene. The volume of inoculum added makes up a pre-established moisture content of 20%. The contamination was done in three levels, low, medium and high, each done in duplicate, for the two inoculum Tk-6020 and Tk-802/850. To serve as control, three flasks were also made without inoculum, but with the contamination and the same water content. And lastly there was a bottle without inoculum and contamination, just the same water content. On the total there were sixteen sealed flasks kept in a thermostatic camera at 25°C. The concentration of benzene was measured over time with gas chromatography, using the GC-2010 Plus Gas Chromatographer of Shimadzu. These measurements where done daily in the beginning, then a little more sporadically as the concentration of Benzene became lower. To do these measurements it was necessary to do a calibration curve for the benzene, in order to relate the peak areas obtain by the gas chromatographer to the concentration of the respective pattern for Benzene. Table 8 show the concentration of benzene in each flask.

Table 8: Initial data on the bioremediation flask.

	Level of contamination	Flask	Soil Weight (kg)	Applied contamination (µl)	Concentration (mg/kg)	Inoculum (ml)
Tk-6020	H	1	199.29	230	202.10	39.86
	H	2	199.35	230	202.10	39.87
	M	3	199.35	25	21.97	39.87
	M	4	199.13	25	21.97	39.83
	L	5	199.52	1	0.88	39.90
	L	6	199.33	1	0.88	39.87
Tk-802/850	H	7	198.86	230	202.10	39.77
	H	8	199.01	230	202.10	39.80
	M	9	199.31	25	21.97	39.86
	M	10	199.63	25	21.97	39.93
	L	11	199.53	1	0.88	39.91
	L	12	199.39	1	0.88	39.88
Blanks	Level of contamination	Flask	Soil Weight (kg)	Applied contamination (µl)	Corrected contamination (mg/kg)	humidity (ml)
	H	13	199.29	230	202.10	20%
	M	14	199.67	25	21.97	20%
	L	15	199.38	1	0.88	20%
	NULL	16	199.21	0	0.00	20%

After the end of the bioremediation test, soil samples from the medium contamination level were analysed by a High Vacuum Scanning Electron Microscopy (SEM). Samples of the soil were placed in aluminium stubs and were coated with an Au/Pd thin film for 100 s, with a 15mA current using the SPI Module Sputter Coater equipment. The samples were viewed with an SEM/EDS system (Quanta 400 FEG ESEM / EDAX Genesis X4M, FEI Company, USA).

3.2 Computational work

3.2.1 Mathematical model of bioremediation

To confirm that the results obtained in the laboratory were satisfactory, a mathematical simulation of expected results was performed. The model used was elaborated from the Jaques Monod model for biodegradation, which in turn is based on Michaëlis-Menten equation of enzymatic kinetics (1913).

The model was specially designed for petroleum compounds (those that are the object of experimental study in this work) and is based on the assumption that the decrease in the concentration of the contaminant over time occurs essentially in two ways, by abiotic decay and

biodegradation. The model is composed by a system of three ordinary differential equations, describing the time variation of the main involved variables in the process: biomass and biodegradable and non-biodegradable fractions of the contaminate, and presents itself as the following formulas:

Equation nº1

$$\frac{dX_1}{dt} = K_1 X_1 X_2 - K_2 X_1^2$$

X_1 - microorganism concentration [ML^{-1}].

K_1 and K_2 - kinetic parameters [$\text{M}^{-1}\text{L}^3\text{T}^{-1}$].

X_2 - biodegradable substrate fraction [ML^{-3}].

t – time.

Equation nº2

$$\frac{dX_2}{dt} = \frac{-1}{Y} K_1 X_1 X_2$$

Y - biological yield or substrate conversion factor.

Equation nº3

$$\frac{dX_3}{dt} = -K_3 X_3 + K_4$$

X_3 - fraction of substrate not degradable by microorganisms [ML^{-3}].

K_3 - kinetic parameter [T^{-1}].

K_4 – constant [$\text{ML}^{-3}\text{T}^{-1}$].

Equation nº4

$$S = X_2 + X_3$$

S - total substrate concentration [ML^{-3}]

3.2.2 Mathematical model of phytoremediation

Plants stick to the soil through their roots, which in addition to providing support also play a vital role in absorbing water and mineral nutrients present in the soil. For this reason, the roots have a huge surface area. And it is through the roots that contaminants are absorbed in the phytoremediation process, to then be carried by the plant by its vascular system, xylem and phloem, through the sap.

When we combine Bio and Phyto degradation, we have a process called rhizodegradation, which can be described as the degradation of a contaminant in the soil through microbial activity that is amplified in the presence of the plant's roots.

The mathematical model of phytoremediation is based on the following formulas:

1. Partition coefficients between plant tissues and air, or water, that help us understand the fate of contaminants in the soil-plant-air system.

Equation n°5

$$K_{PW} = \frac{C_{plant}}{C_{water}} = (f_w + f_L \cdot a \cdot K_{ow}^b) \frac{\rho_P}{\rho}$$

K_{pw} - Partition coefficient between plant tissues and water (kg contaminate / m³ plant [C_{plant}] to be divided by kg contaminate/ m³ of water [C_{water}]).

f_w - Mass fraction of water.

f_L - Mass fraction of lipids.

a - density correction factor, which is worth 1.22 ($\rho_{water} / \rho_{octanol}$).

b - correction factor due to the difference between lipids and octanol (depends on the plant).

K_{ow} - octanol-water partition coefficient.

ρ_P - plant density.

ρ - water density.

2. Absorption by roots.

The partition coefficient between the roots and the soil is determined by dividing the partition coefficient between the plant and the water.

Equation n°6

$$K_{RS} = \frac{K_{PW}}{\rho_S K_S + \varepsilon}$$

K_{RS} - Partition coefficient between roots and soil.

ρ_S - soil density.

ε - void fraction filled with water.

K_S - coefficient that depends on soil moisture, estimated from K_{co} (carbon monoxide transfer coefficient), if the fraction of soil organic matter is known.

Equation n°7

$$K_S = \frac{[Bz\ soil]}{[Bz\ water]}$$

3. Translocation in the transpiratory stream.

Equation n°8

$$m = Q \cdot C \cdot TSCF$$

m - mass flow carried to the stem and leaves within the xylem (MT⁻¹).

Q - transpiratory debt (L³T⁻¹).

C - concentration in soil water.

TSCF is related to the octanol-water partition coefficient by the following formulas.

Equation n°9

Briggs (1982)

$$TSCF_B = 0.784 \exp \left[\frac{-(\log K_{ow} - 1.78)^2}{2.44} \right]$$

Equation n°10

Hsu (1990).

$$TSCF_H = 0.7 \exp \left[\frac{-(\log K_{ow} - 3.07)^2}{2.78} \right]$$

From the equation that defines the TSCF, it is possible to estimate the absorption of contaminants during Phyto-rehabilitation. First-rate kinetics can be admitted estimating the time required for rehabilitation to reach programmed targets.

Equation n°11

$$k = \frac{m}{M_0}$$

k - 1st order kinetic constant for absorption (T⁻¹).

M₀ - initial contaminant mass.

So the estimate of the mass that remains in the soil at any time t is expressed by the equation n°12:

Equation n°12

$$M = M_0 e^{-kt}$$

M – mass that remains in the soil.

t – time.

To calculate the time needed to reach a pre-established concentration in the soil, we will have:

Equation n°13

$$t = \frac{\ln \frac{M}{M_0}}{k}$$

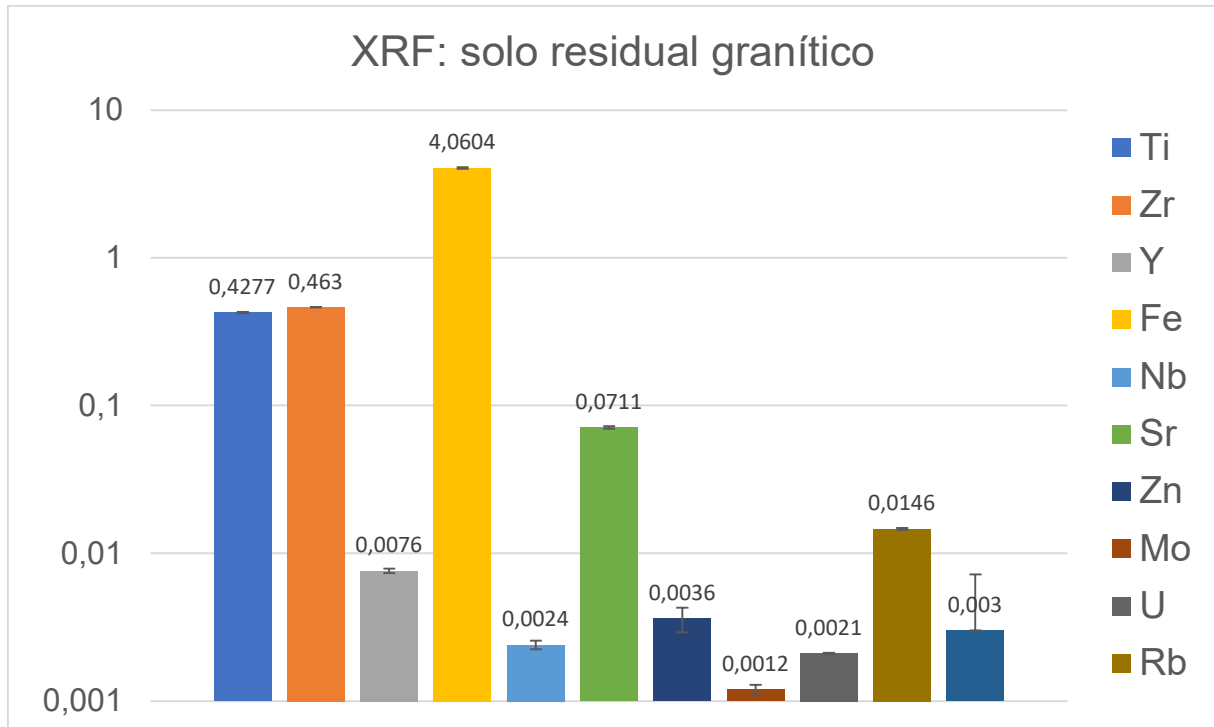
M – mass allowed in the soil.

t – time needed to reach the proposed concentration.

4. Results

4.1 Soil characterization

A chemical analysis of the granitic residual soil by X-ray fluorescence (XRF) was done using an XRF analyser by Oxford Instruments X-Met7500. The results are shown in Graphic 1.

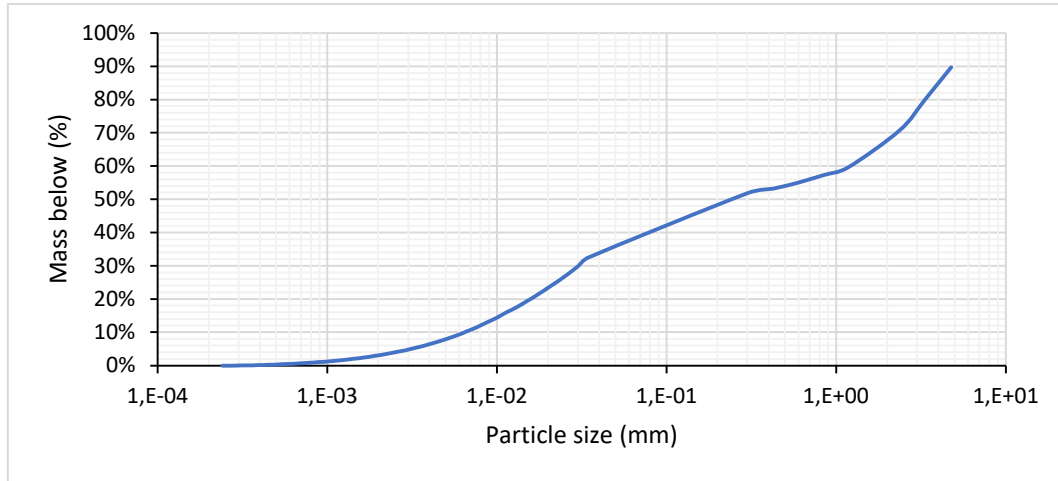


Graphic 1: Results of X-ray chemical analysis.

These results show that, as expected, the soil inherited some characteristics from its parental rock, but others differ due to alterations suffered by the rock in the process of becoming a soil.

The conductivity and pH (5.8) values of the tested soil revealed that it was a non-saline, being slightly acidic. Both these measures were done when the soil was first collected. The soil showed a porosity of 33.33%.

The results of the particle size analysis by sieving are as in the following graphic 2.



Graphic 2: grain size distribution of the granitic residual soil.

The resulting images of the High Vacuum Scanning Electron Microscopy (SEM) test on the soil samples from the medium contamination level after the bioremediation test, and their respective graphics, can be seen below (Figures 5).

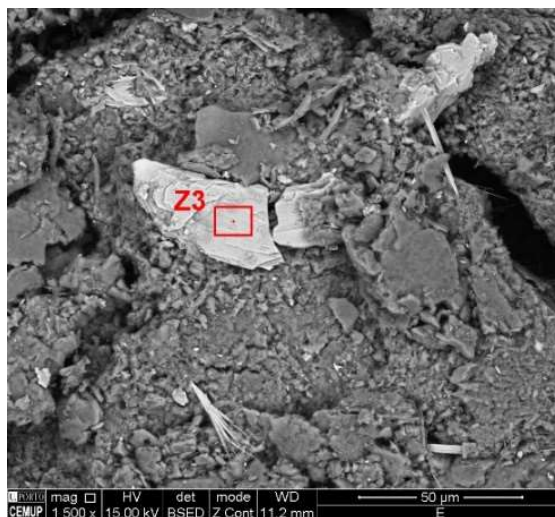
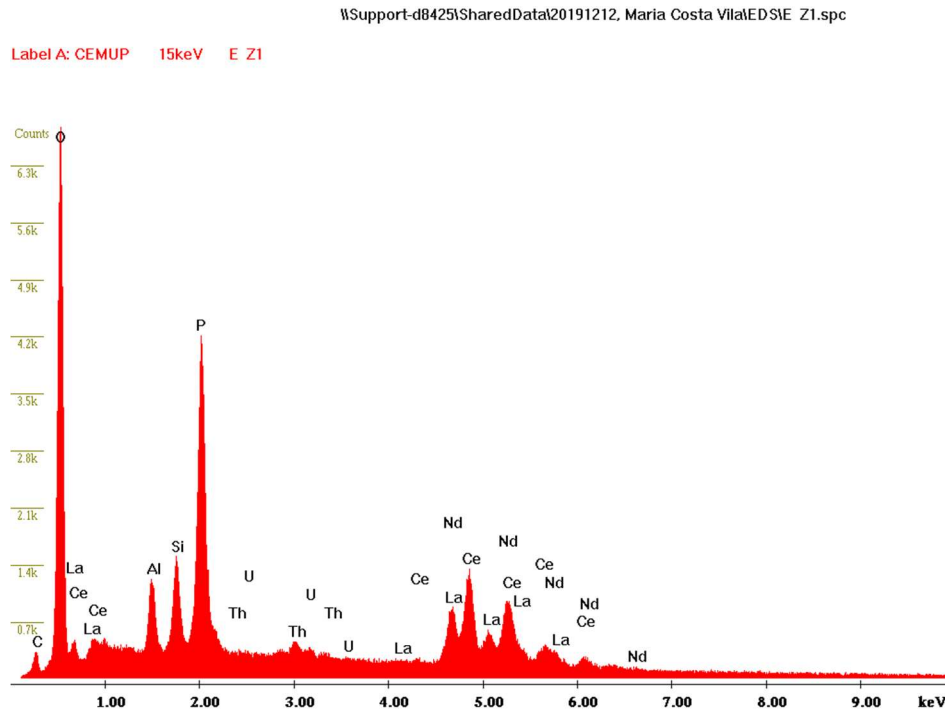
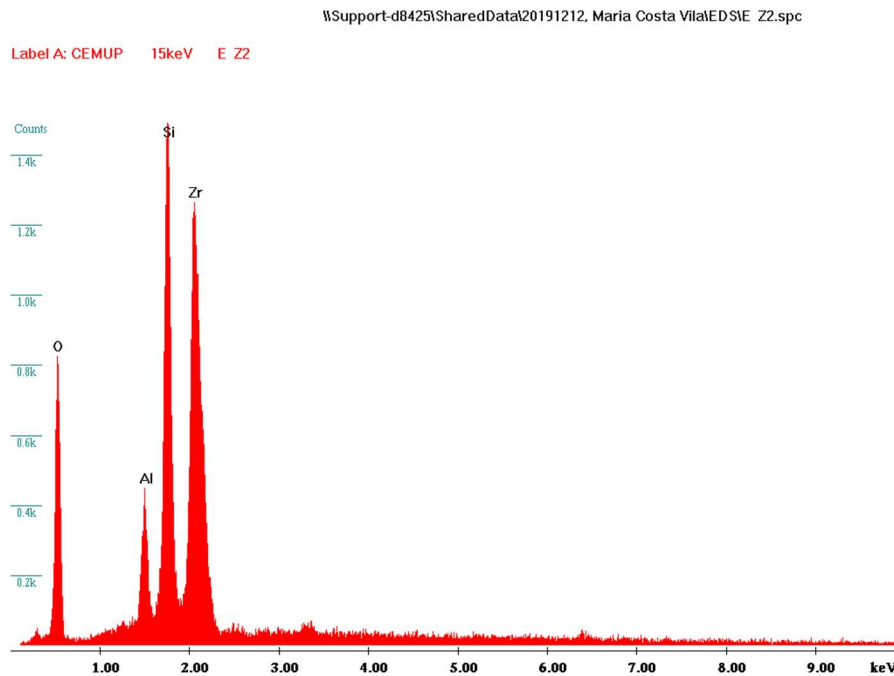


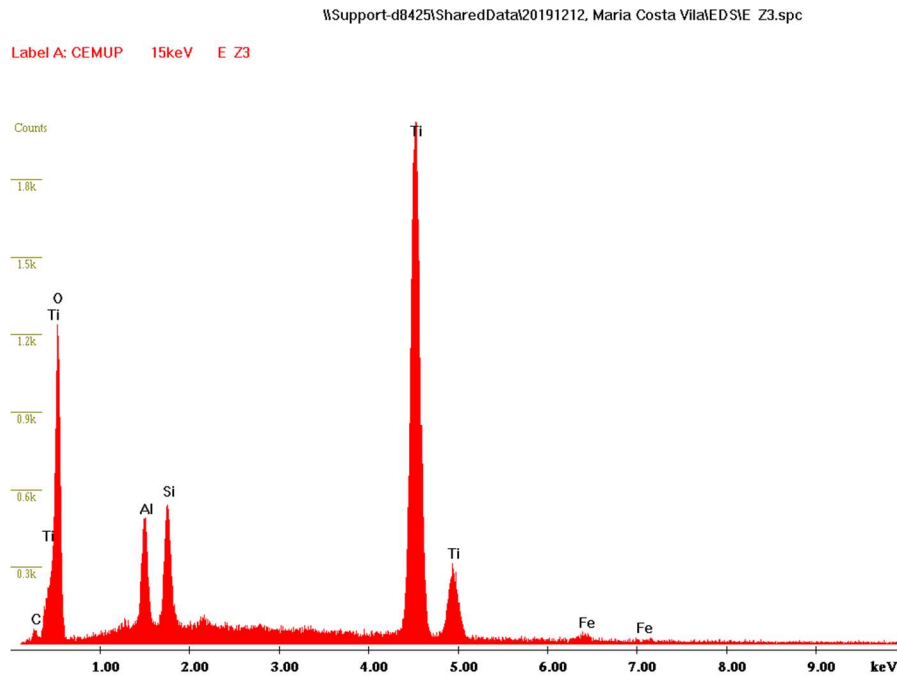
Figure 5: images generated by SEM. Z1 - monazite particle, Z2 – zircon, and Z3 - zirconium oxide.



Graphic 3: chemical elements found in the sample, referring to the image Z1.



Graphic 4: chemical elements found in the sample, referring to the image Z2.



Graphic 5: chemical elements found in the sample, referring to the image Z3.

4.2 Inoculum characterization

The quantification and characterization of the microbial population that was found in the soil samples from the refinery, revealed around four different types of bacteria, one was white and comparatively bigger, another also white but smaller, a translucent one and a slightly yellow, all smooth looking. Both soil samples showing similar communities of bacteria.

Figure 6 show the petri dishes with the colonies of bacteria from the two different soils.

TK-6020

TK-802/850

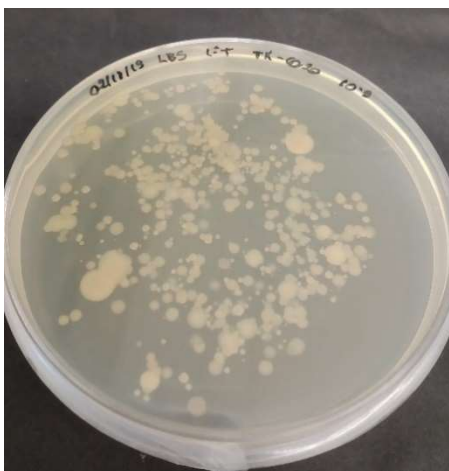
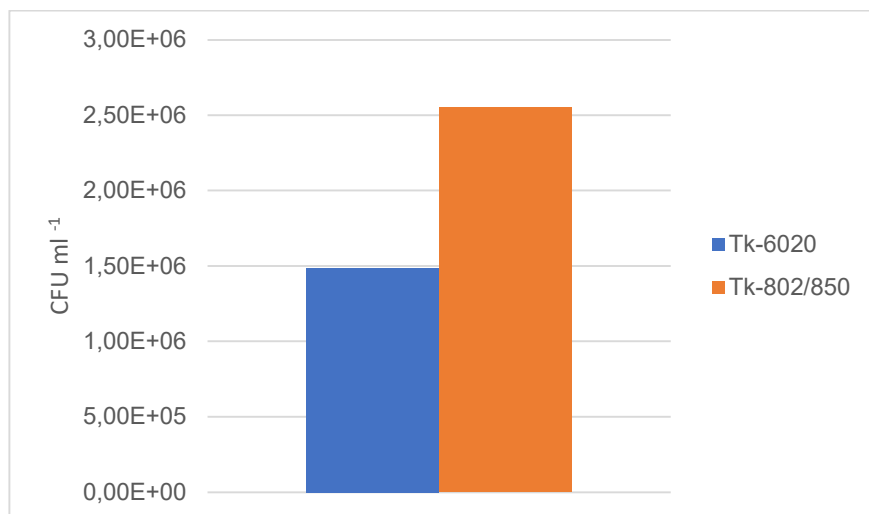


Figure 6: Microbial consortium obtained from soil samples Tk-6020 and Tk-802/850.

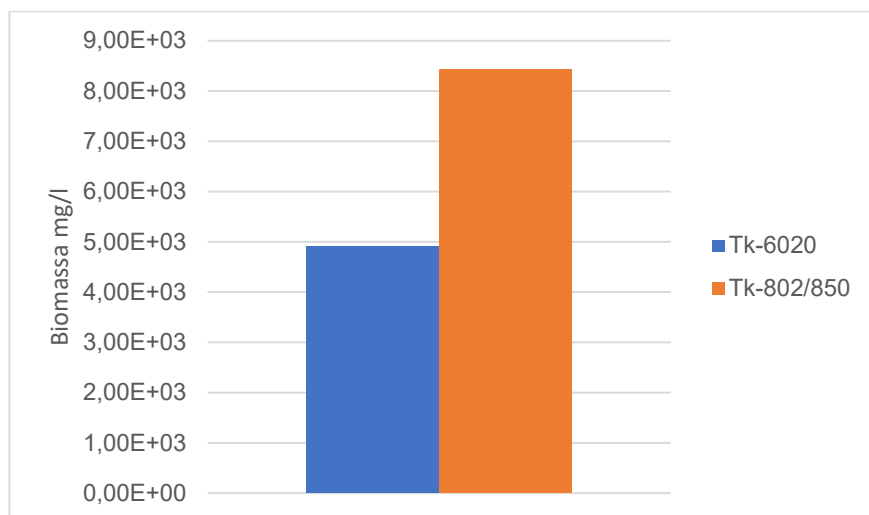
The colony counting (CFU) on Petri dishes shows the following results:



Graphic 6: Results of the counting of CFU of the microbiotic population.

This graphic show us that the soil collected from near an aromatics tank have a greater abundance of microorganisms.

To obtain the microorganism mass present in the inoculum, a 10ml sample was centrifuged at 3000rpm for 10min, and the residual sample was subsequently weighed. The microorganism mass obtain is as shown in the following graphic (Graphic 7):



Graphic 7: Biomass of Microbial population.

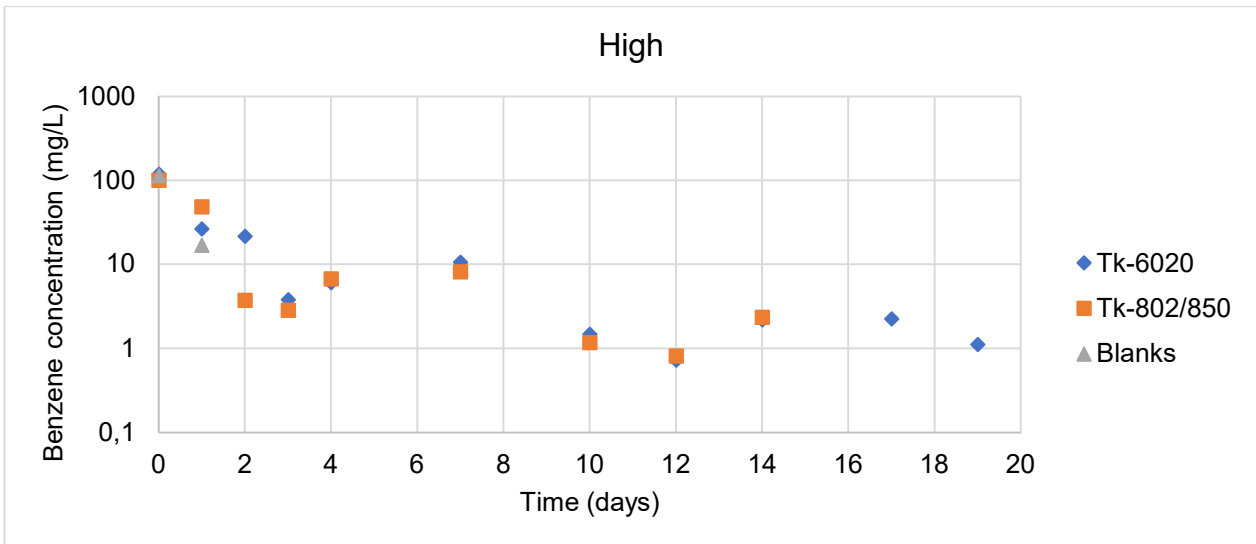
4.3 Bioremediation tests

The purpose of carrying out bioremediation tests on soils contaminated with benzene was to verify the behaviour of the two different microbial consortium developed in liquid medium when applied to unsaturated soils and assess the time required for them to reach minimum levels of contamination admitted. The results obtained in the sixteen different flasks are in the tables 9, 10 and 11:

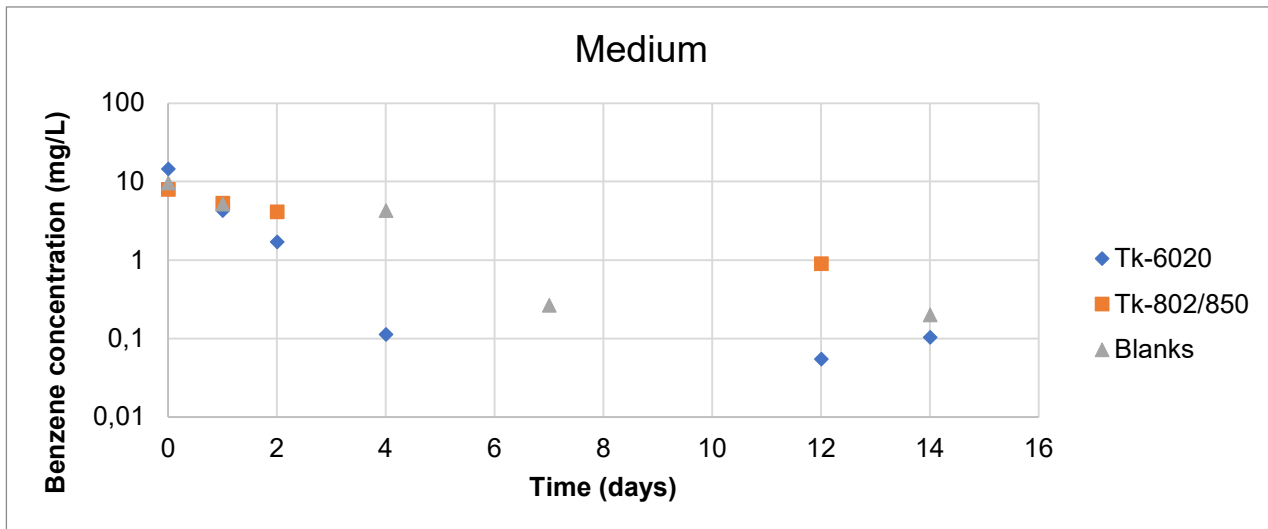
Table 11: Results of the blanks flasks.

Blanks				
Date	Time (days)	benzene concentration in the gas phase		
		High	Medium	Low
		12A	13M	14B
15/11/2019	0	113.199	9.63148	0.11274
16/11/2019	1	16.9053	5.2022	0.03269
17/11/2019	2		4.31186	
18/11/2019	3			
19/11/2019	4	25.3962		
22/11/2019	7	11.6704		
25/11/2019	10			
27/11/2019	12		0.26642	
29/11/2019	14	5,85504		
02/12/2019	17			
04/12/2019	19			

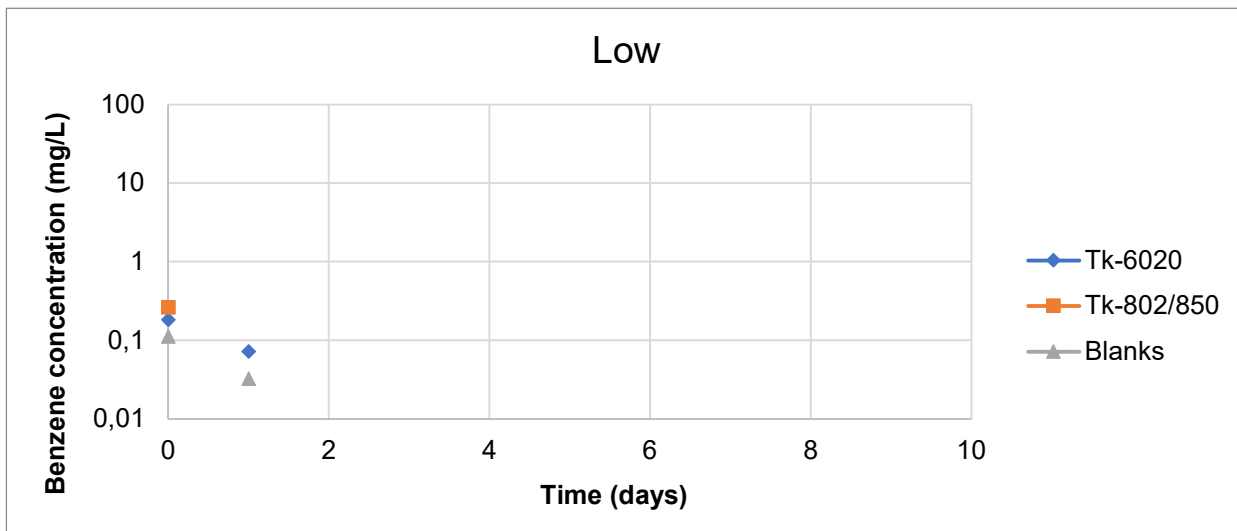
The graphics 8, 9 and 10 show the comparison between the results obtained in the tests with inoculum Tk-6020, Tk-802/850 and the blanks, in the different levels of contamination.



Graphic 8: Comparison of results of the bioremediation flasks with high level of contamination.



Graphic 9: Comparison of results of the bioremediation flasks with medium level of contamination.



Graphic 10: Comparison of results of the bioremediation flasks with high low of contamination.

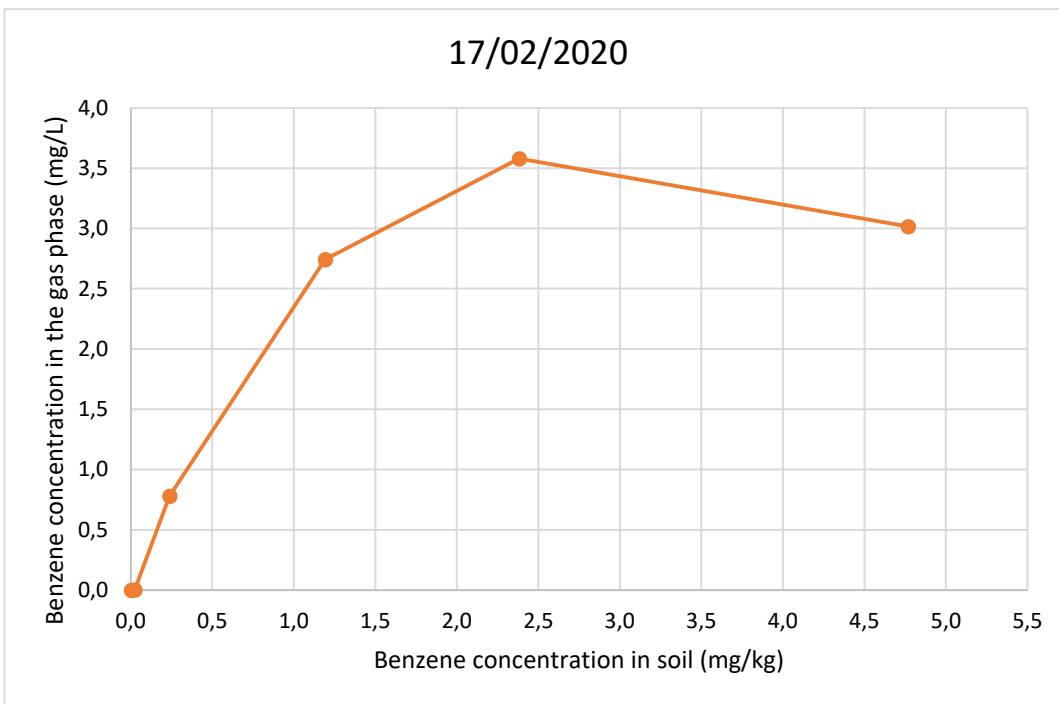
By comparing the results in each level of contamination, and of those in the blanks flasks, it was possible to observe that the inoculum helps to accelerate the degradation of benzene, being the inoculum Tk-6020 slightly more effective than the Tk-802/850.

4.4 Sorption test

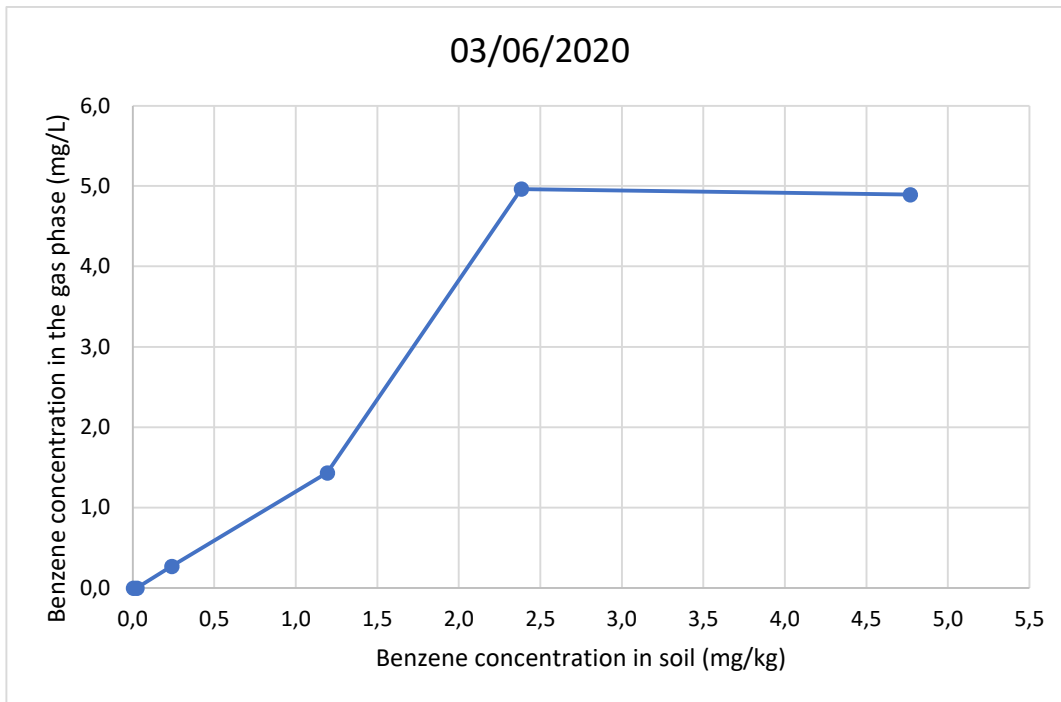
The results obtain in the sorption test were as it follows in the table 12. The experimental study of the relationship between the amount of contaminant adsorbed by the soil and the its concentration in the gas phases, at constant temperature, allows to obtain the equilibrium adsorption isotherms, the graphics 11 and 12 below show these isotherms based in the obtained results.

Table 12: Results of the sorption test for benzene.

Flask	17/02/2020			03/06/2020		
	Concentration in gas phase (mg/l)	Average	Standard deviation	Concentration in gas phase (mg/l)	Average	Standard deviation
1A	-0.3348	-0.2978	0.0369	0.5781	0.1667	0.4113
1B	-0.2609			-0.2446		
2A	-0.0111	0.0013	0.0124	-0.1783	-0.1513	0.0269
2B	0.0138			-0.1244		
3A	0.7075	0.7812	0.0738	0.1270	0.2681	0.1410
3B	0.8550			0.4091		
4A	1.9788	2.7437	0.7649	1.3723	1.4333	0.0610
4B	3.5086			1.4942		
5A	5.7092	3.5803	2.1288	4.9600	4.9648	0.0047
5B	1.4515			4.9695		
6A	1.8933	3.0150	1.1217	5.6837	4.8945	0.7892
6B	4.1367			4.1053		



Graphic 11: Sorption isotherm from the measurements results of 17/02/2020.



Graphic 12: Sorption isotherm from the measurements results of 03/06/2020.

4.5 Bioremediation simulation results

To do the mathematical modelling of the bioremediation test, the values used in the calculation were:

$$Y = 0.35$$

$$K1 = 0.035642 \text{ [(l/mg) days]}$$

$$K2 = 0.032155 \text{ [(l/mg) days]}$$

$$K3 = 0.3243/\text{days}$$

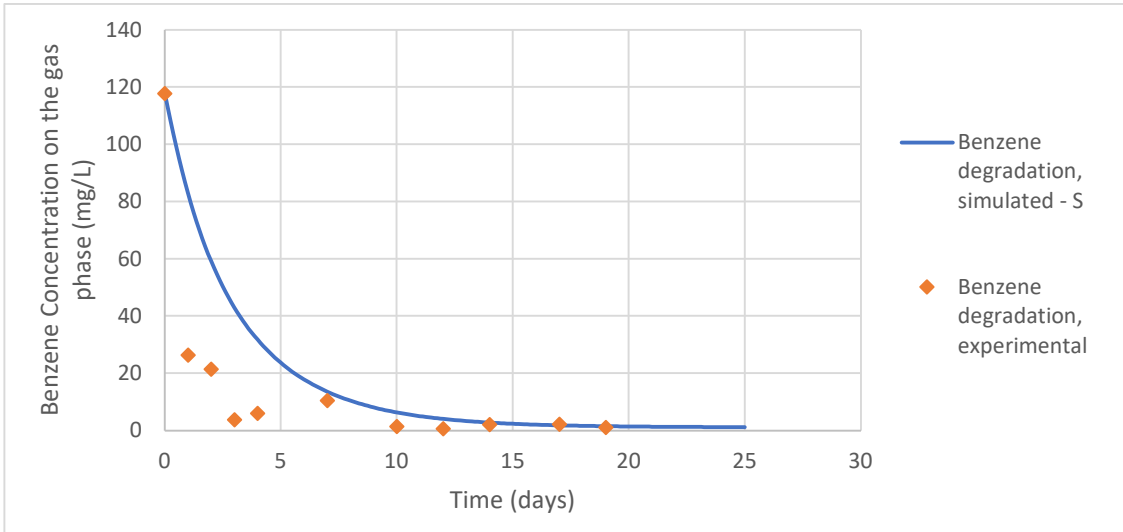
$$K4 = 0.35673 \text{ [(mg/l)/ days]}$$

$$T_{1/2} = 2 \text{ days}$$

$$S = 118 \text{ (mg/l)}$$

$$X1 = 4.91 \text{ (mg/l)}$$

And it presented the following result:



Graphic 13: Result of the bioremediation mathematical modelling.

This graphic (Graphic 13) show us what the result of the bioremediation would have being in perfect conditions. By comparing the results of the test itself, and the mathematical extrapolation curve in this graphic, we can evaluate how efficient the test was. We can see that, although they are not in full conformity, the points follow the general direction of the curve, which shows that the test performed in the laboratory presented acceptable results.

4.6 Phytoremediation simulation results

The values used to calculate the expected results of a phytoremediation were as follows:

Table 13: Pre-established values for the mathematical modelling of phytoremediation.

Soil Details		Benzene Details		Plant Details		Water Details	
fw	0.2	Kow	2.13	fl	2 %	P	0.97 g/cm ³
a	1.22	logKow	0.32838	b	0.77		
ps	1.06 g/cm ³	TSCFB	0.330564	ρP	1		
K_{co}	83 L/kg	TSCFH	10.45516	Q	1 L/day		
f_{co}	0.004	M₀	960 mg				
K_s*	0.332 L/kg	M	0.02 mg				
ε	33 %						
C_s	4.8 mg/kg						
C	14.45783 mg/L						

Being:

K_{CO} – organic carbon partition coefficient (L/kg)

f_{CO} – fraction of organic carbon on soil.

C_s – benzene concentration in the soil.

And the obtain results were:

1. Partition coefficient between plant tissues and air, or water.

$$K_{pw} = 1.273341$$

2. Absorption by roots.

$$K_{RS} = 36.61827$$

3. Translocation in the transpiratory system.

$$m = 151.1589 \text{ mg/day}$$

Based on that, we calculate:

$$k = 0.157457 \text{ d}^{-1}$$

$$t = 68.4564 \approx 69 \text{ days}$$

This means that in order to reduce the initial value of 4.8mg of benzene per kg of soil to the reference value of 0.02mg / kg, 69 days would be needed.

5. Conclusions

As we reach to the end of this study, we can evaluate that, this project's initial proposal, which was to evaluate the effectiveness of a bioremediation of a soil contaminated by benzene, was achieved. The bacteria collected near the refinery's storage tanks proved to be able to degrade benzene in the contaminated soil, showing that the process of decontamination by bioremediation is indeed an effective and applicable process under these circumstances.

Therefore, we obtained positives results that enabled us to move on to the second proposal of this study, which was to perform a phytoremediation on the same contaminated soil. This second objective, however, could not be achieved due to the interruption of laboratory activities, due to the covid-19 pandemic. However, the projection of a mathematical model of such remediation provided us with an ideological scenario of how this phytoremediation would have occurred and gave us important information to better understand how this process of removing contaminants from the soil by the plant occurs.

That being said, there is still a lot to study and learn about contaminates soils recovery processes, as this is a problem of great impact today. And this study opens the way for new investigations, especially the study of phytoremediation that could not be completed.

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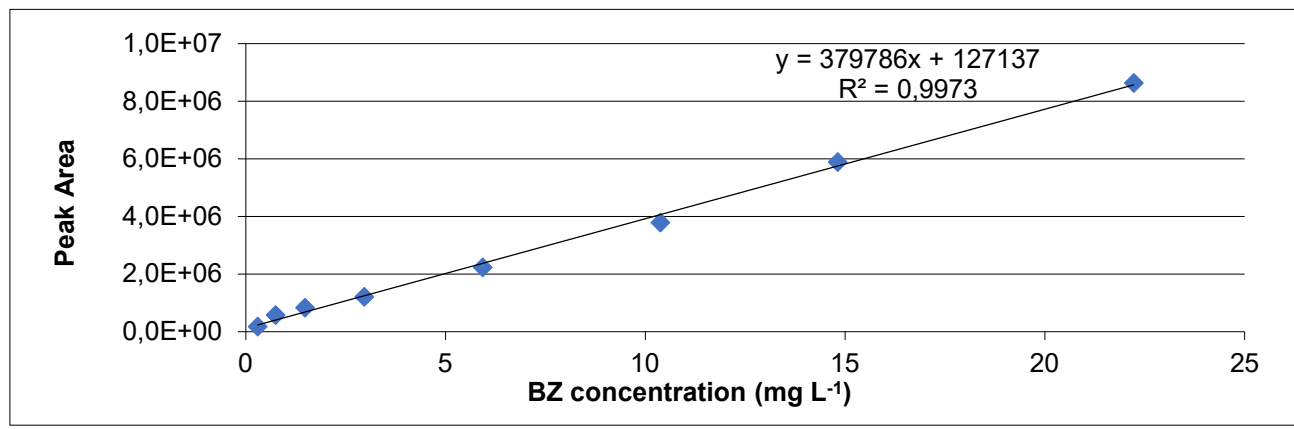
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Attachment 1. Benzene calibration curve.

Benzene calibration		Vol. Flask =		592 mL		dBZ = 0,877				
Vol BZ(μL)	0,2	0,5	1	2	4	7	10	15	20	25
BZ (mg L ⁻¹)	0,2963	0,7407	1,4814	2,9628	5,9257	10,3699	14,8142	22,2213	29,6284	37,0355
Area GC	173482,7	629558,4	817213,2	1095066,3	2168882,7	3694893,4	5613029,3	8053182,3	10940409,7	13948579,2
	156857,8	564496,8	855589,3	1272661,8	2197851,2	3725005,8	5912091,8	8589367,7	12084410	14166909,4
	190052,8	554599,2	823677,6	1278705,5	2342750,3	3959966,7	6134964,7	9255710,9	12682032,9	14440726,9
Average	1,73E+05	5,83E+05	8,32E+05	1,22E+06	2,24E+06	3,79E+06	5,89E+06	8,63E+06	1,19E+07	1,42E+07
Standard Deviation	16597,5	40722,3	20546,2	104323,3	93153,0	145130,5	261892,9	602437,1	884980,3	246594,6
RSD (%)*	9,6	7,0	2,5	8,6	4,2	3,8	4,4	7,0	7,4	1,7



Attachment 2. Chemical composition report by XRF.

Name	Class	Date	Time	Duration			
solo_residual	Mining Mode	07/11/2019	16:09:25	30.5 s			
Element	Ti %	Fe %	Zn %	Rb %	Sr %	Y %	Zr %
solo_residual	0.4298	4.1187	0.0044	0.0148	0.0722	0.0076	0.0474
solo_residual	0.4314	4.0011	0.0036	0.0143	0.0691	0.0073	0.0462
solo_residual	0.4219	4.0615	0.0028	0.0147	0.0719	0.0080	0.0452
Average	0.4277	4.0604	0.0036	0.0146	0.0711	0.0076	0.0463
±	0.00417	0.04804	0.00068	0.00022	0.00140	0.00027	0.00090
Element	Nb %	Mo %	Pb %	U %			
solo_residual	0.0024	0.0014	0.0000	0.0021			
solo_residual	0.0023	0.0012	0.0089	0.0021			
solo_residual	0.0026	0.0012	0.0000	0.0021			
Average	0.0024	0.0012	0.0030	0.0021			
±	0.00016	0.00009	0.00421	0.00002			
Reference:							

Attachment 3. Granulometric analysis by sedimentation report.



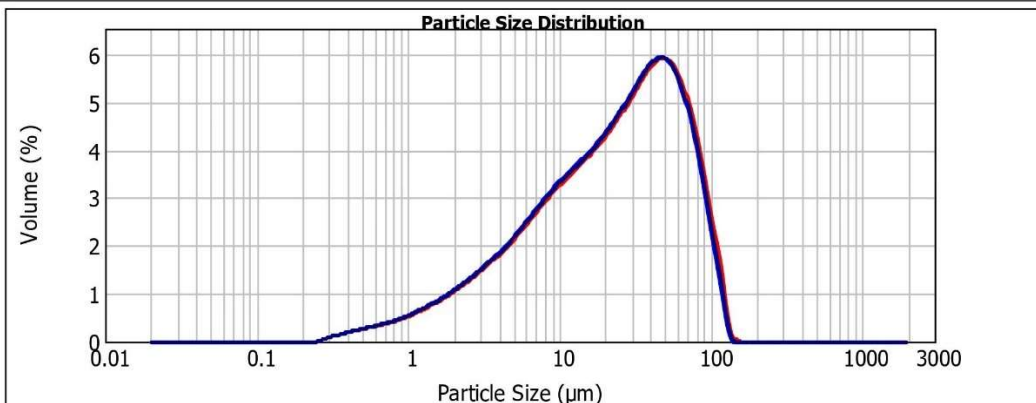
Result Analysis Report

Sample Name: a4 - Average **SOP Name:** **Measured:** 8 de novembro de 2019 14:56:13
Sample Source & type: **Measured by:** cmof **Analysed:** 8 de novembro de 2019 14:56:15
Sample bulk lot ref: **Result Source:** Averaged

Particle Name: Silica 1.0 **Accessory Name:** Hydro 2000G (A) **Analysis model:** General purpose **Sensitivity:** Normal
Particle RI: 1.544 **Absorption:** 1 **Size range:** 0.020 to 2000.000 um **Obscuration:** 15.56 %
Dispersant Name: Water **Dispersant RI:** 1.330 **Weighted Residual:** 0.511 % **Result Emulation:** Off

Concentration: 0.0154 %Vol **Span :** 2.934 **Uniformity:** 0.92 **Result units:** Volume
Specific Surface Area: 0.827 m²/g **Surface Weighted Mean D[3,2]:** 7.254 um **Vol. Weighted Mean D[4,3]:** 31.739 um

d(0.1): 3.227 um d(0.5): 23.781 um d(0.9): 73.011 um



— a4, 8 de novembro de 2019 14:56:13 — a4, 8 de novembro de 2019 14:57:29
— a4, 8 de novembro de 2019 14:58:46 — a4 - Average, 8 de novembro de 2019 14:56:13

Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.010	0.00	0.105	0.00	1.096	0.57	11.482	3.25	120.226	0.40	1258.925	0.00
0.011	0.00	0.120	0.00	1.259	0.66	13.183	3.42	138.038	0.00	1445.440	0.00
0.013	0.00	0.138	0.00	1.445	0.76	15.136	3.59	158.489	0.00	1659.587	0.00
0.015	0.00	0.158	0.00	1.660	0.86	17.378	3.78	181.970	0.00	1905.461	0.00
0.017	0.00	0.182	0.00	1.905	0.98	19.953	4.00	208.930	0.00	2187.762	0.00
0.020	0.00	0.209	0.00	2.188	1.10	22.909	4.26	239.883	0.00	2511.886	0.00
0.023	0.00	0.240	0.01	2.512	1.23	26.303	4.55	275.423	0.00	2884.032	0.00
0.026	0.00	0.275	0.08	2.884	1.37	30.200	4.84	316.228	0.00	3311.311	0.00
0.030	0.00	0.316	0.08	3.311	1.52	34.674	5.11	363.078	0.00	3801.894	0.00
0.035	0.00	0.363	0.12	3.802	1.69	39.811	5.30	416.869	0.00	4365.158	0.00
0.040	0.00	0.417	0.16	4.365	1.87	45.709	5.55	478.630	0.00	5011.872	0.00
0.046	0.00	0.479	0.25	5.012	2.07	52.481	5.20	549.541	0.00	5754.399	0.00
0.052	0.00	0.550	0.29	5.754	2.28	60.256	4.80	630.957	0.00	6606.934	0.00
0.060	0.00	0.631	0.33	6.607	2.48	69.183	4.41	724.436	0.00	7585.776	0.00
0.069	0.00	0.724	0.38	7.586	2.69	79.433	3.33	831.764	0.00	8709.636	0.00
0.079	0.00	0.832	0.43	8.710	2.89	91.201	2.37	954.993	0.00	10000.000	0.00
0.091	0.00	0.955	0.50	10.000	3.07	104.713	1.44	1096.478	0.00		
0.105	0.00	1.096	0.50	11.482	3.07	120.226	1.44	1258.925	0.00		

Operator notes: