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HYDROCARBON BIOREMEDIATION STUDIES

Research Dissertation Report of the Master's Degree in Biological and Chemical Engineering

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HYDROCARBON BIOREMEDIATION STUDIES

JURY

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“*You never know what results will come from your action. But if you do nothing, there will be no results”*

Mahatma Gandhi

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RESUMO

Na economia moderna movida a combustíveis fosseis, todos os produtos petroquímicos são bastante importantes para a sociedade, mas devido ao seu uso excessivo são considerados um dos principais poluentes no Mundo, causando efeitos nocivos e por vezes irreversíveis no meio ambiente. A atuação adequada e atempada em caso de contaminação é essencial e por isso, a procura de soluções que visam remediar o efeito causado por estes compostos quando se encontram indevidamente nas águas e nos solos, continua a ser um assunto pertinente e atual.

Entre as diferentes estratégias disponíveis para remoção e tratamento destes contaminantes, a biorremediação através da utilização de organismos vivos, apresenta-se como uma das viáveis, quer pela sua viabilidade económica quer pela abordagem mais sustentável pelo uso de tecnologia verde.

Nesta dissertação, primeiramente realizou-se uma pesquisa bibliográfica metódica, que a conduz à submissão de um artigo científico intitulado *Bioremediation of soils contaminated with hydrocarbons: one overview.*

Posteriormente, realizaram-se estudos experimentais, referentes à verificação da existência e caracterização de organismos hidrocarbonoclásticos em amostras de solo obtidas durante o mês de outubro de 2020 na Mata da Machada e na Praia da Alburrica, concelho do Barreiro. Os estudos laboratoriais prosseguiram com a determinação da taxa de biorremediação de hexano, gasolina, tolueno, por culturas simples ou mistas das colónias selecionadas. A determinação da biodegradabilidade dos hidrocarbonetos recorreu à utilização do indicador redox 2,6-diclorofenolindofenol através do método espetrofotométrico utilizando suspensão celular.

As colónias foram caracterizadas morfologicamente e bioquimicamente, pela realização do Teste Gram, onde na amostra Mata da Machada identificou-se, através das sete colónias, uma Gram-Negativa, duas Gram-Positivas e quatro que continham estirpes Gram + e Gram -. Na amostra Praia da Alburrica, através das cinco colónias obtidas, identificou-se duas Gram-Positivas e três com estirpes Gram + e Gram -.

O crescimento em meio seletivo de *Pseudomonas* CN Agar Base, permitiu identificar *Pseudomonas aeruginosa* na amostra Mata da Machada e na amostra Praia da Alburrica.

Os resultados demonstraram que na amostra da Mata da Machada, apesar de ser um local protegido e uma reserva natural apresentam bactérias, incluindo, *Pseudomonas aeruginosa*, que conseguem degradar os hidrocarbonetos estudados. Nas amostras da Praia da Alburrica, sendo uma zona fluvial, a existência destas bactérias que conseguem degradar hidrocarbonetos era mais provável, tendo acontecido esse desenvolvimento através da utilização dos hidrocarbonetos estudados. A degradação dos hidrocarbonetos ocorreu mais facilmente através das interações positivas entre organismos.

Palavras-chave: microrganismos hidrocarbonoclásticos, hidrocarbonetos, biodegradabilidade, DCPIP, espetrofotometria.

ABSTRACT

In the modern economy powered by fossil fuels, all petrochemical products are very important to society, but due to their excessive use they are considered one of the main pollutants in the world, causing harmful and sometimes irreversible effects on the environment. Proper and timely action in case of contamination is essential and, therefore, the search for solutions that aim to remedy the effect caused by these compounds when they are unduly found in water and soil, remains a relevant and current issue.

Among the different strategies available for the removal and treatment of these contaminants, bioremediation through the use of living organisms is one of the viable ones, both for its economic viability and for a more sustainable approach through the use of green technology.

In this dissertation, first, a methodical bibliographic research was carried out, which led to the submission of a scientific article entitled Bioremediation of soils contaminated with hydrocarbons: one overview. This research allowed to select the methodology of the experimental studies.

Subsequently, experimental studies were carried out, referring to the verification of the existence and characterization of hydrocarbonoclastic organisms in soil samples obtained during the month of October 2020 in Mata da Machada and Praia da Alburrica, municipality of Barreiro. Laboratory studies continued with the determination of the rate of bioremediation of hexane, gasoline, toluene, by single or mixed cultures of selected colonies. The biodegradability determination of hydrocarbons resorted to the use of the redox indicator 2,6-dichlorophenolindophenol through the spectrophotometric method using cell suspension.

Colonies were morphologically and biochemically characterized by performing the Gram Test, where in the Mata da Machada sample, one Gram-Negative, two Gram-Positive and four containing Gram + and Gram - strains were identified through the seven colonies. In the Praia da Alburrica sample, through the five colonies obtained, two Gram-Positive and three with Gram + and Gram - strains were identified.

The growth on selective medium of *Pseudomonas* CN Agar Base allowed the identification of *Pseudomonas aeruginosa* in the sample Mata da Machada and in the sample Praia da Alburrica.

The results showed that in the Mata da Machada sample, despite being a protected site and a natural reserve, there are bacteria, including *Pseudomonas aeruginosa*, which manage to degrade the studied hydrocarbons. In the samples from Praia da Alburrica, being a river area, the existence of these bacteria that manage to degrade hydrocarbons was more likely, and this development occurred through the use of the studied hydrocarbons. Degradation of hydrocarbons occurred more easily through probably due positive interactions between organisms.

Keywords: hydrocarbonoclastics microorganisms, hydrocarbons, biodegradability, DCPIP, spectrophotometry.

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SYMBOLS AND ABREVIATIONS

AMG – Alburrica Mix Gasoline

AMH – Alburrica Mix Hexane

AMT – Alburrica Mix Toluene

BH - Bushnell-Haas

BTEX - Benzene, Toluene, Ethylbenzene, Xylene

CN - Cetrimide nalidixic acid

DCPIP - 2,6-dichlorophenolindophenol

LB – Luria- Bertani

G – gasoline

H – Hexane

MMG – Mata Mix gasoline

MMH – Mata Mix Hexane

MMT – Mata Mix Toluene

NaCl - Sodium chloridem

nm - Nanometer

O.D – Optical density

PAHs - polyaromatic hydrocarbons

T – Toluene

1. Introduction

Many of the organic and inorganic chemical pollutants are subject to the enzymatic action of several living organisms, which promote their biodegradation. The productive use of biodegradative processes to remove or detoxify pollutants present in the environment and which threaten public health, usually as contaminants in soil, water, or sediments, is called bioremediation. Environmental approaches that involve bioremediation are generally well accepted by public opinion and are increasingly emerging as viable strategies for an efficient, ecological and sustainable treatment of contaminated environments.

Environmental pollution caused by organic contaminants in soil and surface waters has a major impact on ecosystems, since these hydrophobic contaminants interact with soil particles, but due to their low water solubility and high interfacial tension, they cannot be easily removed. However, the presence of contaminants, exerts an environmental pressure that induces the alteration of the microbiome, selecting and promoting the growth of strains capable of metabolizing polluting agents. Many bacterial strains, found in polluted environments, have the ability to use hydrocarbons as the sole source of carbon and energy, being called hydrocarbonoclastic microorganisms. For this reason, bacteria have a high potential to be used in the recovery of contaminated environments and have been presented as the microorganisms with greater viability in the biodegradation of persistent polluting particles.

The study developed aimed to select microorganisms with potential for hydrocarbon bioremediation from soil samples collected in two contrasting environmentals, one where is already supposed to exist soil contaminated with hydrocarbons and the other collected from one environmentally protected natural zone. The observed colonies with potential for bioremediation were morphologically characterized, and the bioremediation rate was monitored throughout the natural attenuation process.

The presentation of the research study and main findings of this dissertation is organized in seven chapters. The first chapters highlight the context, the relevance of the topic and also the objectives set.

The chapter 2, entitled as a theoretical framework, contains two parts: part A where the literature review that supported the writing of the paper *Bioremediation of soils contaminated with hydrocarbons*: *one overview* is presented; and part B where the theoretical fundamentals of the methodologies used in laboratory work were developed.

Chapter 3 describes the planning and experimental procedures. In chapter 4 all the results obtained, and their due analysis and discussion, are mentioned and in chapter 5, the main findings and all conclusions drawn during the thesis are presented.

The “Future Perspectives”, shown in chapter 6, intend to indicate possible paths and approaches for future methodologies and strategies that can be applied for possible optimization of bioremediation processes.

2. Theoretical framework

2.1. Part A: Article – bibliographic review related to bioremediation

The bibliographic review was done to contribute to the dissemination of information available on this pertinent topic. The methodology applied to research of the bioremediation state of the art and the main theoretical foundations found are presented below.

2.1.1. Introduction

Soil pollution is an unbalance of soil environmental mainly caused by the presence of xenobiotics with anthropocentric origin. The sources of pollutants and contaminants are diverse, and despite all efforts and global awareness to reduce its use, the petroleum hydrocarbons are still between the most common chemicals involved in soil and water pollution. This alteration in soil ecosystem has very wide consequences since beyond the land degradation and water contamination, within and underlying the soil, the xenobiotics also enters the food chains, being bioaccumulated in different organisms.

Initially soil decontamination processes were based in physical and chemical treatments such as pump and treat, extraction of soil vapor and thermal desorption. However, there were a lot of concerns related to these strategies as they entailed a lot of costs, as well as doubts about their effectiveness, since, for example, pollutants were sometimes inconveniently transferred from the ground to the air and often lead to incomplete decomposition of pollutants (Sarkar *et al*., 2020).

Bioremediation emerged as a different approach, based on the study of soils as dynamic ecosystems that support an enormous population of microorganisms, such as bacteria and fungi, with the potential to degrade and metabolize the most varied chemicals. The bioremediation studies were boosted by the Exxon Valdex environmental catastrophe that happened in 1989, when there was a massive spill of about 11 million gallons of crude oil in

Gulf of Alaska (Pepper, *et al*., 2015). At this time, not only emerged a global awareness that the hydrocarbons damages persist for decades, but also that the approach to reduce their effects has to be holistic, including the removal, immobilization and use of the native microorganisms' community with hydrocarbonoclastic potential to metabolize it. Since that time the scientific community has deepened its investigations about the ability of organisms to use hydrocarbons, mainly the toxic ones such as persistent pollutants with low hydrophilicity, as a carbon and energy metabolic source (Sarkar *et al*., 2020).

Increasingly, the bioremediation approach, using living organisms or enzyme-mediated transformation is providing several opportunities to solve problems related to contamination of solid or liquid waste, since it´s well accepted, with low cost and low environmental impact (Sarkar, *et al.,* 2020; Smułek *et al*., 2020; Poeta *et al*., 2018; Rizi *et al*., 2017). Also, Osmakava *et al*., in their work published in 2020 about the high-impact of policymaking program to stimulate scientific progress based on bio-based exploration, highlighted the positive impact of bioremediation, and indicated it as a path to forward. However, despite all these advantages, bioremediation is still a time demanding process that depends not only of pollutants chemical and physical properties, but also on the degradative capacity of organisms and so it is also dependent of all the limiting factors that affect the growth and their survival (Sarkar *et al*., 2020). Addressing soil bioremediation in general and in the limiting factors that still reduce the performance and limit the application of this technology, this aims to contribute to summarize and highlight the most important soil decontamination topics, published over the last 10 years.

2.1.2. Methodology

A thorough literature search was conducted using the Online Knowledge Library: B-on and the Science Direct database (January 2021). Studies were identified combined together using the "AND" set operator using the following terms: "soil bioremediation, hydrocarbons, biodegradability, metagenome, bioremediation rate". The first cut-off used was current studies selecting references only from the last 10 years.

Despite B-on being a virtual library that guarantees access to several databases including the Science Direct database, allowing access to the text of a wide range of publications in full text, only 15 of all citations appeared in both platforms. From the 133 published citations found, 59 were excluded for not checking all the inclusion criteria, being 74 studies eligible for inclusion while appearing to be relevant to the study question (Figure 1).

All potentially relevant studies, 11 review articles, 50 research articles and 13 book chapters were obtained and evaluated in detail by authors.

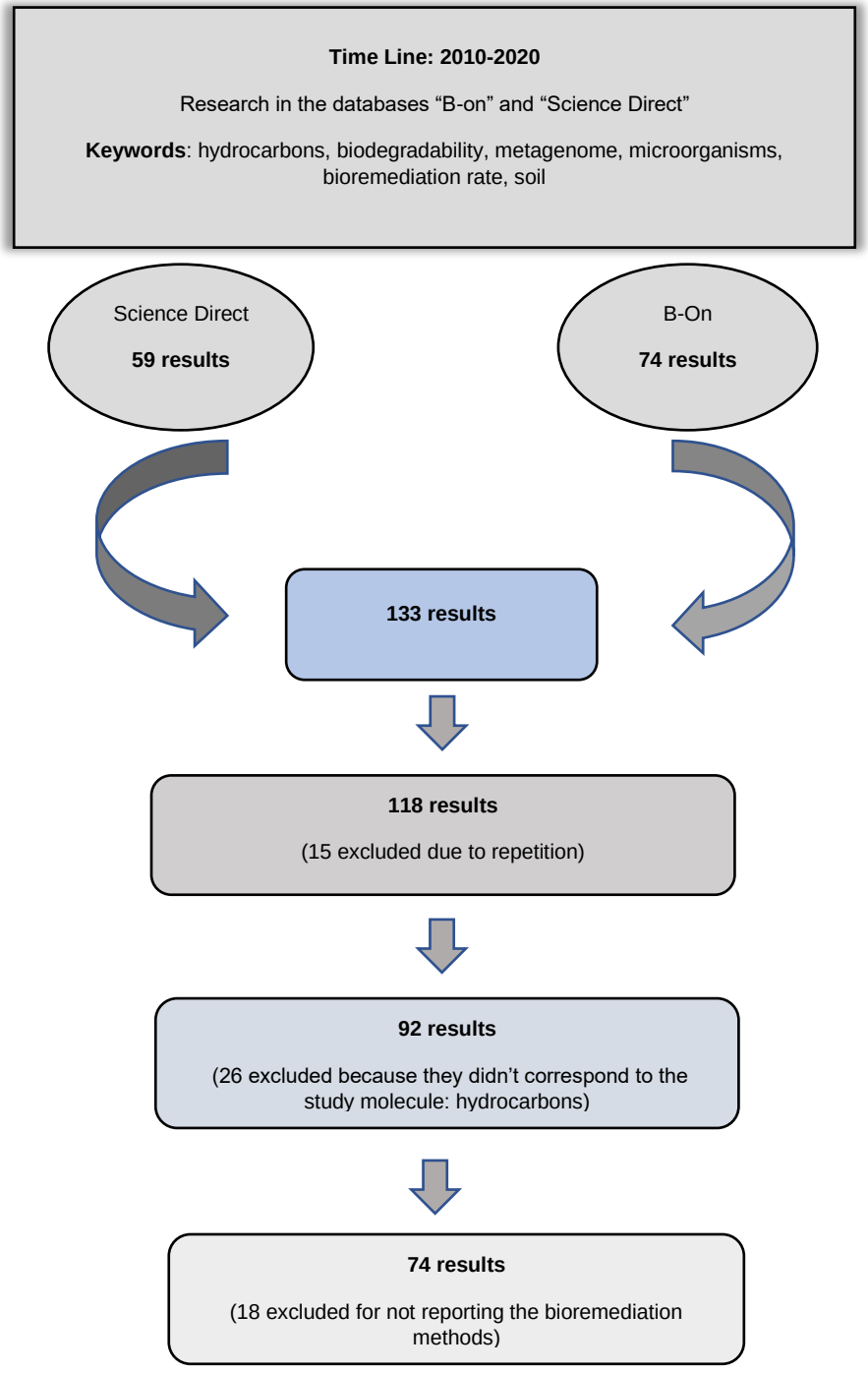

Figure 1 - Flowchart of criteria used to select the final 74 studies assigned in this study.

2.1.3. Main findings

The presentation and discussion of the main findings throughout this study are organized ranging from the more general concepts as hydrocarbon pollution, bioremediation methods and strategies and limiting factors to the assessment of the strengths, constraints and driving challenges of soil bioremediation programs.

2.1.3.1. Hydrocarbon Pollution

The environmental contamination with hydrocarbon is intrinsically related to the toxic organic substances present in petroleum and represents the most frequent world spread environmental contaminant. Crude oil and refined petroleum products are extensively used as raw material for different industries, daily activities and for energy production. The most noticeable sources of oil contamination are related with its extraction, processing in refining installations, dumping and accidents during its transportation.

The crude oil consists of a complex mixture containing more than 17,000 identifiable chemical components (Cafaro *et al.*, 2013; Napp *et al.*, 2018; Sarkar *et al.*, 2020; Jariyal *et al.*, 2020). The major components in crude oil, are the non-polar hydrocarbons such as aliphatic, alicyclic, and aromatic hydrocarbons. Their low polarity makes them persist in the environment for a long time and have a great ecological impact on the soil ecosystems. There is also a minor fraction of compounds (< 15% by mass) that contains heteroatoms such as nitrogen (N), sulphur (S), and oxygen (O) and which have greater water solubility and greater toxicity to aquatic ecosystems.

Despite the presence of hydrocarbons has always a negative environmental impact, its severity is related with the type and volume of pollutants, local geography and hydrogeological complexities, climate and season, species and biological communities, degradation activity (geochemical and microbial), local economic and amenity considerations, and the adequacy and timely application of clean-up methods. For this reason, the design, implementation, monitoring, and/or performance assessment of bioremediation technologies needs a holistic approach, a multidisciplinary team and is time demanding. The empowerment to provide an adequate and fruitful answer require an organization, systemization and share of the existing knowledge with the scientific community.

2.1.3.2. Bioremediation types

There is a multiplicity of organisms with bioremediation potential, which are able to metabolize and not suffer harmful effects due to the presence of the contaminating agent in their habitat.

These organisms or their enzymes, define the types of bioremediation and the technological approach needed to implement the process. Most of these organisms are extremophiles and in adverse situations take advantage of environmental pressure. When the appropriate environmental conditions are met, their growth can be limited only by the source of nutrients, so despite their number significantly increase in areas of oil spill, it also decreases when the pollutant disappears, being a self-regulated growth. Various organisms have been isolated from heavily polluted coastal areas, variety of oil spill or soil contaminated by petroleum. The kingdom to which living organisms involved in the process belong. define the type of bioremediation.

2.1.3.3. Bacterial bioremediation

The use of the hydrocarbonoclastic bacteria is the most common choice for the petroleum hydrocarbons degradation, either in aquatic or terrestrial environments recovery. Despite all the biotechnological potential, these bacteria are widely distributed in nature, and significantly produce biosurfactants with emulsification properties that are extremely useful to increase the solubility in water of nonpolar compounds, allowing their use as food and energy source. In 2013, Mahjoubi *et al*., isolated 125 strains, adapted to grow on minimal medium supplemented with crude oil. The diversity of the bacterial collection was analysed by 16S rRNA sequencing and by identifying statistically significant differences amongst the metagenomes of the microbial communities found in contaminated environments (Ali *et al.*, 2020; Acosta & Marques, 2016; Gutiérrez *et al.*, 2020; Kayanadath, 2019; Li *et al.*, 2018).

Zhang, C., *et al.* (2020) presented an interesting study where agricultural wastes, such as wheat bran and swine wastewater, were used as a source of nutrients and consequently boost the growth of microorganisms with hydrocarbonoclastic potential to remediate oil-contaminated soil.

Mycoremediation

In this case, bioremediation is carried out by fungi that degrade substrates into smaller molecules by extracellular fungal enzymes. These molecules are subsequently absorbed and metabolized in the cells. There are several advantages in mycorremediation, namely its high growth rate, its ability to penetrate the pollutant and survival relatively under conditions of stress, pH and nutrients. In addition, the nutritional stress condition stimulates the fungal enzyme machinery, offering significant benefits (Agrawal *et al.*, 2020).

Mixed cell culture system

Several studies show that for the biodegradation of petroleum hydrocarbons, a microbial consortium with a succession of species is usually necessary and that the use of mixed bacterial culture is more advantageous compared to pure cultures due to synergistic interactions between microbial species. The use mixed cultures (bacteria, fungi, algae and plants) it is also referred as advantageous since its significantly increase the capacity to degrade both aliphatic and aromatic hydrocarbons (Acosta-González and Marqués, 2016; Auffret *et al*., 2014; Brakstad *et al*., 2018; Bacosa and Liu, 2017; Fasca *et al*., 2017; Imam *et al.,* 2019; Sarkar *et al.*, 2020; Shankar *et al*., 2014; Koo *et al*, 2014).

Current environmental remediation techniques rely on consortia of bacteria, microalgae, and fungi. Consortia are beneficial because by acting on several species at the same time the degradation of pollutants occurs in a faster and more efficient way.

In general, microbial consortia present adaptive strategies to survive extreme environments. Consortia between kingdoms maintain stability and strengthen defenses against external tensions through changes in physical and spatial structure, but with more variety, complexity, and possibilities for interactions. These can physically interact through aggregation, surface charge or immobilization (Zhang *et al.*, 2018).

Phytoremediation

Phytoremediation defines the use of plants and their associated microorganisms to assimilate, transform, metabolize, detoxify, and degrade pollutants. Remediation of soil or water is carried out by this process based on vegetation through a variety of mechanisms, such as phytoextraction, phytovolatilization, rhizodegradation rhizofiltration, phytostabilization and phytodegradation (Agrawal *et al*., 2020; Wang *et al*., 2020; Gkorezis *et al.*, 2016). Several benefits of phytoremediation have been established mainly due to the fact that we are introducing producers that can also improve the aesthetics of brownfields or other contaminated sites. But this strategy also has several limitations, such as more demanding environmental conditions, such as temperature, nutrient availability, the choice of the species, the introduction of new species that can persist in the environment and the time required to achieve the remedial goals (Ramírez-García *et al.* 2018; Santos *et al.* 2011).

Zooremediation

As the name implies, this remediation depends on the animals' activity to remove environmental pollutants. Various animals such as arthropods, water filters and earthworms

can be used for soil remediation purposes. The use of earthworms, also called vermiremediation, depends on the worm's ability to successfully survive in the presence of a wide variety of organic and inorganic pollutants. Earthworms have the potential to remedy pollutants through various processes, such as biotransformation, biodegradation, or bioaccumulation, however their use must be restricted to controlled areas, to prevent the dispersion of the contaminants into food webs. In general, the use of zooremediation is limited due to the ethical, human, and ecological safety concerns associated with the use of animals. The only publication found reports the study carried out by Chacina *et al.* 2016 using earthworms Eisenia fetida, along with other microorganisms all components of biopreparation but with controversial results, while it was possible to obtain a 99% reduction in hydrocarbons in just 22 weeks, it was also found that 30% of the earthworm species died after 14 days because of the toxic impact of the diesel fuel.

2.1.3.4. Genetically engineered microorganisms

While advances in molecular biology have had a profound effect on the understanding of biological remedial processes and are used extensively in the research community, their use in the operational clean-up community is limited at present. There is, however, tremendous potential for use of these technologies to improve the design, implementation, field performance, and monitoring of remediation technologies.

One important phase of bioremediation processes is the identification of the microbial community present in contaminated environments, using molecular biology tools to evaluate the 16S rRNA genes. These molecular techniques are extremely important since they allow to obtain enriched microbial pools capable of efficiently degrading pollutants in the environment (Birell *et al*., 2019; Imam *et al*., 2019).

Much of contemporary synthetic biology research relies on the use of bacterial chassis for plugging-in and plugging-out genetic circuits and new-to-nature functionalities (Nikel *et al*., 2018).

Several types of organic pollutants can be degraded by the action of microbial enzymes. Biodegradation reactions that are mediated by those enzymes are responsible for activating certain energy-generating metabolic pathways. These species have the ability to use molecular oxygen (aerobic microorganisms) or nitrate (anaerobic microorganisms) as the final electron acceptor in the electron transport chain (Agrawal *et al*. 2020; Eibes *et al*., 2015). In addition, some microorganisms excrete enzymes that are functional only in the extracellular environment. Some of the genes that encode these crucial biodegradative enzymes are

present in plasmids and can be used to transform other bacteria and developed novel strains able to produce and secrete enzymes with high specificity for a specific pollutant. The genetically engineered or modified organisms are a great value in whole process, since in most common circumstances in an oil polluted site indigenous microorganisms are unable or do not act their full capability to degrade the pollutants.

2.1.3.5. Bioremediation methods

Bioremediation methods can be broadly classified as *ex situ* and *in situ* (Figure 2). This terminology is associated with the local of the treatments, ex situ involves the physical removal of the contaminated matrix for treatment process. In contrast, in situ techniques involve treatment of the contaminated material in place with the least possible disturbance allowing the ecosystem to revert to its original conditions. Both have their advantages and disadvantages based on the nature and extent of the contamination. *In situ* treatments are the most cost efficient and they are recommended whenever the excavation of contaminated material is dangerous. *Ex situ* methods have a relatively high operating costs and space requirements, but they allow a better control of environmental conditions, a more easily monitorization, higher effectiveness and reliability (Acosta-González and Marqués, 2016; Eibes *et al*., 2015; Sarkar *et al*., 2020).

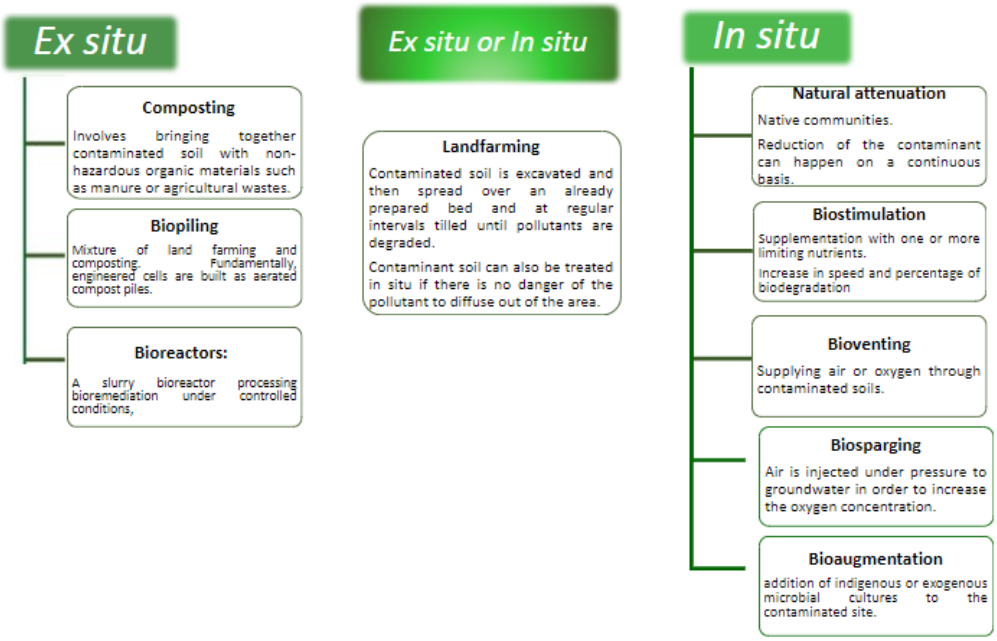

Figure 2 - Diagram indicating the methods associated with *in situ* and *ex situ* strategies

In situ bioremediation practices

Natural mitigation consists of using natural processes caused by native communities to degrade contaminants and reduce their concentrations to acceptable levels without the input of external bioremediation enhancers (e.g., electron donors, electron acceptors, other microorganisms or nutrients). One of the main advantages of this approach is that there is no need to add nutrients to the soil or the adequacy of any environmental condition, that the reduction of the contaminant can happen on a continuous basis. This is due to the natural adaptation of native microorganisms in the contaminated soil. In this case, these microorganisms start to use polluting organic compounds in their metabolism as a carbon source. However, the use of this technology as the main option in bioremediation requires some caveats, since the conditions of the environment, including the type of concentration of contaminants, may not contribute to the reduction of contaminating substances and consequently increase the risks of contamination of people and animals. As the natural attenuation processes usually take months and years, the results obtained from biodegradation are very slow and even unpredictable, so, consequently, it was necessary to perform the monitored natural attenuation also called biostimulation (Andrade, *et al.* 2011; Gkorezis *et al.*, 2016).

Biostimulation or accelerated natural attenuation is a bioremediation strategy that aims to achieve a balance of nutrients for the growth and functioning of the native community. Supplementation with one or more limiting nutrients, such as nitrogen, phosphorus, and sulphur, in hydrocarbon-laden locations is important, as it helps in accelerating microbial growth by increasing the hydrocarbon degradation capacity. Biostimulants can be water-soluble inorganic nutrients, oleophilic fertilizers, as well as oxygenation. With the addition of nutrients and the optimization of conditions, they allow to increase the growth rate and the metabolic activities of microorganisms, with the consequent increase in speed and percentage of biodegradation. In this context, biostimulation may have lower costs compared to other bioremediation techniques (Andrade *et al.*, 2011; Gupta *et al.*, 2016; Lo *et al.*, 2020; Sarkar *et al.*, 2020).

When it is necessary to provide air or oxygen to existing soil microorganisms in order to optimize the aerobic biodegradation of contaminants, the process is called Bioventing if it is applied in the unsaturated zone or biosparging, if the air or oxygen injected are not confined to the unsaturated zone, but rather extends remediation to the saturated zone of the soil matrix. In both process nutrients, if needed, can also be injected.

Bioincrease or bioaugmentation aims to add specialized microorganisms to the environment to help remove pollutants. Bioaugmentation agents are characterized by their ability not only to degrade the contaminant of interest, but also to be resilient to the prevailing environmental conditions, as well as their genetic stability and ability to survive in competition with the native community.

Landfarming is an engineered bioremediation system particularly useful for remote sites due to minimal equipment requirement, which generally uses passive aeration by tilling or ploughing the contaminated soil in order to reduce contaminant levels. The equipment employed and the procedures are similar to those used in agriculture, hence its designation in general terms can be understood as a technique where the microorganisms are cultivated to be able to degrade the hazardous compounds. More associated with ex situ treatments where after excavation, the soil is deposited on a large flat impermeable surface to increase interaction between polluted soil and atmosphere in order to improve the aerobic microbial activity in the soil. This technology can also be applied in situ as long as the contaminant cannot migrate to aquifers or other areas.

***Ex situ* bioremediation practices**

In addition to landfarming there are others *ex situ* bioremediation technologies such as composting, biopile and bioreactors, that have been successfully used in the treatment of hydrocarbon contaminated soils.

The simplest and most usual of the *ex situ* bioremediation techniques in treating of hydrocarbon contaminated soil is composting where the organic compounds are degraded by microorganisms into innocuous, in innocuous byproducts.

The biopile, also called biocells, bioheaps, biomounds or static-pile composting, have been proved to be an efficient way to remediate soils contaminated with petrochemical contaminants (Germaine et al., 2014). In biopile technique is a combination between landfarming and composting that provides a favourable environment for indigenous aerobic and anaerobic microorganisms and also controls physical losses of the contaminants by leaching and volatilization (Kumar *et al.,* 2011; Mani & Kumar 2014).

2.1.3.6. Limiting Factors

Throughout all the bibliography studied it is unanimous that the success of bioremediation using the native microorganism’s community is very limited by the unbalanced levels of

nutrients and / or physicochemical conditions prevalent in the contaminated sites. The growth of the microorganisms for bioremediation is often very specific and demanding. To optimize bioremediation rates, it is necessary, whenever possible, to mitigate all factors that may be or become limiting of the process. For this reason, it is necessary to know the biotic factors associated with the microorganism specific needs and the abiotic factor that can be intrinsic to the pollutants, such as, diffusion, solubility, partitioning and volatility, and largely affect their bioavailability and as such their metabolization. And also, the environmental factors - nutrients, salinity, pressure, temperature, pH, water availability, osmotic stress and the presence of contaminants.

The possible unbalance of essential nutrients is considered the critical factor in reducing the rate of metabolism of organisms, therefore, the natural bioremediation. With an ideal ratio of 100: 10: 1 (carbon: nitrogen: phosphorus) for aerobic microorganisms, an excess of carbon in the form of hydrocarbons can cause a shortage of nitrogen and phosphorus. Oxygen is generally used as a final electron acceptor in microbial metabolism, and both, oxygen, and molecular oxygen, are essential in the main hydrocarbon degradation pathways of aerobic bacteria. In anoxic bacteria the most common electron acceptors in the natural environment are nitrates, manganese, iron and sulphate (Santos et al., 2010; Daghio *et al.*, 2017; Heyob *et al.*, 2017).

The biodegradation that involves these degrading microorganisms has an optimal pH range of 6.5 to 8.5, since in this range of pH values most enzymes from non-extremophile microorganisms have the three-dimensional conformation of their active site, with greater affinity for the substrate, leading to higher reaction rates and therefore higher rates of bioremediation (Joriyal *et al*., 2020; Pepper *et al*., 2015).

Temperature is one of the factors that most controls the degradation of hydrocarbons, since it influences not only the enzymatic activity and all metabolism, but also the solubility of the gas and the physical and chemical properties of the hydrocarbon components. At low temperature values the viscosity of the oil increases and the volatilization of the lighter fractions decreases, at higher temperatures it increases the solubility of the hydrocarbons and their diffusion rate, as well as decreasing the viscosity. Investigations are being carried out to study the enzymes of microorganisms that live in cold environments, since recently, the genes that code for the cytochrome P450 alkane hydroxylases have been identified in the genomes of psychrophilic organisms. These enzymes have the ability to convert n-alkanes to simpler and harmless

alcohols. The bioremediation of n-alkanes by cold-adapted organisms occurs at a temperature of 5 ºC, thus making it possible for these enzymes to be able to clean up spills of contaminants at low temperatures, such as in the Arctic (Brakstad *et al*., 2018; Brown *et al*., 2020; Fasca *et al.*, 2017; Lofthus *et al.*, 2018; Siddiqui, 2015).

Another factor that is also extremely important and that influences hydrocarbon degradation is the presence of surfactants that aim to increase the bioavailability of hydrophobic pollutants. Several microorganisms are able to produce a wide variety of secondary metabolites, some with amphiphilic polar character and lower interfacial tension that allow the solubilization of hydrophobic substances in water. These biosurfactants play a vital role in increasing the rate of degradation, facilitating solubilization of nonpolar compounds. Generally, most hydrocarbon-degrading microorganisms perform in situ synthesis of a wide range of surfactants, which are called biosurfactants. These biosurfactants have more advantages than chemical surfactants because they are much less toxic, more biodegradable, selectivity and specificity under extreme conditions. (Ibrar and Zhang, 2020; Imam *et al.*, 2019; Smułek *et al.*, 2020).

All the information presented reinforce that to apply a successful bioremediation program a multidisciplinary approach is needed, usually led by professionals with knowledge of biology and technology, supported by hydrogeologists, soil scientists and engineers. deepening this knowledge is important to adequately respond to pressure of the world's environmental pollutant crisis.

2.2. Part B: Theoretical foundations of methods used in determining the bioremediation rate

2.2.1. Soil Sampling

The sampling plan is extremely important in analysing contaminated soils. When the collection is carried out, there must be an adequate judgment about the sample, if it is representative and so can cover the present population diversity. The soil samples are extremely fragile as they isolate the microorganisms from the rest of the matrix that is present under survivable conditions. Thus, it is necessary to follow a set of procedures to correctly perform the sample collection and obtain reproducible procedures.

Soil microbiota is considered very important as it is a determining factor in the dynamics of the soil as an ecosystem, and deeply affects the biotransformation of selected analytes. Furthermore, cross-contamination of microorganisms from other sources must be avoided, so

the use of sterilized and clean sampling devices (spoons, spatulas, shovels, etc.) is essential during the collection of soil samples (Imam, *et al.*, 2019; Carter and Gregorich, 2008).

Sampling from deeper soils, e.g., with low oxygen or anaerobic conditions, are highly susceptible to being harmed, so nitrogen sampling containers to avoid oxygen contamination is recommended (Imam, *et al.*, 2019; Carter and Gregorich, 2008).

For the collection of raw samples, the sampling conditions must be properly decided to ensure that a good analysis will be carried out later. Glass flasks with a lid are recommended for this type of harvest. The headspace should be avoided or filled with sample to minimize any volatile losses. Plastic bags have also been reported for the purpose of sampling soil/sediment samples in some studies (Imam, *et al.*, 2019; Carter and Gregorich, 2008).

Storage of samples should be at 4°C immediately after sampling (Imam, *et al.*2019).

2.2.2. Microbial Growth

Cell growth is defined as the increase in all cellular constituents. This increase may be associated with an increase in the size of a cell or number of cells because of cell multiplication.

Most microorganisms multiply using asexual division by binary fission or budding (Nicolau, 2014; Ribeiro, 2000).

Thence, during a cell division cycle corresponds to the generation or doubling time, all measurable cellular components double, following the number of cells and the amount of biomass present. This only happens if they have the proper nutritional and environmental conditions

2.2.2.1. Microbial growth curve

Microbial growth is often monitored based on the curve that represents the growth of a population of a given microorganism, in a continuous system or in batch (batch culture). When the growth of a cell population is monitored over time and the calculation of the logarithmic of the number of cells per unit volume or the measurements of culture optical density (OD), concentration of microbial biomass or logarithm of the number of bacterial cells as a function of time can be graphically represented. obtaining aa characteristic growth curve, as shown in figure 3 (Bruslind, 2021; Najafpour, 2007; Ribeiro 2000).

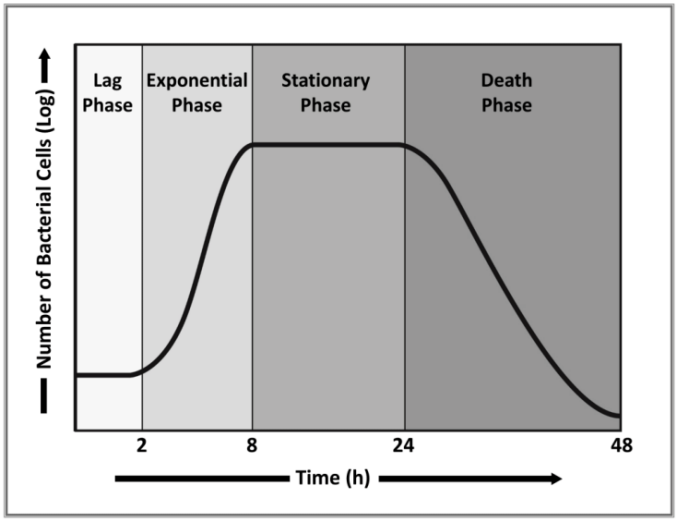

Figure 3 - Different stages present in a typical growth curve

(Garrison and Huigens, 2017)

In the latency phase (Lag Phase), after inoculation of the culture medium, the microorganism cells are in an adaptation period to the new medium conditions. This phase can have a longer or shorter duration depending on the physiological state of the culture used as inoculum and the growth conditions.

In the exponential phase, after a period of acceleration, the specific growth rate of the microbial population becomes constant, that is, the cells undergo division and their number doubles after a certain period. During this phase, when all nutrients are available in excess, the microorganisms divide and the population grows at a specific maximum growth rate that depends on the microorganism's genetic potential, the composition of the culture medium and the growth conditions.

During the deceleration phase, there is a decline in the maximum specific rate of growth, due to a decrease in the concentration of one or more nutrients essential to cellular metabolism and/or an increase in the concentration of products of metabolism that are toxic to the cells (Bruslind, 2021; Najafpour, 2007).

In the stationary phase, the depletion of an essential nutrient and/or accumulation of metabolism-inhibiting products cause population division to stop. However, even with a lack of nutrients, cells can remain viable for some time due to endogenous reserves, which they use in maintenance processes. However, sooner or later, there is a decline in the concentration of viable cells during the cell death phase.

During this phase of decline or death, there is an irreversible loss of cell division capacity, thus resulting in death. This leads to a decrease in the concentration of viable cells in the microbial population over time (Bruslind, 2021; Najafpour, 2007).

2.2.2.2. Quantitative analysis of microbial growth

The determination of the concentration of a cellular component over time, cell concentration or biomass allows experimentally obtaining the growth curve of the microbial population and calculating the specific growth rate (equation 1) and the generation or duplication time (equation 2) of the microorganism in question (Looser *et al.*, 2015; Pepper, *et al.* 2015).

Equation 1 - Specific growth rate (μ)

$$\mu = \frac{dN}{dt}$$

Where:

- N is the number of cells at time t.

Equation 2 - Duplication time

$$Duplication\ time = \frac{ln\ (2)}{Growth\ rate}$$

Equation 3 shows how to calculate the growth rate.

Equation 3 - Growth rate

$$Growth\ rate = \frac{\ln(\frac{N(t)}{N(0)})}{t}$$

Where:

- *N(t)* is the number of cells at time *t;*
- *N(0)* is the number of the cells at time *0;*
- *gr* is the growth rate;
- *t* is the time (usually in hours).

To draw the microbial growth curve, it is then necessary to make a quantitative assessment of the evolution of cell number over time. This can be based on direct methods such as:

- Total cell count.
- Viable cell Count - Colony Forming Units (CFU's)

Or in indirect methods such as the spectrophotometric analysis of the Optical Density (O.D) of the crop (Pepper *et al.,* 2015; Pommerville, 2018).

The total cell count is based on filling with a suspension of cells to be counted, in a cavity located between a slide and a coverslip. This cavity, located in the center of the sheet, is divided into squares of known and well-defined dimensions and with a known volume. For this method, the microscope is used, thus being able to count the number of cells present in each square, that is, the number of cells per unit of volume is known. The slide used for this type of counting is often called a hemocytometer (Badievau *et al.* 2018; Pepper *et al.*, 2015; Pommerville, 2018).

The usual way to carry out a viable cell count is to determine the number of cells capable of forming colonies in a solid culture medium with a composition suitable for the growth in question. Each colony is assumed to originate from a single viable cell, therefore, the results obtained are usually expressed in Colony Forming Units (CFU's) and the method is commonly called counting CFU's (Looser *et al.* 2015; Pepper *et al*., 2015; Pommerville, 2018).

In the spectrophotometric analysis of the Optical Density of a microbial culture, the assessment of the turbidity of a microbial culture is a method quick and indirect, of estimating cell concentration. A beam of light focused on a microbial suspension is partially deflected (light scattering) by the cells and the percentage of unshifted light (transmittance, T) is measured. The amount of light that passes through the cell suspension depends on the cells concentration in the suspension and their size, the wavelength and intensity (I_0) of the incident light, and the diameter of the tube containing the cell suspension. The optical density of the suspension corresponds to the absorbance, which is determined by the following equation:

Equation 4 - Optical density

$$O.D.= \log \left(\frac{I_0}{I}\right) \text{ or } O.D.=\log \left(\frac{I}{I_0}\right)$$

Where I_o is the intensity of the incident light, and I is the intensity of the light transmitted through the cell suspension

Wavelengths frequently used for measuring the O.D of bacterial or yeast cell suspensions are 540, 600 or 640 nm (De Caro and Haller 2018; Pommerville, 2018: Ribeiro, 2000).

2.2.3. Colony Morphology in Microbiology

Describing the appearance of colonies has been an important tool in microbiology to describe different material strains there are different criteria to characterize the appearance of these colonies. As morphology is influenced by the type of medium and growing conditions, great care must be taken in describing the parameters. A good determination of morphology depends on a correct inoculation and sample concentration as it is necessary that the colonies are well separated (Badieyan *et al.,* 2018; R. Caprette, 2016).

Some morphological characteristics are represented in Figures 4 to 8.

- Size

Figure 4 - Classification of the size of colonies.

(Badieyan et al., 2018)

- Shape

Figure 5 - Classification of the shape of colonies according with the boundary morphology.

(Badieyan *et al.,* 2018)

- Elevation

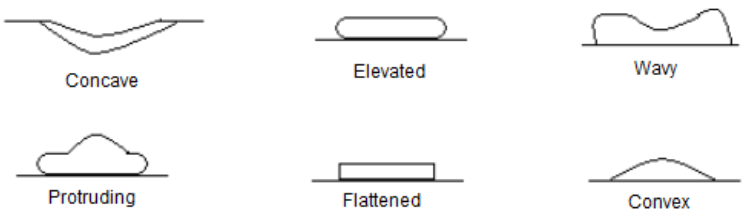

Figure 6 - Classification of the colonies according with their elevations in solid media.

(Badieyan *et al.,* 2018)

- Margin

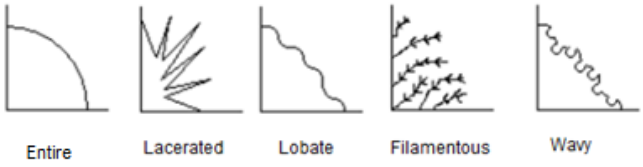

Figure 7 - Classification of colonies according with their margin.

(Badieyan *et al*., 2018)

- Structure

Figure 8 - Classification of the structure of colonies.

(Badieyan *et al.,* 2018)

- Glow: transparent, translucent, opaque;
- Color: colorless, pigmented.

2.2.4. Gram stain

Gram stain was developed by Christian Gram in 1884 and modified by Hucker in 1921, which aims to differentiate bacteria into Gram-positive and Gram-negative bacteria, which helps in the classification and differentiation of microorganisms. The main difference between these two types of bacteria is the composition of the cell wall, where the Gram-positives have a thick layer of peptidoglycan, and the Gram-negative ones, a thin layer of peptidoglycan, over which there is a layer composed of lipoproteins, phospholipids, proteins, and lipopolysaccharide (Qi*et al*, 2019; Ribeiro, 2000).

Due to this difference, when the reagents used for staining are applied, there are different reactions. Upon addition of the primary dye, crystal violet, Gram-positive bacteria retain it after application of the mordant iodine forming a complex within the peptidoglycan, so when the bleach is applied to the cells, the crystal violet-iodine complex remains within the cell, causing the color to remain purple (Qi *et al*, 2019; Ribeiro, 2000).

In the case of gram-negative bacteria, as mentioned above, they do not contain a thick layer of peptidoglycan, which is freely distributed between the inner cell and the outer membranes cells. The application of acid-alcohol bleach dehydrates the outer membrane, leaving holes in the membrane causing the bleach to wash and remove the complex present in the cells, leaving them colorless. For these Gram-Negative bacteria to be visible it is necessary to add a secondary color, safranin, to turn them pink. Figure 9 schematically shows the Gram stain process (Qi *et al*, 2019; Ribeiro, 2000).

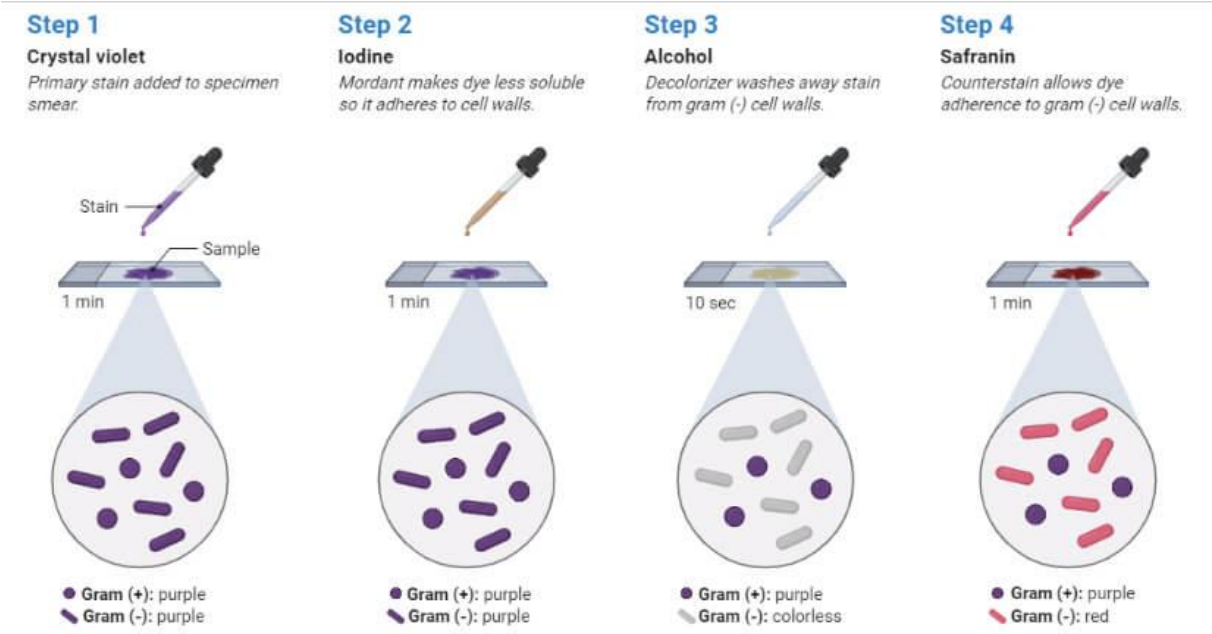

Figure 9 - Diagram of the Gram stain main steps.

(Qi, *et al*, 2019)

2.2.5. Identification for *Pseudomonas aeruginosa*

Microorganisms of the genus *Pseudomonas* are Gram-negative *bacilli*, mobile by means of a polar flagellum, with oxidative metabolism. They have aerobic respiration, with oxygen being the only final electron receptor, and in their absence, they can use nitrates as the final electron acceptor, but never adopting a fermentative metabolic pathway. These do not develop under acidic conditions (pH <4.5) (Soares, 2018).

The selective medium Pseudomonas CN Agar Base is used to identify *Pseudomonas aeruginosa*, based on the detection of pyocyanine production. The peptone and casein present provide nitrogen, vitamins, minerals, and amino acids essential for growth. Cetrimide is a quaternary ammonium compound that inhibits most of the growth of microorganisms, allowing the growth of *Pseudomonas aeruginosa* with production of pyocyanine. The addition of

nalidixic acid to cetrimide allows a greater recovery of *Pseudomonas aeruginosa,* with the synthesis of pyocyanine being more easily visualized, while the contaminating microbiota is strongly inhibited. Magnesium chloride and potassium sulfate favor the production of this pigment. Glycerol is used as a carbon and energy source. Finally, agar is used as a solidifying agent.

There are strains of *Pseudomonas aeruginosa* that grow in this selective medium, but do not have the capacity to produce pyocyanin, however they fluoresce or acquire a reddish-brown color, due to the production of other pigments (worstrubrine and pyomelanin). Therefore, the analysis of these microorganisms under the action of UV light (wavelength 360 ± 20nm) is important to verify the fluorescence emission (Soares, 2018; Ribeiro 2000).

2.2.6. Biodegradability test using 2,6-dichlorophenol indophenol (DCPIP)

Biodegradation tests are based on the use of the redox indicator 2,6-dichlorophenolindophenol (DCPIP). The functional principle consists in detecting the oxidation of the carbon source supplied to the microorganisms, which during the metabolization process oxidize the hydrocarbon and the electrons that are released are transferred to acceptors. When adding the redox indicator DCPIP to the Bushnell-Hass (BH) culture medium, it will act as an electron acceptor capable of confirming the degrading activity of microorganisms on the carbon source. The test is considered positive when the DCPIP indicator changes color from blue, oxidized state, to colorless, reduced state (Chikere, *et al.*, 2019; Sanches, 2009).

To analyze the reduced percentage of hydrocarbons, absorbance analysis in the spectrophotometer is used (Chikere, *et al.*, 2019; Sanches, 2009).

2,6-Dichlorophenolindophenol (DCPIP, DCIP or DPIP), which is part of the Hill reagent family, is a chemical compound used as a redox indicator. When oxidized it is blue with a maximum adsorption at 600 nm, when reduced it is colorless. It acts as an electron acceptor, receiving them from the oxidation process, mediated by microorganisms that use hydrocarbon as substrate (Chikere *et al*., 2019; Sanches, 2009).

2.2.7. Spectrophotometry

Spectrophotometry is any process that uses light to measure the chemical concentrations of a sample. It is based on the analysis of electromagnetic radiation emitted or absorbed by substances (Burgess, 2007 De Caro and Haller, 2015).

One of the most used types of spectrophotometry is ultraviolet – visible (UV-Vis), which is based on the adsorption of light by an substance or sample, but in this case, the sample is illuminated with electromagnetic rays of various wavelengths in the visible and adjacent bands, i.e., ultraviolet and part of the lower infrared region of the spectrum. Depending on the substance, light is partially absorbed. Remaining light, i.e., transmitted light, is registered as a function of wavelength by a suitable detector, providing the ultraviolet/visible spectrum of the sample. As each substance absorbs light in a different way, there is a unique and specific relationship between the substance and its spectrum (De Caro and Haller, 2015; Burgess, 2007).

The UV/Vis spectrophotometer measures the intensity of light passing through a sample solution in a cuvette and compares it to the intensity of light before passing through the sample. The main components of a UV/Vis spectrophotometer are a light source, a sample holder, a dispersive device to separate the different wavelengths of light (monochromator) and a suitable detector (Figure 10) (De Caro and Haller, 2015; Burgess, 2007).

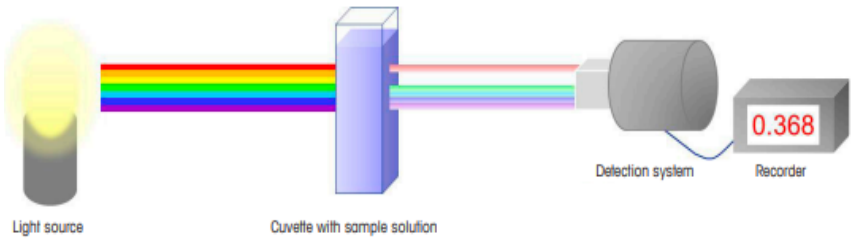

Figure 10 - Diagram of the spectrophotometer components

(De Caro et al., 2015)

The intensity of light, when passing through a clear cuvette with a sample, is attenuated proportionally to the concentration of the sample, e.g. a more concentrated sample solution will absorb more light. Furthermore, the attenuation is also proportional to the length of the cuvette, that is, the longer it is, the greater the absorption of light Both factors can be summarized by expressing absorbance (A) as a function of concentration and cuvette length. In particular, the absorbance is equal to the product of the extinction coefficient (ε), the concentration (c) and the cuvette length (d) (Equation 5) (De Caro *et al*., 2015):

Equation 5 - Lambert-Beer Law

$$A = \varepsilon \times c \times d$$

This relationship is called the Lambert-Beer Law, where:

- Sample concentration (c) is given in mol/L or g/ml.
- Cuvette length (d) is given in cm.
- Extinction coefficient (ε) is a specific constant that describes how much the substances absorbing a given wavelength (L/(cm × mol) or ml/(cm × g)).

3. Methodology

This work aims to isolate and characterize microorganisms that degrade hydrocarbons. For this, soil samples from Mata da Machada and Praia da Alburrica were used to obtain the organisms and subsequently the study of biodegradability. Figure 11 shows the flowchart of the experimental steps.

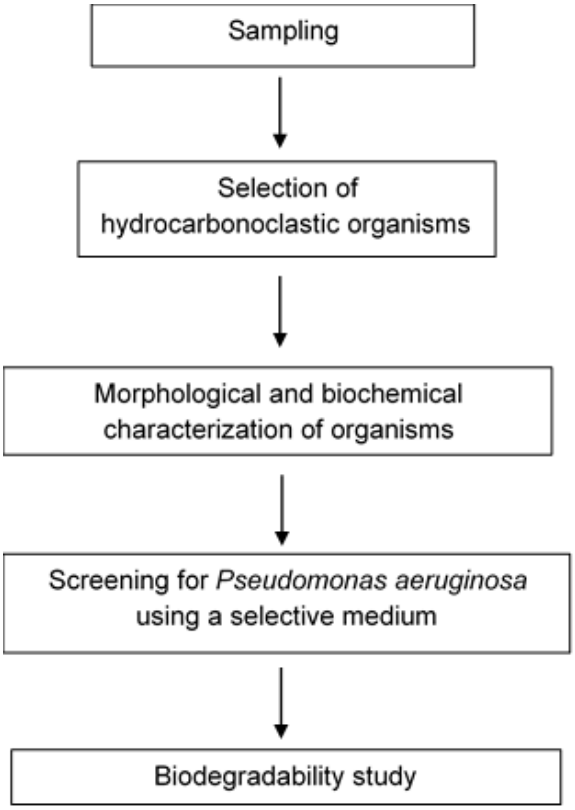

Figure 11 - Flowchart with the experimental steps.

3.1. Sampling

The soil samples were collected in Mata da Machada and Praia da Alburrica with the following coordinates respectively: 38.606133, -9.025713; 38.656527, -9.087775. Figure 12 shows the collection areas through location maps.

Figure 12 - Collection areas through location maps: a) - Mata da Machada; b) - Praia da Alburrica (Google Maps)

Figure 13 shows the collection area of Mata da Machada.

Figure 13 - Collection site of Mata da Machada.

Figure 14 shows the collection area of Praia da Alburrica.

Figure 14 - Collection site of Praia da Alburrica.

For sampling, it is used sterilized materials, gloves, and plastic bag. After the samples are stored at 4ºC.

3.2. Selection of hydrocarbonoclastic organisms

3.2.1. Growth of hydrocarbonoclastic organisms

In carrying out this activity, 10 g of the sample are weighed and added to 100 mL of a sterile saline solution (0.9% NaCl w / v), shaking vigorously for 15 minutes. The mixture is left to stand for the suspension to land in the glass.

3.2.1.1. Growth of hydrocabonoblastic organisms in liquid medium

First, growth is performed on Luria-Bertani (LB) medium with the addition of 2 % of sample to the final volume of medium for 24 hours at a temperature of 30 ºC at 150 rpm.

After growth in LB, growth is then carried out in BH medium (annex 1) plus hydrocarbons as a carbon source. Two different amounts of hydrocarbon, 100 µL and 600 µL, were used to study the growth of microorganisms and the biodegradability rate.

From LB medium growth it was removed 1.2 mL to inoculate 60 mL BH medium. The growth was performed in erlenmeyers 100 mL of capacity in the presence of different hydrocarbons. These are incubated at a temperature of 30 ºC at 150 rpm until growth stabilization through

the verification and reading of the optical densities (O.D.). The blank of this assays were BH medium without inoculation.

3.2.1.2. Growth of hydrocabonoblastic organisms in solid medium - Dilution method

Initially, three sterile test tubes are prepared with 9.9 ml of 0.9% NaCl. To the first test tube is added 0.1 ml of the microbial suspension of the growths carried out in point 3.2.1.1. Vigorous agitation is performed in the vortex for a few seconds. After stirring, 0.1 ml of this dilution is pipetted into a plate with nutrient agar. From the dilution performed, 0.1 ml is removed into the new tube with 9.9 ml of 0.9 % NaCl, where again the agitation and addition in the nutrient agar medium is carried out. This process is repeated once more. The dilutions performed are 1: 100, 1: 10000 and 1: 1000000. Petri dishes are incubated at 30 ºC for 48 hours. Figure 15 shows the process to be performed schematically.

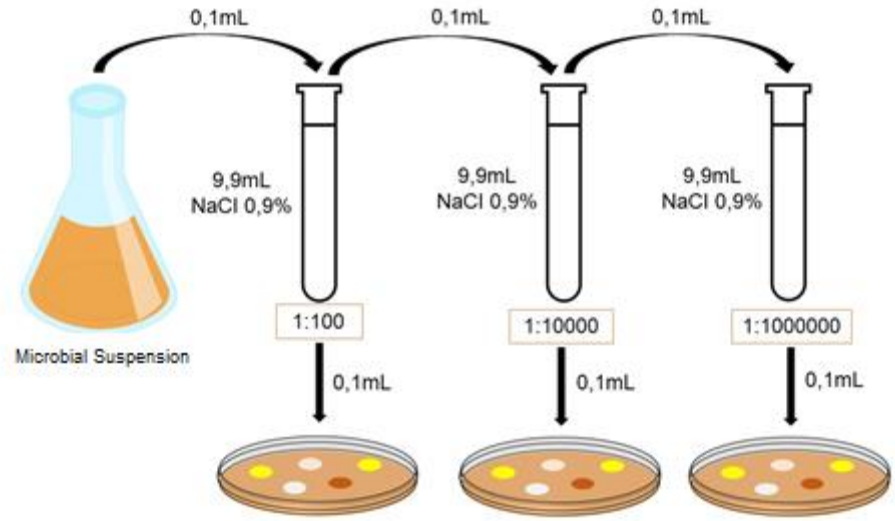

Figure 15 – Serial Dilution method.

3.3. Morphological and biochemical characterization of organisms

3.3.1. Morphological characterization of organisms

This characterization allows us to differentiate the species that are present in the Petri dishes through their visualization.

Through the morphological characteristics referred to in point 2.2.3, it is possible to understand which type of organisms degraded the hydrocarbons.

3.3.2. Biochemical characterization of organisms

3.3.2.1. Gram stain

In the preparation of the smear, first, dry and flambé a clean slide in the flame of the Bunsen burner and identify the side of the slide where the smear will be made. Then, the chosen colony is chopped and placed on the slide. After, the smear is made in an oval shape, very thin and uniform, using a sterile loop. Then, the blade is dried near of the flame. The fixation is done by passing the slide (opposite the smear), 5 times in the flame of the Bunsen burner in a fast way.

The method is started by covering the entire slide with a violet crystal solution and wait for one minute. Then, a quick wash is done with distilled water or simply let the dye drain. Then the lugol solution is added until covered for one minute. The blade is tilted, absolute alcohol is dripped for 30 seconds. A quick wash is carried out again under running water or only the alcohol is drained. Then, the slide is covered with safranin where it is necessary to wait another 30 seconds. Finally, wash the slide with distilled water and let it dry without ever rubbing it.

3.4. Identification for *Pseudomonas aeruginosa* using a selective medium

The preparation of the Petri dishes starts with the weighing of 25.3 g of the medium (*Pseudomonas* CN Agar Base (Dehydrated Culture Media)) for microbiology - PanReac AppliChem)) on a scale for a 1 L Schott Flask with 500ml of distilled water and 5 ml of glycerol. It mixes well. The flask must be heated with agitation until it begins to boil and left for 1 minute. Then it is sterilized in the autoclave at 121 ºC for 15 minutes and distributed over the Petri dishes (*Pseudomonas* CN Agar Base (Dehydrated Culture Media)) for microbiology - ITW Reagents, 2020).

Already with the Petri dishes with the medium, 0.1 mL of cell suspension of the colonies of interest is added. Plates should be placed in an incubator for 48 hours at 37 ºC. After this time, the plates where growth has occurred are placed under UV light (360 nm) to check the fluorescence (*Pseudomonas* CN Agar Base (Dehydrated Culture Media)) for microbiology - ITW Reagents, 2020).

3.5. Biodegradability study

The evaluation of the hydrocarbon biodegradation in the reference followed carries out this study in test tubes, but in this case 2 ml eppendorfs were used, with the proportions necessary to use the same quantities being used. The total volume used for these eppendorfs is 1.5 ml.

In this study, biodegradation using cell suspension is evaluated. The cell suspension carried out consists of growing in 50 mL erlenmeyers of each type of colony in 10 mL of LB medium

for 24 hours at 30 ºC and 250 rpm. After 24 hours, the growth is centrifuged for 10 minutes at 10000 rpm, later discarding the supernatant, and keeping the pellet and this was resuspended in 1.5 mL of 0.9 % (m/v) NaCl.

Three types of eppendorfs performed are representing in Figure 16.

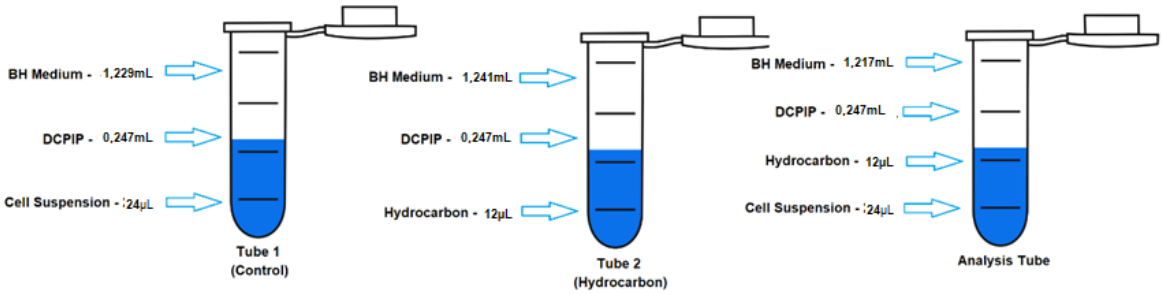

Figure 16 - Constitution of media used in the study of biodegradability using cell suspension.

After inoculation, manual homogenization is carried out and placed in an incubator at 30 ºC under agitation. The incubation time varied between 3 and 5 days for each isolated microorganism. Although visual observation was carried out every 24 hours. After the mentioned days, the absorbance was major at a wavelength of 604 nm.

4. Results

The samples used for analysis were collected in Mata da Machada and Praia da Alburrica and the hydrocarbons used were gasoline, hexane, and toluene. The mean value of the final O.D. after in first grow in LB medium was 1.72.

4.1. Microbial Growth

4.1.1. Mata da Machada

In the Mata da Machada sample, two growths were carried out in the 24-hour LB medium.

Figure 17 shows all the growths obtained with the Mata da Machada sample and the hydrocarbons, gasoline, hexane, and toluene, in the quantities of 100 µL and 600 µL for 30 hours.

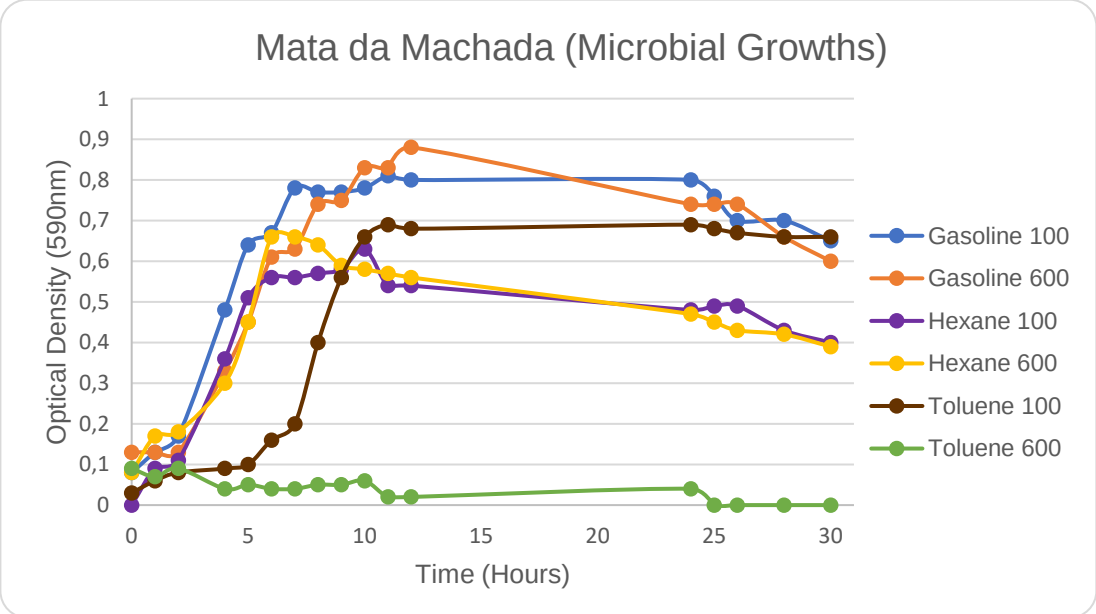

Figure 17 - Microbial growths observed in the sample Mata da Machada in BH medium with hydrocarbons, hexane, gasoline, and toluene, with the quantities of 100 µL and 600 µL for 30 hours.

As can be seen the growth of the colonies selected from Mata da Machada sample having 600 µL toluene did not develop. This can be justified because this hydrocarbon is very toxic, more

complex (aromatic) and the amount used was very high, so, consequently, the microorganisms were unable to grow.

The maximum optical densities observed in each growth are quite different and the time in which they occurred as well. Comparing the growth in the presence of 100 µL and 600 µL of gasoline, the maximum OD values obtained were 0.81 in the 11th hour and 0.88 in the 12th hour, respectively, which indicates that the increase in the concentration of the pollutant did not show greater toxicity, as it did not limit growth. For growths with 100 µL and 600 µL hexane there is already a small discrepancy in time of the maximum optical density, where 0.63 was obtained in the 10th hour and 0.66 in the 6th hour, respectively, that is, in growth with the hexane hydrocarbon 600 µL the peak optical density was obtained much faster than in growth with 100 µL hexane. The increase in hexane concentration was not harmful, but in the presence of this pollutant, the maximum OD was only 78% and 75% of the value obtained with gasoline at the same concentration. Finally, in the growth of the sample with toluene 100 µL, the obtained optical density was 0.69 in the 11th hour.

In the exponential phase of microbial growths, it is observed that not all had this phase at the same time, and in the growths with 600 µL gasoline, 100 µL hexane and 600 µL hexane, it occurred between the 2nd and 6th hours. In the case of 100 µL gasoline to obtain values between the 2nd and the 7th hour. Finally, the exponential phase of the microbial growth of the sample with the toluene hydrocarbon 100 µL was between the 7th and 10th hour, having taken much longer to reach than the other mentioned growths.

In addition to the time in which the exponential phase takes place, the specific growth rate (µ) is a very important data to be analyzed in growths as it is defined as a measure of the number of generations formed in an exponentially growing population, per unit of time. This is represented by the slope presented in the linear regression in the exponential phase of a growth.

Figure 18 depicts the linear regressions performed for the exponential growth phases with the Mata da Machada sample and the hydrocarbons with the respective equations and calculated R^2.

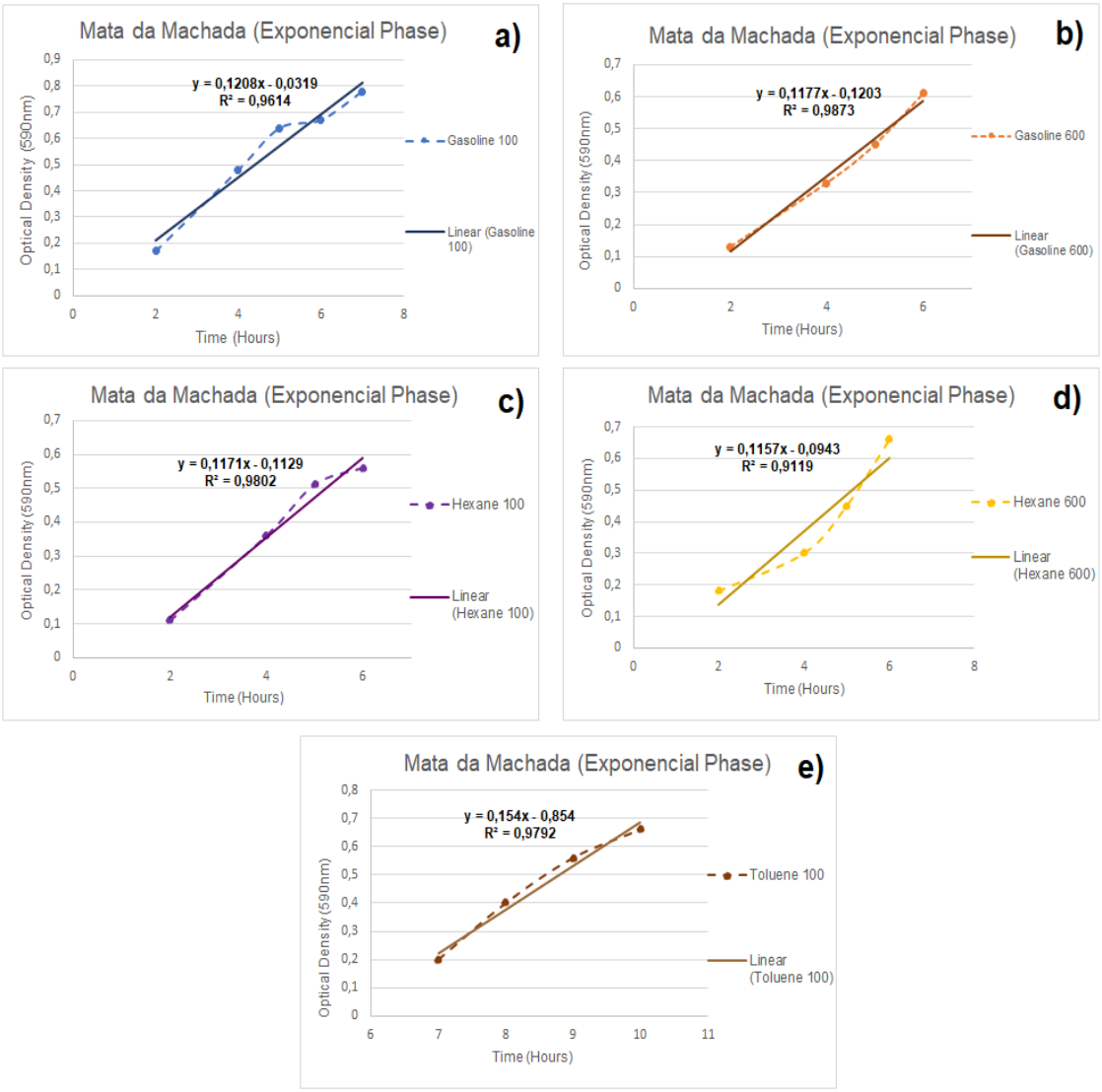

Figure 18 - Analytical determination of the rate of microbial growth from Mata da Machada soil sample in the presence of 100 µL gasoline (a), 600 µL gasoline (b), 100 µL hexane (c), 600 µL hexane (d) and 100 µL toluene (e).

As can be seen from the previous figure, the specific growth rates obtained in the presence of 100 µL and 600 µL gasoline were 0.121 h^{-1} and 0.118 h^{-1}, respectively, showing that the growth in the presence of 100µL gasoline obtained a little more than generations formed over time. In the case of growths in the presence of hexane 100 µL and 600 µL the values obtained were: 0.117 h^{-1} and 0.116 h^{-1}, respectively, proving that in this situation also the hydrocarbon (hexane) in the quantity of 100 µL presents a little more than forming generations over time than in the quantity of 600 µL. Finally, in the growth with the presence of toluene 100 µL, it obtained a specific rate of 0.154 h^{-1}.

4.1.2. Praia da Alburrica

With the Praia da Alburrica sample, two growths were also performed in the 24-hour LB medium, and mean value of the final OD was 1.66. These suspensions that were placed in BH medium having the pollutants hexane, gasoline, and toluene as carbon source in three independent assays.

Figure 19 shows all the growths obtained with the Praia da Alburrica sample and the hydrocarbons, hexane, gasoline, and toluene, in the quantities of 100 µL and 600 µL for 30 hours.

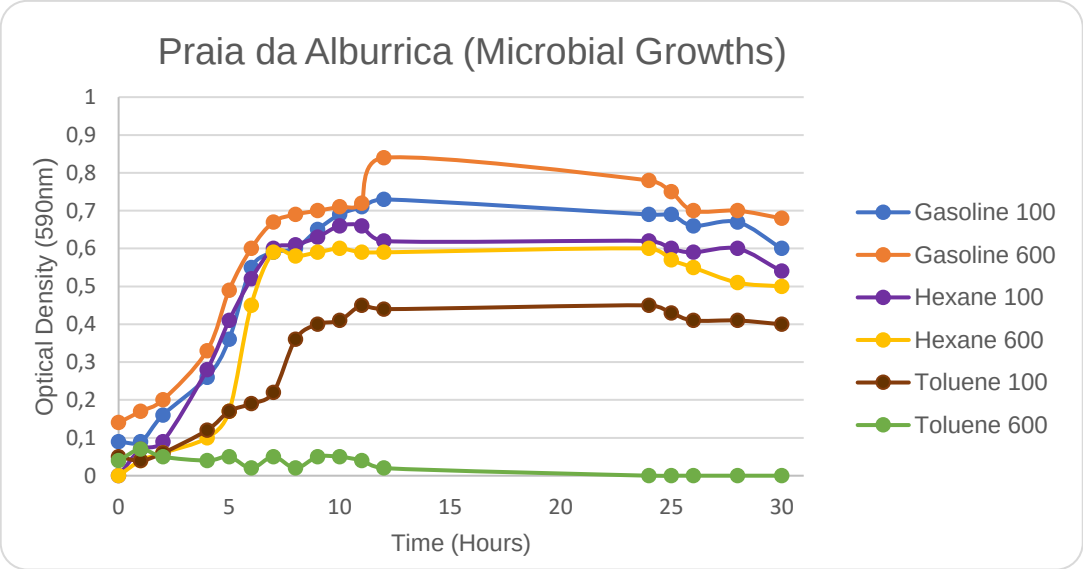

Figure 19 - Microbial growths observed in the sample Praia da Alburrica in BH medium with hydrocarbons, hexane, gasoline and toluene, with the quantities of 100 µL and 600 µL for 30 hours.

As was the case previously in Mata da Machada, the microbial growth of Praia da Alburrica with toluene 600 µL did not develop, thus confirming that the toxicity and complexity of the hydrocarbon may be one of the main factors for no growth of any kind.

In the microbial growths with the Praia da Alburrica sample, it was also found that the maximum optical densities obtained are quite different and the time in which they occur as well. In the growth with 100 µL and 600 µL gasoline, the values obtained were 0.73 and 0.84, respectively, both in the 12th hour, thus demonstrating that in this case the growth that was in the presence of 600 µL gasoline had more cell density at its maximum. For growths with hexane 100 µL and 600 µL the values obtained were 0.66 and 0.60, respectively, both in the 10th hour, proving that in growth with hexane 100 µL the maximum density in the same hour was higher. Finally,

in the growth of the sample with toluene 100 µL, the obtained optical density was 0.45 in the 11th hour.

In the exponential phase of microbial growths, it is observed that not all of them had this phase at the same time, while growths with 600 µL gasoline and 100 µL hexane occurred between the 2nd and 7th hour. In the case of 100 µL gasoline between the 1st and the 6th hour. In the growth in the presence of 600 µL hexane, the exponential phase occurred between the 5th and the 7th hour. Finally, the exponential growth phase with the 100 µL toluene hydrocarbon was between the 7th and the 9th hour.

As with the Mata da Machada sample, the specific growth rate (µ) was calculated. Figure 20 shows the linear regressions performed for the exponential growth phases with the sample Praia da Alburrica and the hydrocarbons with the respective equations and R^2 calculated.

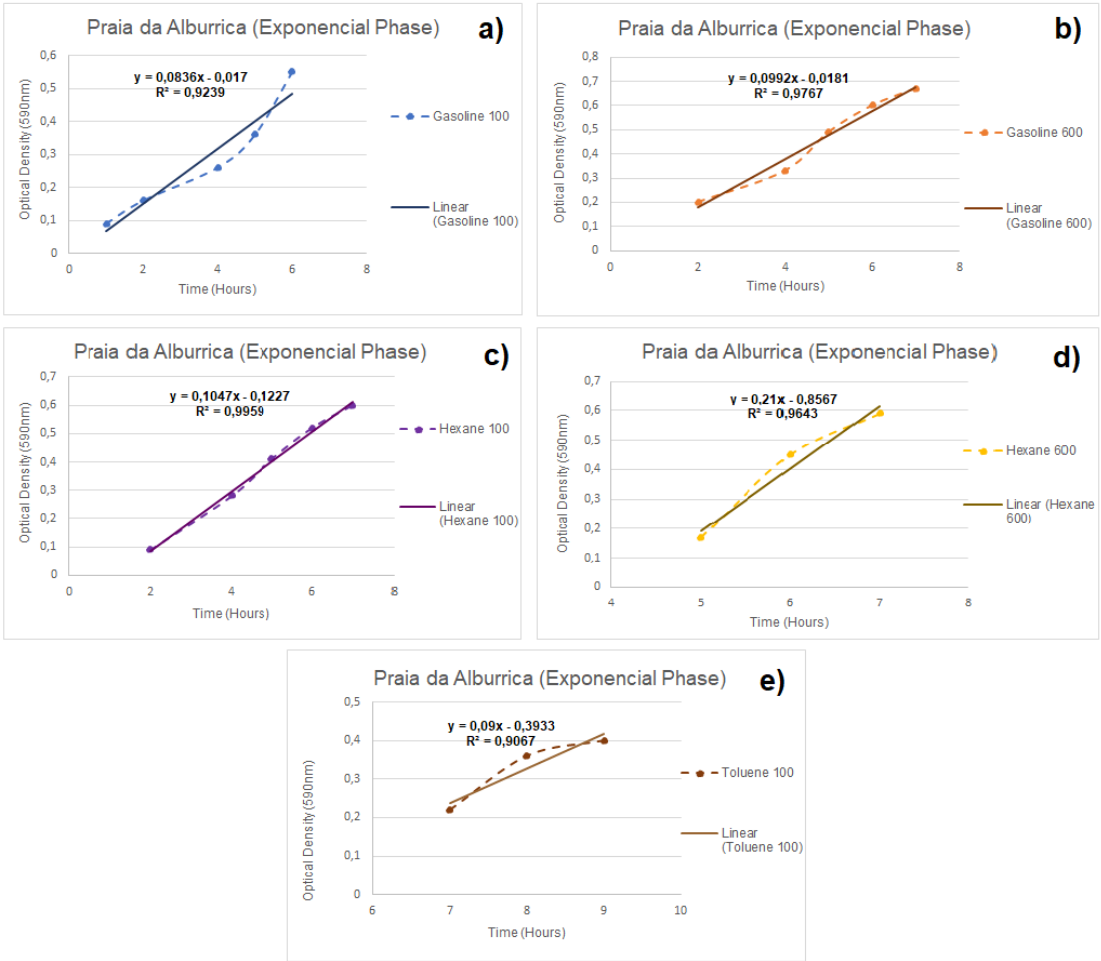

Figure 20 - Analytical determination of the rate of microbial growth of colonies obtained from da Praia da Alburrica in the presence of 100 µL gasoline (a), 600 µL gasoline (b), 100 µL hexane (c), 600 µL hexane (d) and 100 µL toluene (e).

As can be seen from figure 20, the specific growth rates obtained in the presence of 100 µL and 600 µL Gasoline were 0.083 h^{-1} and 0.099 h^{-1}, respectively, thus verifying that there are more generations that form growth in 600 µL gasoline. In the case of growths in the presence of hexane 100 µL and 600 µL the values obtained were: 0.105 h^{-1} and 0.210 h^{-1}, respectively, proving that in this situation also the hydrocarbon (hexane) in the amount of 600 µL presents a little more than forming generations over time than in the quantity of 100 µL. Finally, when growing with the presence of toluene 100 µL, it was obtained a specific rate of 0.090 h^{-1}.

For a better understanding of the results, Table 1 presents the specific growth rates, the maximum optical densities, and the respective time in which they occurred and the duration of the exponential phase.

Table 1 - Microbial growths characteristics, for the two soil samples Mata da Machada and Praia da Alburrica, at the presence of different pollutants and different concentration.

			Maximum O.D	Maximum O.D Hour	Exponential Phase Time (h)	Specific Growth Rate (µ) (h^{-1})
Mata da Machada	gasoline	100 µL	0.81	11th	(2nd – 7th) 5h	0.121
		600 µL	0.88	12th	(2nd - 6th) 4h	0.118
	Hexane	100 µL	0.63	10th	(2nd – 6th) 4h	0.117
		600 µL	0.63	6th	(2nd – 6th) 4h	0.116
	Toluene	100 µL	0.69	11th	(7th – 10th) 3h	0.154
		600 µL	------	------	------	------
Praia da Alburrica	gasoline	100 µL	0.73	12th	(1st – 6th) 5h	0.084
		600 µL	0.84	12th	(2nd – 7th) 5h	0.099
	Hexane	100 µL	0.66	10th	(2nd – 7th) 5h	0.105
		600 µL	0.60	10th	(5th - 7th) 2h	0.210
	Toluene	100 µL	0.45	11th	(7th – 9th) 2h	0.090
		600 µL	-----	------	------	------

The maximum Absorvance obtained, was for the growth of Mata da Machada inocula and 600 µL gasoline (0.88). Except for hexane, all other growths with the Mata da Machada colonies had higher specific values of growth rate and higher final OD values. Regarding the time when the highest optical density value was obtained, the growths of Mata da Machada and Praia da

Alburrica are quite similar, except for the growth of Mata da Machada with 100 µL gasoline and 600 µL hexane.

The duration of the exponential phase, in almost all growths, started between the 1st and the 2nd hour, showing that the microorganisms managed to adapt well to the environment in the presence of the hydrocarbon. There are three exceptions, those in the growth of the Praia de Alburrica with hexane 600 µL proving that probably in this sample the microorganisms took more time to develop in the presence of this quantity of hydrocarbon and in both growths with toluene 100 µL where it is possible to verify that the toxicity and the complexity of this hydrocarbon hinders the development of microorganisms, leading to the exponential phase being later.

The specific growth rates obtained show that the growth with Mata da Machada sample developed much better except for the growth of Praia de Alburrica with hexane 600 µL having more forming generations over time than the growth with Mata da Machada but its exponential phase was just 2 hours.

4.2. Growth dilution study

In this study, the dilution method was used, to verify the microorganisms that developed in the previous growths and that managed to degrade the hydrocarbons. The solid medium used was the nutrient agar. The inoculated plates were incubated for 48 hours at 30 ºC. The dilutions performed were: 1: 100, 1: 10000 and 1: 000000.

4.2.1. Mata da Machada

The dilutions obtained and those chosen for the growths with the sample from Mata da Machada in the presence of the hydrocarbons used with the quantities 100 µL and 600 µL are represented in figure 21. In the growth in the presence of toluene 600 µL as it was not obtained growth, it was not possible to perform the dilutions or the following studies.

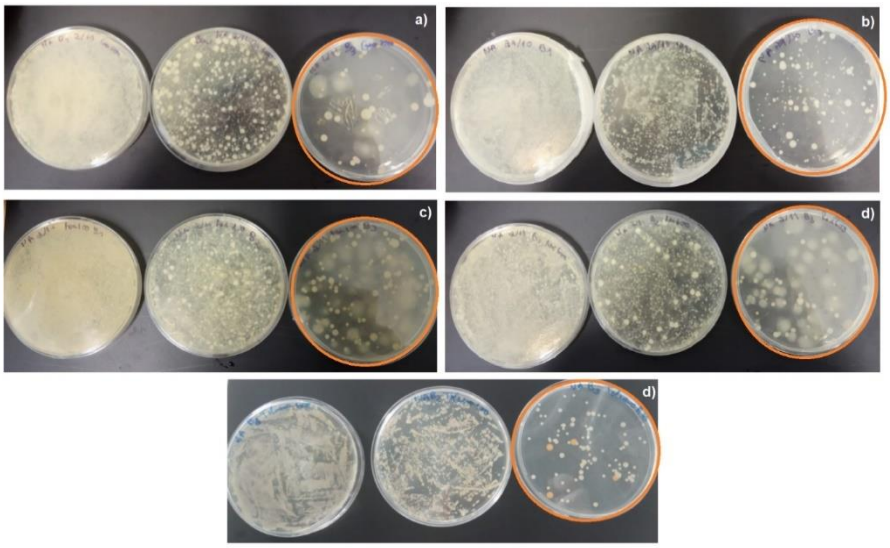

Figure 21 - Images of Petri dishes after 48 hours of growth dilutions obtained through growth with the sample from Mata da Machada in the presence of 100 µL gasoline (a), 600 µL gasoline (b), 100 µL hexane (c), 600 µL hexane (d) and 100 µL toluene (e).

As can be seen from the previous figure in all cases, the 1: 1000000 dilution was chosen because it is the most viable in terms of counting and visualizing the colonies.

4.2.2. Praia da Alburrica

As in the Mata da Machada sample, the dilutions obtained and chosen in the presence of the hydrocarbons, mentioned above, will be presented in figure 22. With this sample it was also not possible to develop any type of growth in the presence of 600 µL toluene, therefore, consequently, the study of dilutions and the following studies were not carried out.

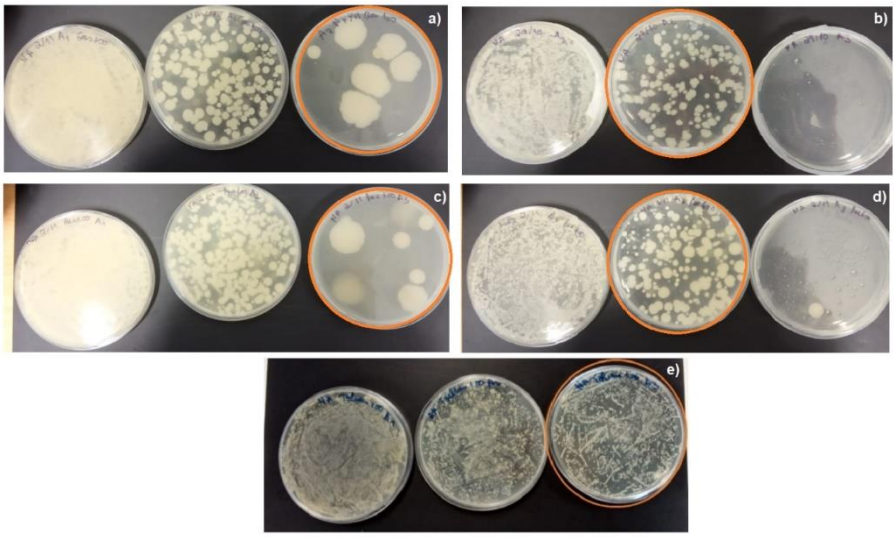

Figure 22 - Images of Petri dishes after 48 hours of growth dilutions obtained through growth with the sample from Praia da Alburrica in the presence of 100 µL gasoline (a), 600 µL gasoline (b), 100 µL hexane (c), 600 µL hexane (d) and 100 µL toluene (e).

In figure 22, the growth with the Praia da Alburrica sample in the presence of hydrocarbons with the amount of 100 µL the dilution chosen is 1: 1000000, in the case of growths with the sample in the presence of hydrocarbons with the amount of 600 µL the chosen dilution is 1: 10000.

4.3. Study of the effect of quantity and type of pollutant in colonies growths

After choosing the dilution used for the analyzes, the study of the amount and type of pollutant was carried out, that is, the growth in each plate was verified, colony morphology, biochemical characterization, and the choice of the best quantity of each contaminant. for the development of microorganisms.

4.3.1. Mata da Machada

In the sample from Mata da Machada, seven different colonies were identified, being identified as types 1, 2, 3, 4, 5, 6 and 7.

4.3.1.1. Gasoline 100 µL

The dilution used for the growth of the sample from Mata da Machada with 100 µL gasoline was 1: 1000000. In this plate, a total of forty-one colonies (4.1×10^6 UFC/mL) were obtained with four different types of colonies, as shown in figure 23.

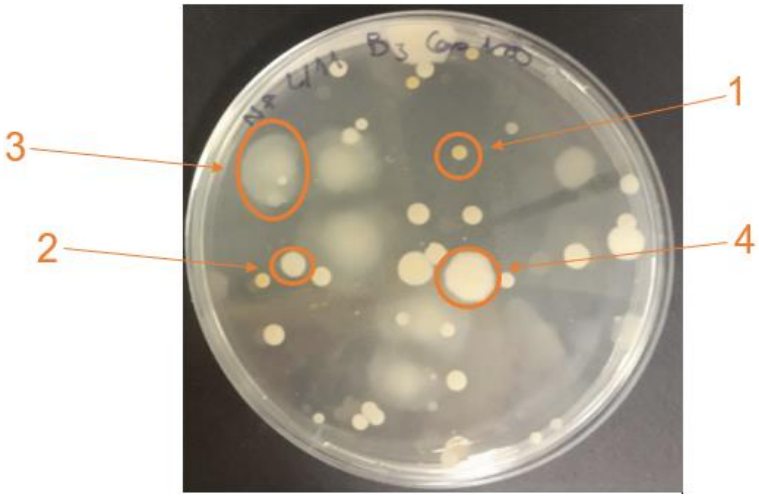

Figure 23 - Colonies obtained from the growth of Mata da Machada colonies with 100 µL gasoline.

Table 2 shows the morphological characteristics of each of the types of colonies identified in the previous Petri dish, which are types 1, 2, 3 and 4.

Table 2 - Morphological characterization of colonies type 1, 2, 3 and 4

Morphological Characterization								
Type of colony	**Colony No.**	**Size**	**Form**	**Elevation**	**Margin**	**Structure**	**Glow**	**Color**
1	7	Small	Circular	Elevated	Entire	Smooth	Opaque	Pigmented (Yellow)
2	22	Small/Medium	Circular	Elevated	Entire	Smooth	Opaque	Pigmented (White with pink nuances in the center)
3	9	Medium/Large	Irregular	Elevated	Entire	Smooth	Translucent	Pigmented (White)
4	3	Medium	Irregular	Elevated	Lobate	Smooth	Opaque	Pigmented (White)

In the biochemical characterization, Gram Test, in colony type 1, Gram Negative bacilli were obtained, as shown in figure 24.

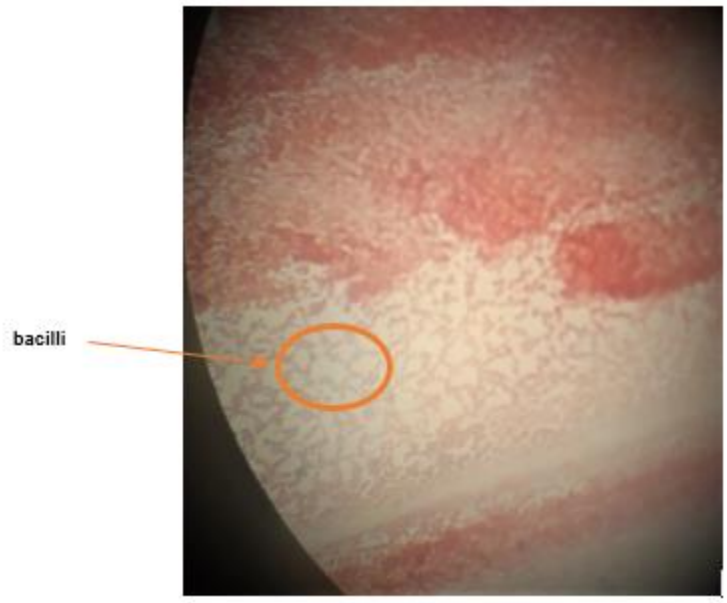

Figure 24 - Gram Test for Colony Type 1.

In the case of colony type 2 in the Gram Test, Gram Positive bacilli and Gram-Negative cocci were obtained (figure 25). The presence of two different types of microorganisms will be represented in other colony analyzes, because of interactions that occurred for the development of microorganisms seems to be a very common situation due to the environmental pressure and stress caused by the introduction of hydrocarbons in the medium, which is also the only one carbon source for the consumption of these microorganisms.

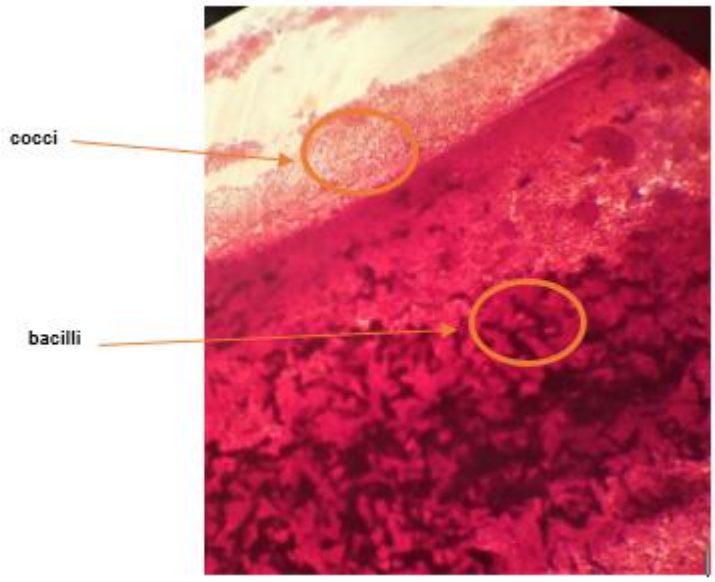

Figure 25 - Gram Test for Colony Type 2.

The biochemical characterization obtained in the type of colony 3 was also two different types of microorganisms, having been Gram-Negative cocci and Gram-Positive cocci, as shown in figure 26.

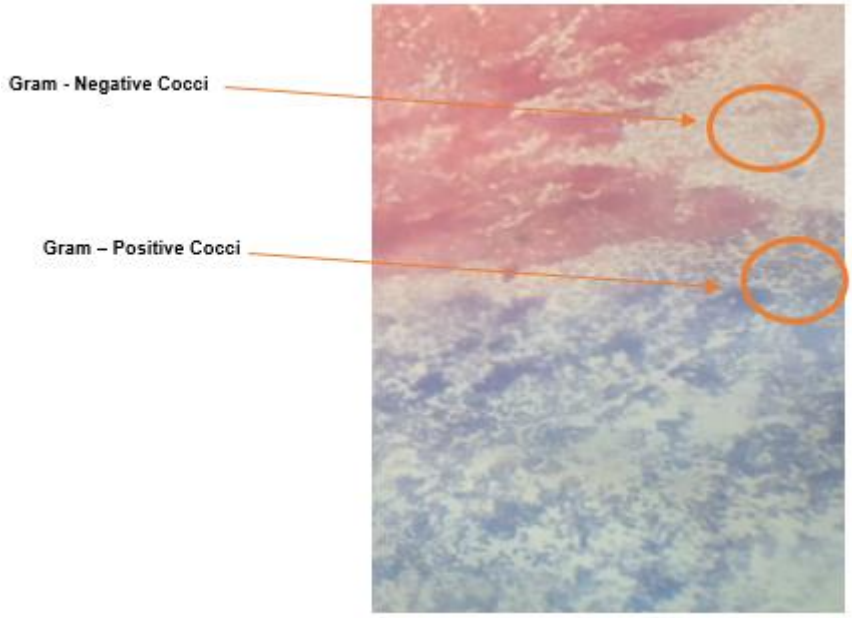

Figure 26 - Gram Test for Colony Type 3.

Finally, in the type 4 colony, the results obtained in the Gram test were bacilli and bacilli with spores Gram Positive, as shown in figure 27.

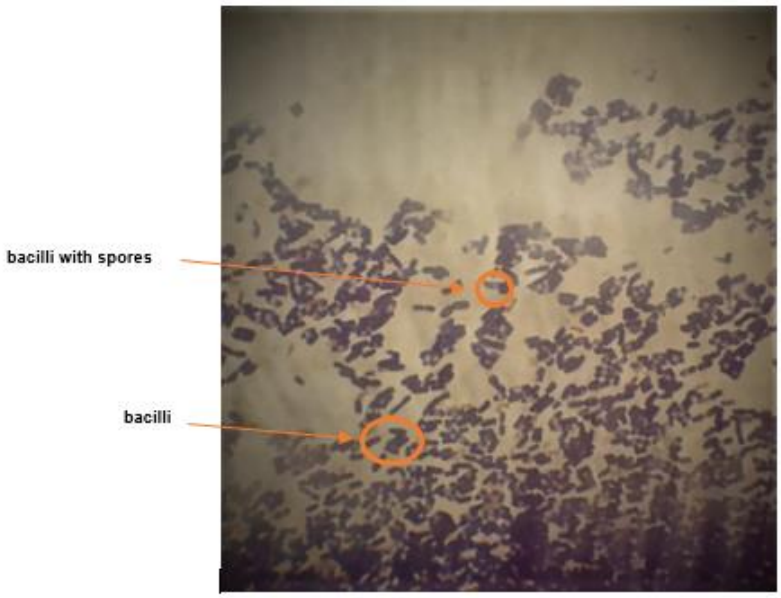

Figure 27 - Gram Test for Colony Type 4.

4.3.1.2. Gasoline 600 µL

In the growth of the Mata da Machada sample with the 600 µL gasoline hydrocarbon, a 1: 1000000 dilution was used. In the Petri dish, a total of one hundred sixty-nine colonies (1.69×10^7 UFC/mL) and three different types of colonies were obtained, identified as types 2, 4 and 5, as shown in Figure 28.

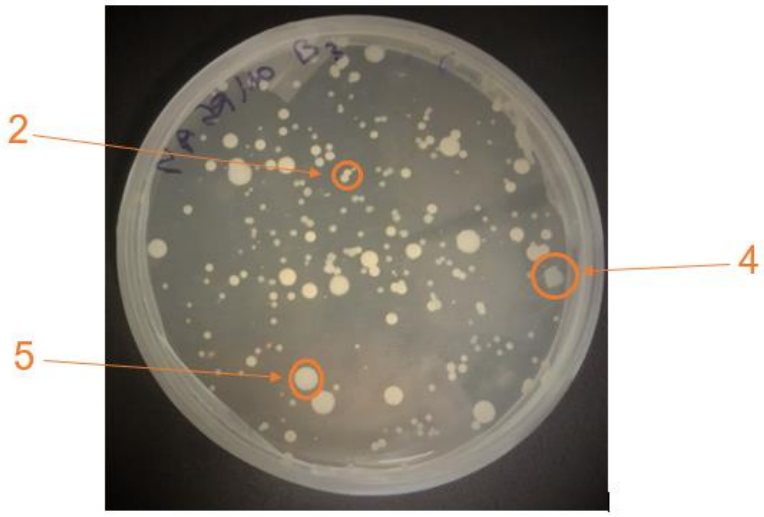

Figure 28 - Colonies of the growth of Mata da Machada with Gasoline 600 µL.

The morphological characterization of colonies of types 2, 4 and 5 are shown in table 3, and colonies of types 2 and 4 have already been referred to previously.

Table 3 - Morphological characterization of colonies type 2, 4 and 5

Morphological Characterization								
Type of colony	**Colony No.**	**Size**	**Form**	**Elevation**	**Margin**	**Structure**	**Glow**	**Color**
2	151	Small/Medium	Circular	Elevated	Entire	Smooth	Opaque	Pigmented (White with pink nuances in the center)
4	2	Medium	Irregular	Elevated	Lobate	Smooth	Opaque	Pigmented (White)
5	16	Medium	Circular	Elevated	Entire	Smooth	Opaque	Pigmented (White)

In the Biochemical characterization through the Gram Test, colonies of type 2 and 4 have already been mentioned previously and are represented in figures 24 and 26, respectively, having obtained in the colony of type 2 Gram Positive bacilli and Gram-Negative cocci and in the type of colony 4 bacilli and bacilli with spores Gram Positive.

In the case of the type 5 colony, after performing the Gram Test, Gram Positive bacilli were obtained, as shown in Figure 29.

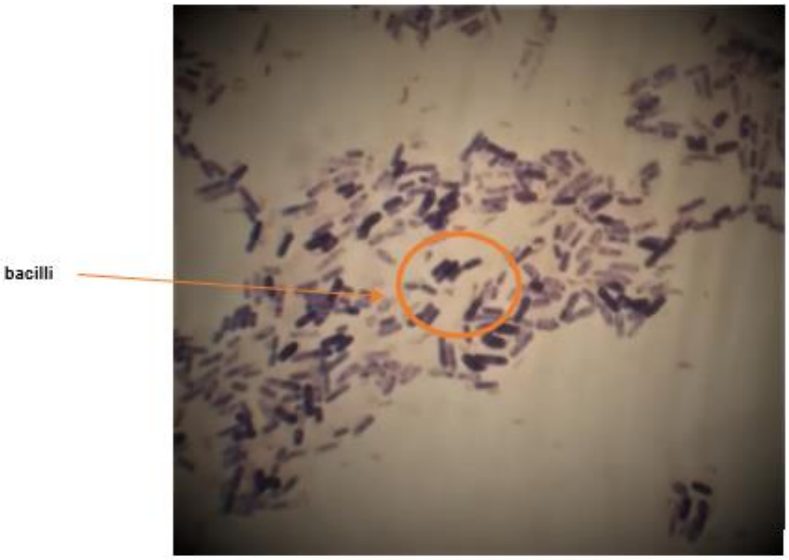

Figure 29 - Gram Test for Colony Type 5.

4.3.1.3. Hexane 100 µL

The plate used contained a 1: 1000000 dilutions of the sample growth of Mata da Machada and the hydrocarbon hexane 100 µL. A hundred and twenty-nine colonies were counted (1.29 × 10^7 UFC/mL), with three different types of colonies, as shown in figure 30.

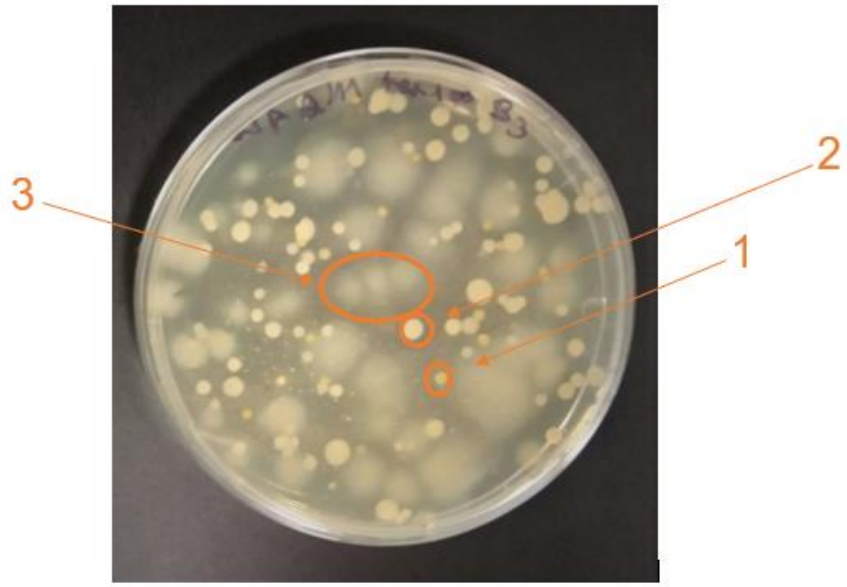

Figure 30 - Colonies of the growth of Mata da Machada with hexane 100 µL.

Table 4 shows the morphological characteristics of each of the types of colonies identified in the previous Petri dish, which are types 1, 2 and 3.

Table 4 - Morphological characterization of colonies type 1, 2 and 3

Morphological Characterization								
Type of colony	**Colony No.**	**Size**	**Form**	**Elevation**	**Margin**	**Structure**	**Glow**	**Color**
1	18	Small	Circular	Elevated	Entire	Smooth	Opaque	Pigmented (Yellow)
2	74	Small/Medium	Circular	Elevated	Entire	Smooth	Opaque	Pigmented (White with pink nuances in the center)
3	37	Medium/Large	Irregular	Elevated	Entire	Smooth	Translucent	Pigmented (White)

In the biochemical characterization, the results obtained from colony types 1, 2 and 3, have already been mentioned, having been obtained for colony type 1, Gram-Negative bacilli as shown in figure 24, for colony type 2, bacilli Gram Positives and Gram-Negative cocci, as shown in figure 25. Finally, in colony type 3, Gram Negative cocci and Gram-Positive cocci were obtained, as can be seen in figure 26.

4.3.1.4. Hexane 600 µL

In the plate used with the 1: 1000000 dilutions performed from the sample growth of Mata da Machada and with the 600 µL hexane hydrocarbon, a total of sixty-nine colonies (6.9 $\times 10^6$UFC/mL) were obtained, with three different types of colonies, being that the types of colonies obtained are the same as those existing on the previous plate, that is, type of colony 1, 2 and 3, as shown in figure 31.

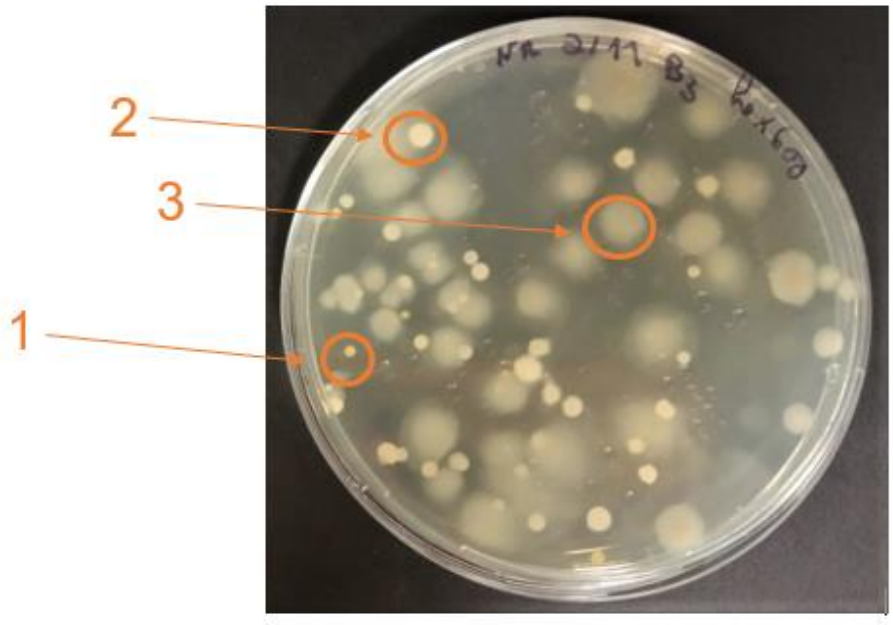

Figure 31 - Colonies of the growth of Mata da Machada with hexane 600 µL.

In the morphological characterization, 3 types of colonies already mentioned were obtained, these being types 1, 2 and 3. The characteristics of these colonies are shown in table 4. There are fifty-four colonies of type 1, twenty-six colonies of type 2 and thirty- eight colonies type 3.

In the biochemical characterization, as occurred in the growth with the presence of 100 µL hexane, the results obtained from colony types 1, 2 and 3, have already been mentioned, obtaining for colony type 1, Gram-Negative bacilli as shown in figure 24, for colony type 2, Gram-Positive bacilli, and Gram-Negative cocci, as shown in figure 25. Finally, in colony type 3, Gram Negative cocci and Gram-Positive cocci were obtained, as can be seen in figure 26.

4.3.1.5. Toluene 100 µL

In the use of Toluene in previous growths with the sample from Mata da Machada, only results with the amount of 100 µL were obtained, therefore, it was only possible to obtain colonies with this amount. The dilution used for the morphological and biochemical characterization was 1: 1000000 where ninety-four colonies (9.4 $\times 10^6$UFC/mL) were obtained with two different types of colonies, types 6 and 7, as shown in figure 32.

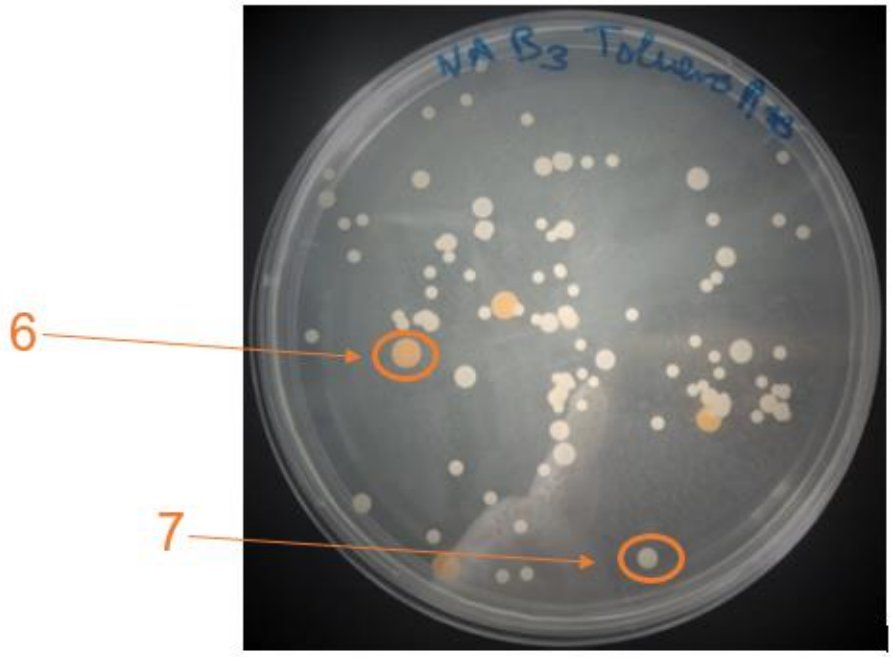

Figure 32 - Colonies of the growth of Mata da Machada with Toluene 100 µL.

The morphological characterization of the colonies of types 6 and 7 presents in the Petri dish of the growth of Mata da Machada with toluene 100 µL is shown in table 5.

Table 5 - Morphological characterization of type 6 and 7 colonies

Morphological Characterization								
Type of colony	**Colony No.**	**Size**	**Form**	**Elevation**	**Margin**	**Structure**	**Glow**	**Color**
6	4	Medium	Circular	Elevated	Entire	Smooth	Opaque	Pigmented (Orange)
7	90	Small/Medium	Circular	Elevated	Entire	Smooth	Opaque	Pigmented (White)

In the biochemical characterization, Gram Test, in the type 6 colony (figure 33), the presence of two different types of microorganisms was again obtained (Gram-Positive Cocci and Gram-Negative Bacilli), confirming once again that the symbiosis in these cases is widely used for the development and growth of microorganisms.

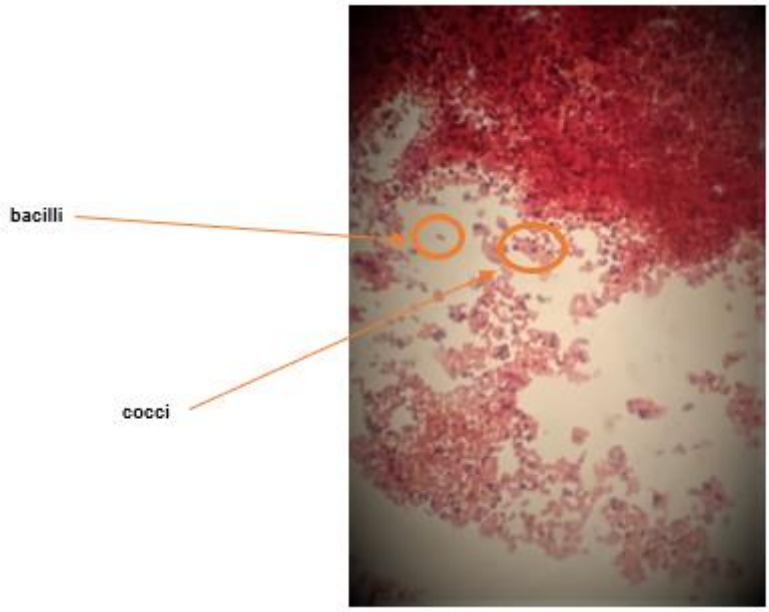

Figure 33 - Gram Test for Colony Type 6.

Colony type 7 (figure 34) shows, as in colony type 6, the development of two different types of microorganisms (Gram-Negative bacilli and Gram-Positive Bacilli), thus assuming that for this type of aromatic compounds and with a high toxicity microorganism to be able to develop in their presence need to perform symbiosis.

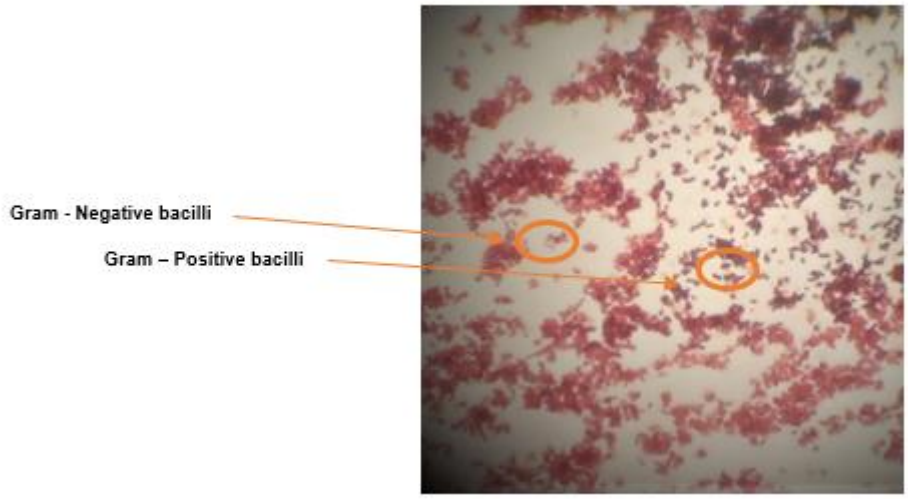

Figure 34 - Gram Test for Colony Type 7.

4.3.2. Praia da Alburrica

In this sample, five different types of colonies were identified, these being the types 8, 9, 10, 11 and 12.

4.3.2.1. Gasoline 100µL

In the growth of the sample Praia da Alburrica with the hydrocarbon gasoline 100 µL, the dilution 1: 1000000 was used. In the Petri dish, a total of seven colonies (7 $\times 10^5$ UFC/mL) of three different types were obtained, identified as types 8, 9 and 10, as shown in Figure 35.

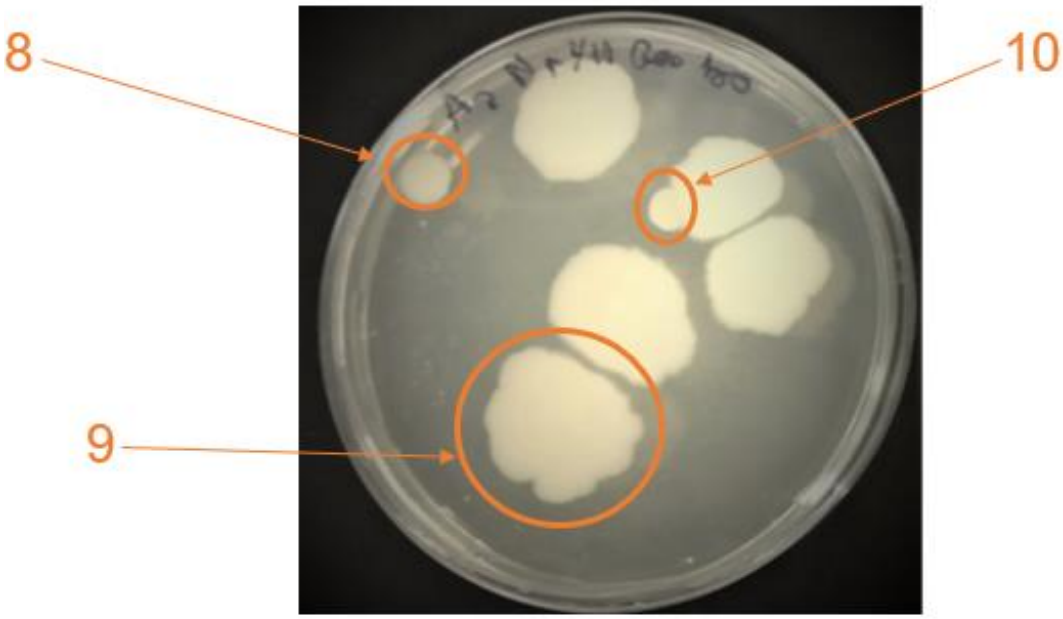

Figure 35 – Praia da Alburrica growth with 100 µl gasoline.

Table 6 shows the morphological characteristics of each type of colony represented on the Petri dish.

Table 6 - Characterization of colonies 8, 9 and 10

Morphological Characterization								
Type of colony	**Colony No.**	**Size**	**Form**	**Elevation**	**Margin**	**Structure**	**Glow**	**Color**
8	1	Large/Medium	Circular	Elevated	Entire	Smooth	Opaque	Pigmented (White)
9	5	Large/Medium	Irregular	Elevated	Wavy	Smooth	Opaque	Pigmented (White)
10	1	Large	Irregular	Elevated	Entire	Smooth	Translucent	Pigmented (Beije)

The biochemical characterization (Gram test), obtained in colony type 8, was gram positive bacilli, as shown in figure 36.

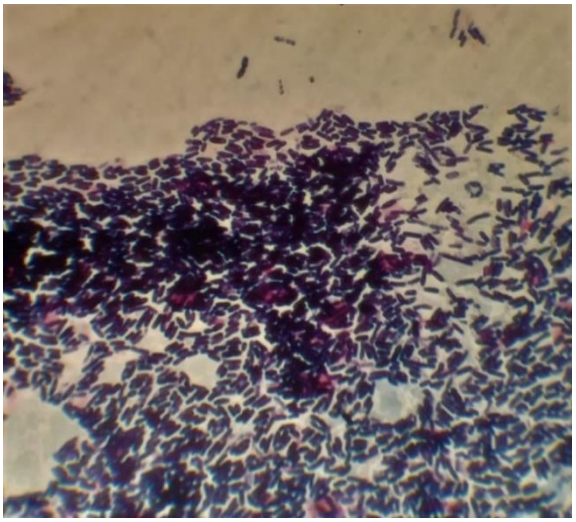

Figure 36 - Gram Test for Colony Type 8.

The colony type 9 presents in its Gram Test result, bacilli, and bacilli with spores Gram Positive. These are represented in figure 37.

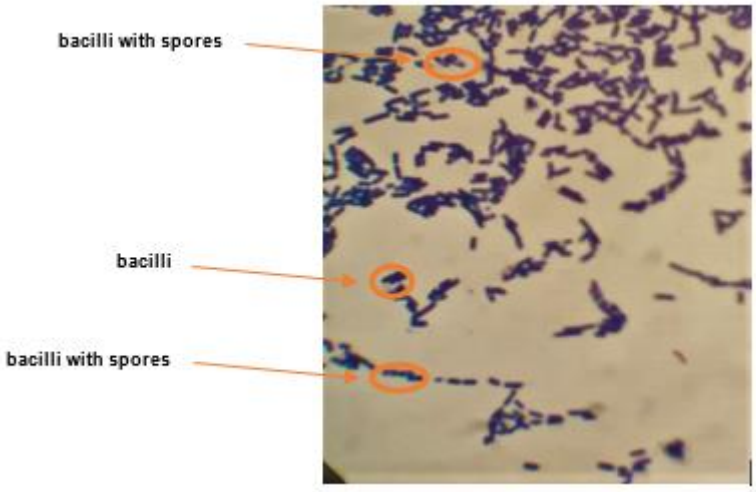

Figure 37 - Gram Test for Colony Type 9.

Finally, in the colony of type 10, a biochemical characterization (Gram Test) of Gram-Positive and Gram-Negative bacilli was obtained, as shown in Figure 38. This appears to be yet another case of symbiosis between microorganisms to be able to remain and survive environmental pressure provided by the presence of hydrocarbon in the medium that in this case is Gasoline.

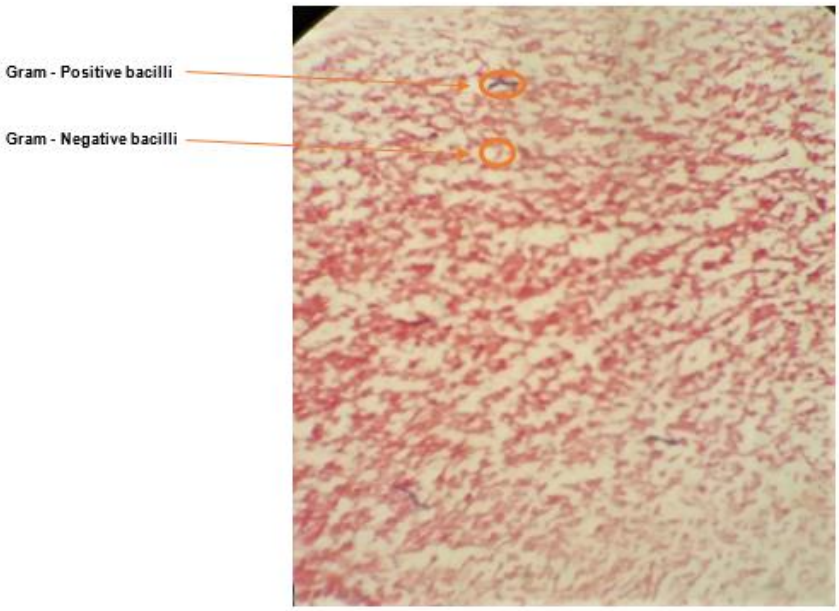

Figure 38 - Gram Test for Colony Type 10.

4.3.2.2. Gasoline 600 µL

In the Petri dish with the dilution 1: 10000 obtained from the growth of the sample Praia da Alburrica and gasoline 600 µL, a total of one hundred and four colonies (1.04×10^5 UFC/mL) were obtained with two different types of colonies, type 8 and 9, as shown in figure 39.

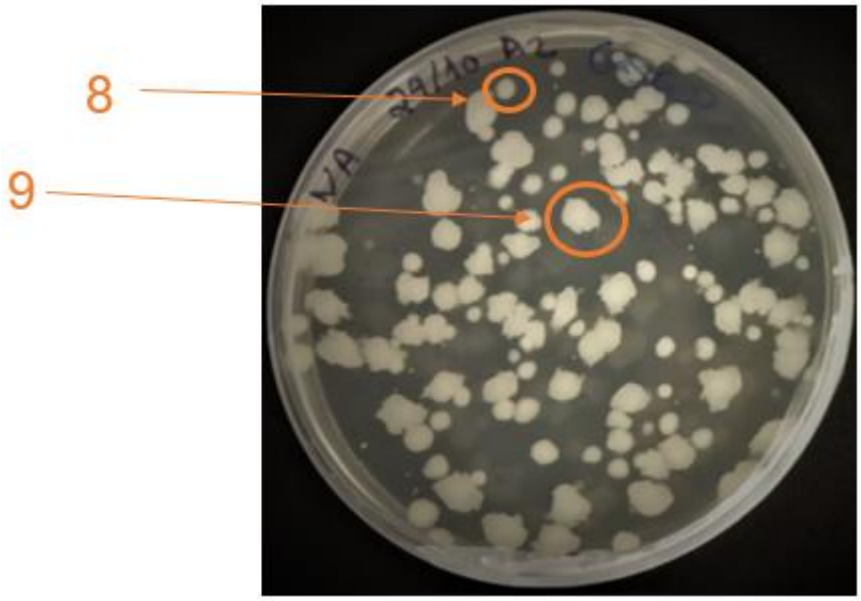

Figure 39 – Praia da Alburrica growth with 600µl Gasoline.

In the morphological characterization, we have two types of colonies previously mentioned. The characteristics of these colonies are shown in table 7.

Table 7 - Morphological characterization of colonies 8 and 9

Morphological Characterization								
Type of colony	**Colony No.**	**Size**	**Form**	**Elevation**	**Margin**	**Structure**	**Glow**	**Color**
8	45	Large/Medium	Circular	Elevated	Entire	Smooth	Opaque	Pigmented (White)
9	59	Large/Medium	Irregular	Elevated	Wavy	Smooth	Opaque	Pigmented (White)

In the biochemical characterization, the results obtained from colony types 8 and 9, as described above, have already been mentioned, having been obtained for colony type 8, Gram-Positive bacilli as shown in figure 36 and for colony type 9, bacilli and bacilli with spores Gram-Positive, as shown in figure 37.

4.3.2.3. Hexane 100 µL

In the plate used with the dilution 1: 1000000 performed from the sample growth of Praia da Alburrica and with the hydrocarbon hexane 100 µL, a total of seven colonies (7 $\times 10^5$ UFC / mL) were obtained, with three different types of colonies, as shown in figure 40.

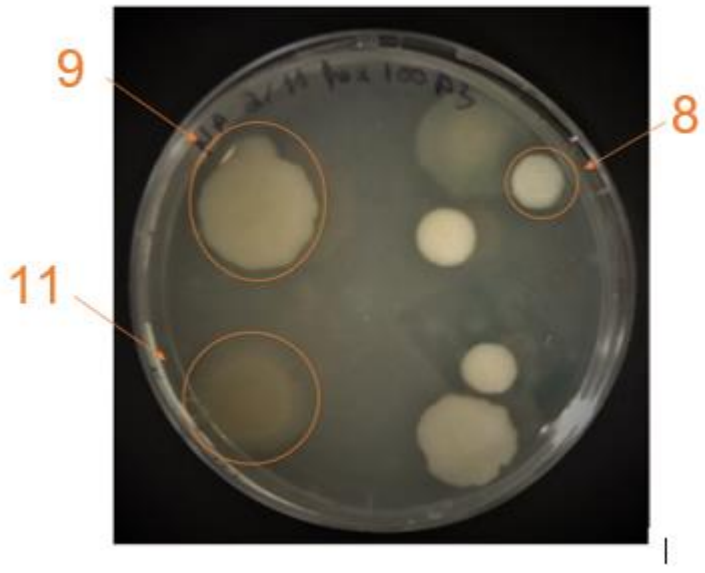

Figure 40 – Praia da Alburrica growth Petri dish with 100µL hexane.

Table 8 shows the morphological characteristics of types 8 and 9, previously mentioned, and type 11.

Table 8 - Morphological characterization of colonies type 8, 9 and 11

Morphological Characterization								
Type of colony	**Colony No.**	**Size**	**Form**	**Elevation**	**Margin**	**Structure**	**Glow**	**Color**
8	3	Large/Medium	Circular	Elevated	Entire	Smooth	Opaque	Pigmented (White)
9	2	Large/Medium	Irregular	Elevated	Wavy	Smooth	Opaque	Pigmented (White)
11	2	Large	Irregular	Elevated	Entire	Smooth	Translucent	Pigmented (Beije)

In the biochemical characterization, the results obtained from colony types 8 and 9, as described above, have already been mentioned, having been obtained for colony type 8, gram positive bacilli as shown in figure 36 and for colony type 9, bacilli and bacilli with gram positive spores, as shown in figure 37.

Finally, in colony type 11, two different types of bacteria were obtained, gram positive and Gram-Negative bacilli, in the same colony as shown in figure 41. As mentioned above, there are two different types of microorganisms can mean that these they developed in the face of an environmental pressure on growth (hydrocarbon), and for that reason they had to perform the symbiosis to survive.

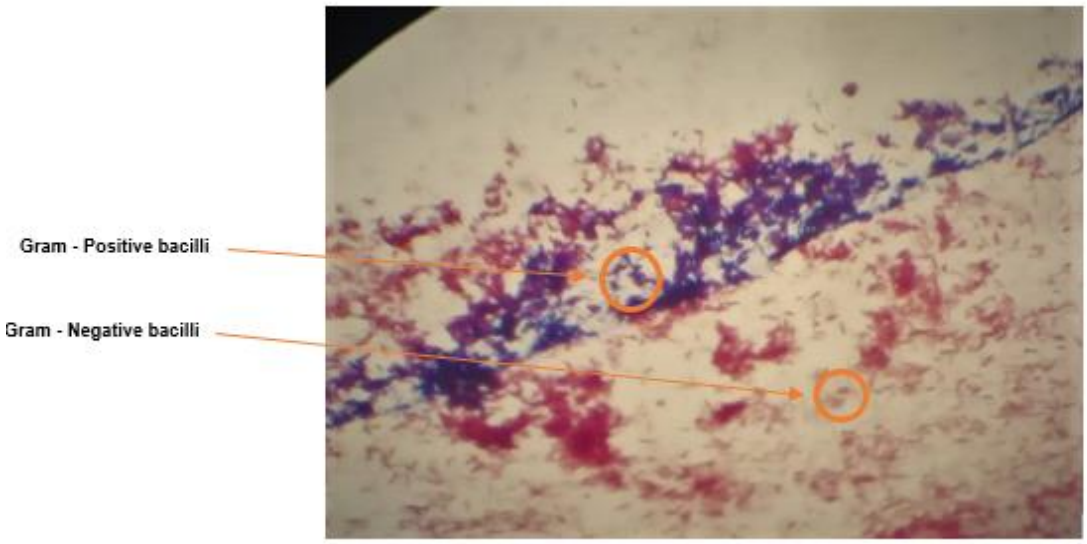

Figure 41 - Gram Test for Colony Type 11.

4.3.2.4. Hexane 600 µL

The Petri dish used with the 1: 10000 dilution made from the sample growth of Praia da Alburrica and with hexane 600 µL contains a total of one hundred forty-five colonies (1.45×10^5 UFC/mL), with type eight colonies, as shown figure 42.

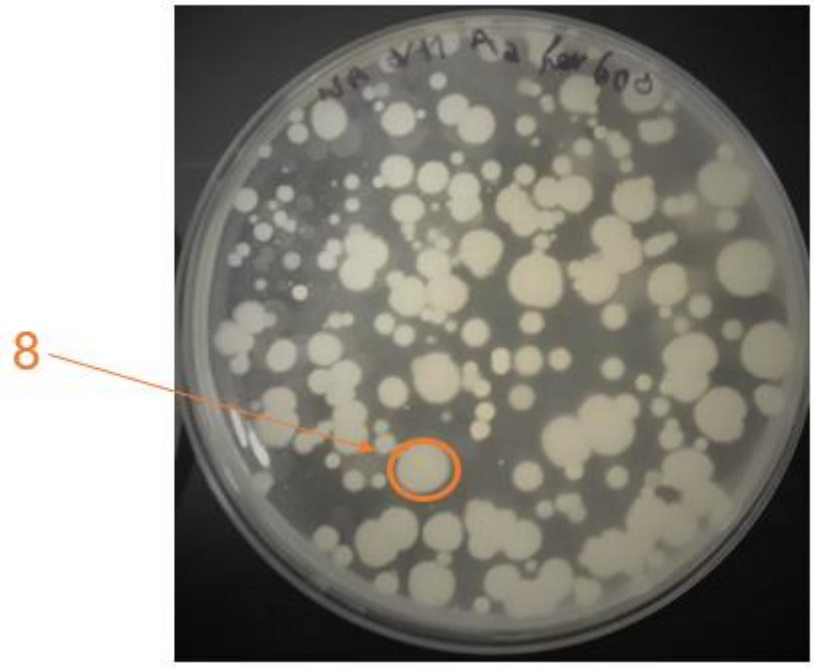

Figure 42 – Praia da Alburrica growth Petri dish with 600 µL hexane.

This type of colony present on the plate is the same as the type 8 shown above, with the same morphological characteristics, as shown in table 9.

Table 9 - Morphological characterization of colony type 8

Morphological Characterization								
Type of colony	**Colony No.**	**Size**	**Form**	**Elevation**	**Margin**	**Structure**	**Glow**	**Color**
8	145	Large/Medium	Circular	Elevated	Entire	Smooth	Opaque	Pigmented (White)

In the biochemical characterization, Gram test, the results obtained are also the same, so the colony shows positive Gram bacilli as shown in figure 36.

4.3.2.5. Toluene 100µL

In the growth of the sample Praia da Alburrica and the hydrocarbon Toluene, as previously mentioned, there was only growth with the amount of 100 µL, therefore, only colonies were obtained when using this amount. The dilution used for the morphological and biochemical characterization was 1:1000000 where an uncountable number of colonies were obtained. The Petri dish contained one type of colony, type 12, as shown in figure 43.

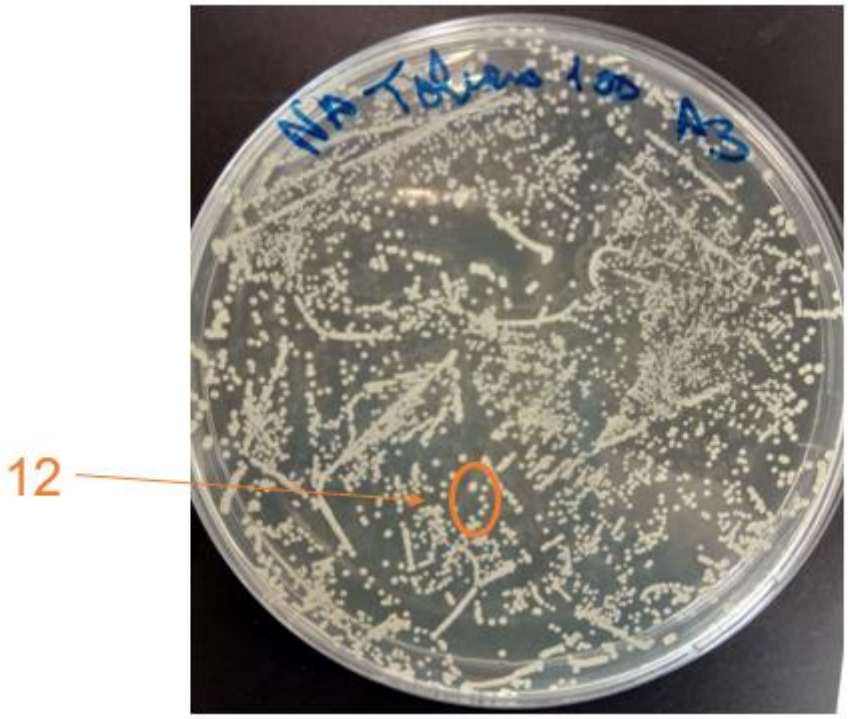

Figure 43 - Praia da Alburrica growth with 100 µl Toluene.

The morphological characterization of the colony of type 12 present in the Petri dish of the growth of Praia da Alburrica with toluene 100 µL is shown in table 10.

Table 10 - Morphological characterization of colony type 12

Morphological Characterization								
Type of colony	**Colony No.**	**Size**	**Form**	**Elevation**	**Margin**	**Structure**	**Glow**	**Color**
12	Uncountable	Small	Circular	Elevated	Entire	Smooth	Opaque	Pigmented (White)

In the biochemical characterization, Gram Test, the presence of two different types of microorganisms was also obtained, proving once again the possibility of symbiosis between organisms for survival in a different and restricted environment. In this case, Gram Positive and Negative cocci are represented, as shown in figure 44.

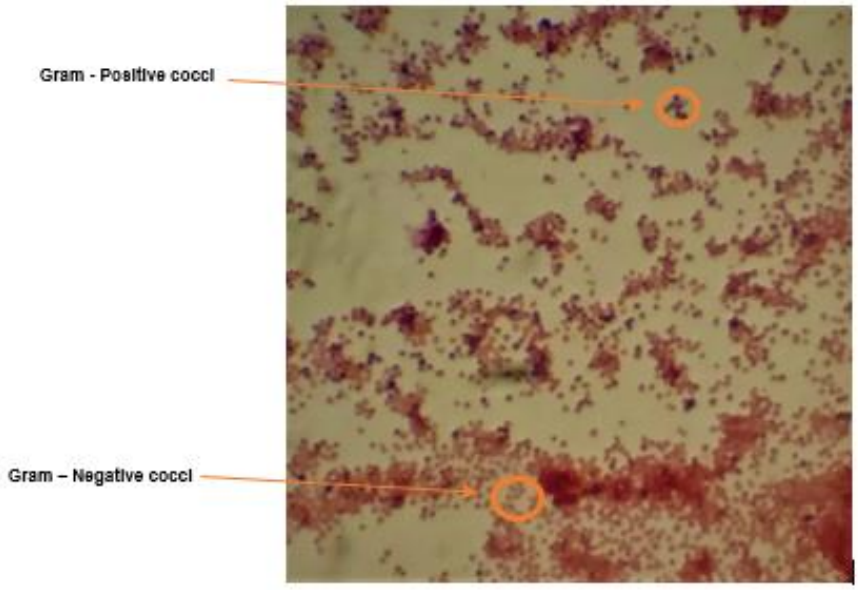

Figure 44 - Gram Test for Colony Type 12.

4.4. Identification of *Pseudomonas aeruginosa* using a selective medium

To carry out the study of biodegradability that will be presented in the next section, it was necessary to carry out a growth of each of the colonies. Colonies with gram-negative bacteria were found to inoculate in the selective medium for *Pseudomonas aeruginosa*, these being colonies 1, 2, 3, 6, 7, 10, 11 and 12. The plates with the selective medium and the colonies were incubated for 48 hours at 37 ºC. After incubation it was found that growth occurred in colonies 1, 2, 3 and 10, as shown in figure 45.

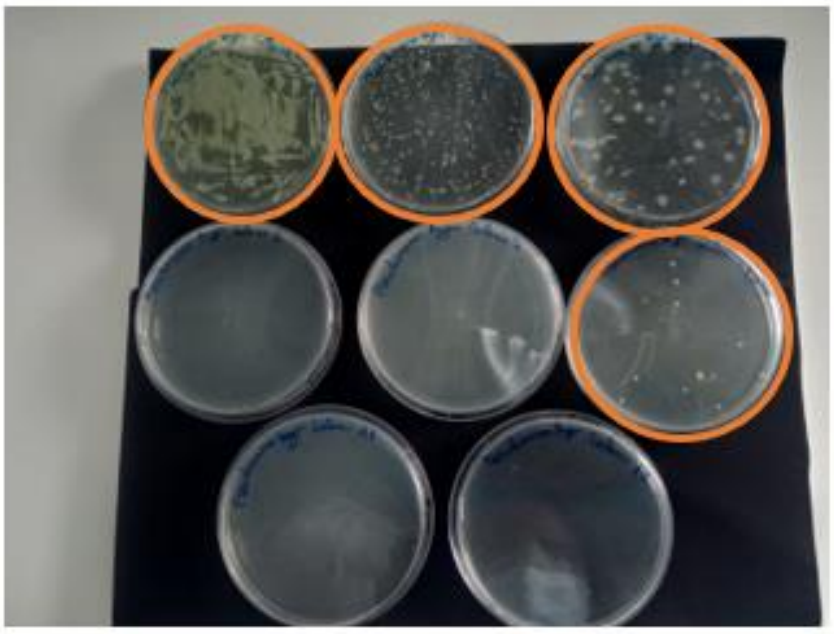

Figure 45 – Identification of the Petri dishes (orange ring) were growth of Pseudomonas *aeruginosa* happened.

The plates where growth occurred were observed under the action of UV light at 360 nm, where green fluorescence was observed in all plates, as shown in figure 46.

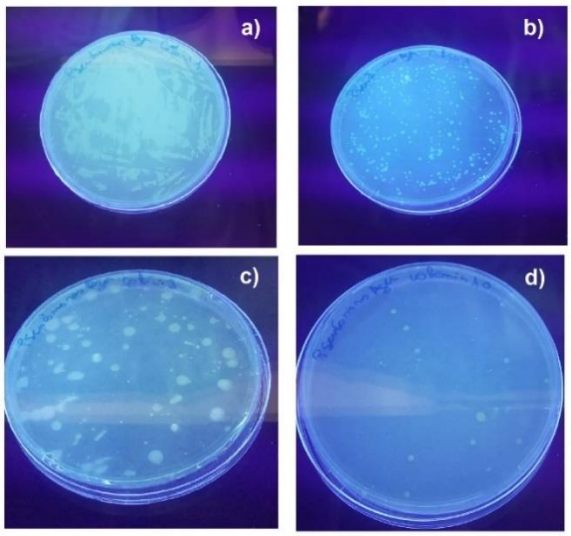

Figure 46 - Observation of the plates under the action of UV light (360 nm): a - type 1 colony; b- type 2 colony; c- type 3 colony; d – type 10 colony.

Through the results obtained previously it is possible to verify that in the colonies presented there is *Pseudomonas aeruginosa*. In the type 1 colony, it is even possible to state that the microorganism present is *Pseudomonas aeruginosa*, as it presents only gram-negative bacilli in its morphology. In the other colonies, there are other types of microorganisms including

Gram-Positives, so in addition to Pseudomonas there are also others bacteria that have not been identified. Of the four plaques where the growth of *Pseudomonas aeruginosa* occurred, three of them colonies present in the sample Mata da Machada and only one in Praia da Alburrica.

4.5. Biodegradability Study

The analysis of biodegradability was carried out using the redox indicator DCPIP. When added to the culture medium, it acts as an electron acceptor released during the metabolization process, where the microorganisms oxidize the hydrocarbon. The color change from blue (oxidized state) to colorless (reduced state). All growth performed with the indicator had a duration of 5 days, where the reference followed mentions a growth between 3 and 5 days.

The formula used to calculate the percentage of biodegradation was as follows:

Equation 6 - Percentage of biodegradation

$$\%\, biodegration\ = 100 - (\frac{Absorbance\ of\ each\ type\ of\ colony}{Absorbance\ of\ hydrocarbon} \times 100)$$

4.5.1. Biodegradability study using cell suspension

In the study of Biodegradability, the colonies of Mata da Machada and Praia da Alburrica were analyzed. The colonies used for this analysis were those obtained in the growth in the presence of hydrocarbons with the quantity of 100 µL, since no growth was obtained toluene 600 µL with 100 µL of hydrocarbon it was possible to do comparisons between all pollutants.

To obtain a greater amount of biomass from each of the colonies, a cell suspension was performed. The biodegradation capacity was analyzed through the colony individually and in a mixture according to the growth in each hydrocarbon.

The 2 ml eppendorfs used in this study were defined as follows: tube 1, which was used for control, contained Medium BH, DCPIP and cell suspension; tube 2, which was used to obtain the absorbance of the hydrocarbons, contained Medium BH, DCPIP and the hydrocarbon with the quantity of 100 µL; the tubes used for the analysis, contained, Medium BH, DCPIP, hydrocarbon with the quantity of 100 µL and the cell suspension. For all the tubes described above, 5 replicates were performed.

4.5.1.1. Mata da Machada

In the study of the biodegradability of gasoline with colonies of type 1, 2, 3, 4 and MMG (Mata Mix Gasoline) the absorbances and percentages of biodegradation obtained are shown in table 11, and the values obtained for the absorbances of Gasoline were as follows: 1.944; 1.936; 1.931; 1.941 and 1.92. Tube 1 is represented as a control because it aims to prove that no type of biodegradation occurs without the presence of the hydrocarbon. Therefore, the values of this tube must always be higher than the absorbance of the samples, because during the incubation process, no changes can occur. All tubes presented in both Mata da Machada and Praia da Alburrica have higher values than those obtained in the samples.

Table 11 - Biodegradation using the cell suspension of colonies 1, 2, 3, 4 and MMG over five days

Mata da Machada				
Colony	**Test Tube**	**Tube 1 (Control)**	**Biodegradation (%)**	**Biodegradation (average and standard deviation) (%)**
1G1	1.274	1.531	36.10	34.59 ± 0,012
1G2	1.262	1.539	34.80	
1G3	1.263	1.541	34.59	
1G4	1.286	1.543	33.75	
1G5	1.289	1.522	32.86	
2G1	1.257	1.611	35.33	33.59 ± 0,012
2G2	1.260	1.617	34.90	
2G3	1.292	1.618	33.09	
2G4	1.289	1.619	33.59	
2G5	1.296	1.625	32.50	
3G1	1.286	1.431	33.80	33.47 ± 0,004
3G2	1.288	1.441	33.47	
3G3	1.291	1.442	33.14	
3G4	1.281	1.432	34.00	
3G5	1.286	1.440	33.02	
4G1	1.216	1.501	37.44	37.49 ± 0,004
4G2	1.204	1.501	37.80	
4G3	1.207	1.504	37.49	
4G4	1.211	1.506	37.60	
4G5	1.215	1.508	36.71	
MMG1	1.141	1.900	41.31	40.96 ± 0,037
MMG2	1.143	1.911	40.96	
MMG3	1.140	1.911	49.04	
MMG4	1.158	1.902	40.34	
MMG5	1.138	1.899	40.73	

In the analysis of this biodegradability study, after the 5 days of incubation, it was not possible to verify a marked color change, so it was necessary to analyze the absorbances to confirm if the biodegradation occurred using the previously described colonies.

This analysis and the following ones were performed on the spectrophotometer at 604 nm as mentioned in the reference followed (Sanches, 2019).

With the absorbance values obtained, it is possible to confirm that there was some biodegradation of gasoline, which was not a very high degradation and for that reason the color change did not occur. The highest percentage obtained in this study was in the mixture of colonies that developed in the presence of gasoline, thus proving that the symbiosis and synergy between microorganisms makes biodegradation easier.

In the study of the biodegradability of Hexane with colonies of type 1, 2, 3 and MMH (Mata Mix Hexane) the absorbances and percentages of biodegradation obtained are shown in table 12, and the values obtained for the absorbances of Hexane were as follows: 1.881; 1.891; 1.885; 1.881; 1.89.

Table 12 - Biodegradation using the cell suspension of colonies 1, 2, 3 and MMH (Mata Mix Hexane) over five days

Mata da Machada				
Colony	**Test Tube**	**Tube 1 (Control)**	**Biodegradation (%)**	**Biodegradation (average and standard deviation) (%)**
1H1	1.404	1.531	25.36	25.41± 0.002
1H2	1.407	1.539	25.59	
1H3	1.411	1.541	25.15	
1H4	1.403	1.543	25.41	
1H5	1.407	1.522	25.56	
2H1	1.399	1.611	25.62	25.78 ± 0.001
2H2	1.402	1.617	25.86	
2H3	1.399	1.618	25.78	
2H4	1.395	1.619	25.84	
2H5	1.407	1.625	25.56	
3H1	1.398	1.431	25.68	26.28 ± 0.003
3H2	1.394	1.441	26.28	
3H3	1.386	1.442	26.47	
3H4	1.390	1.432	26.10	
3H5	1.389	1.440	26.50	
MMH1	1.374	1.899	26.95	26.76 ± 0.002
MMH2	1.385	1.898	26.76	
MMH3	1.387	1.897	26.42	

MMH4	1.380	1.890	26.63	
MMH5	1.384	1.891	26.77	

Once again it was not possible to visually check the color change, but rather through the absorbances obtained. The percentages obtained from the biodegradation of hexane were notably lower than those obtained in the study of biodegradability with gasoline, showing that gasoline is a more easily biodegradable hydrocarbon. In this case, the percentage obtained from the mixture of colonies is also slightly higher than that of colonies, proving once again that the action between microorganisms is beneficial in the degradation of pollutants.

With this study it appears that the colonies of types 1, 2 and 3 are more efficient in the degradation of gasoline than hexane.

Finally, in the study of toluene's biodegradability with type 6, 7 and MMT (Mata Mix Toluene) colonies, the absorbances and percentage of biodegradation obtained are shown in table 13, and the values obtained for the absorbances of Toluene were as follows: 1.951; 1.951; 1.957; 1.961 and 1.960.

Table 13 - using the cell suspension of colonies 6, 7 and MMT (Mata Mix Toluene) over five days

Mata da Machada				
Colony	**Test Tube**	**Tube 1 (Control)**	**Biodegradation (%)**	**Biodegradation (average and standard deviation) (%)**
6T1	1.786	1.811	8.46	8.53 ± 0.005
6T2	1.786	1.821	8.46	
6T3	1.790	1.817	8.53	
6T4	1.780	1.818	9.23	
6T5	1.777	1.820	9.36	
7T1	1.777	1.611	8.92	8.98 ± 0.002
7T2	1.777	1.621	8.92	
7T3	1.778	1.617	9.15	
7T4	1.779	1.618	9.28	
7T5	1.784	1.614	8.98	
MMT1	1.681	1.931	13.84	14.26 ± 0.003
MMT2	1.678	1.938	13.99	
MMT3	1.678	1.931	14.26	
MMT4	1.673	1.930	14.68	
MMT5	1.677	1.930	14.44	

As toluene consists of a more complex hydrocarbon, it was expected that the percentages would be quite low, being necessary for this type of hydrocarbon to add some strategy to the process to increase the biodegradation capacity.

As in the other cases previously presented, the mixture of colonies shows higher values of biodegradation.

4.5.1.2. Praia da Alburrica

In the study of the biodegradability of gasoline with colonies of type 8, 9, 10 and AMG (Alburrica Mix Gasoline) the absorbances and percentages of biodegradation obtained are shown in table 14. The absorbances obtained from gasoline are the same as previously presented (1.944; 1.936 1.931, 1.941 and 1.92).

Table 14 - Biodegradation using cell suspension from colonies 8, 9, 10, and AMG (Alburrica Mix Gasoline) over five days

Praia da Alburrica				
Colony	**Test Tube**	**Tube 1 (Control)**	**Biodegradation (%)**	**Biodegradation (average and standard deviation) (%)**
8G1	1.378	1.901	29.12	28.93 ± 0.003
8G2	1.376	1.901	28.93	
8G3	1.375	1.905	28.79	
8G4	1.378	1.908	29.00	
8G5	1.374	1.900	28.44	
9G1	1.376	1.511	29.22	29.08 ± 0.003
9G2	1.373	1.52	29.08	
9G3	1.372	1.518	28.95	
9G4	1.374	1.528	29.21	
9G5	1.375	1.520	28.39	
10G1	1.390	1.511	28.50	27.84 ± 0.005
10G2	1.397	1.512	27.84	
10G3	1.397	1.518	27.65	
10G4	1.394	1.520	28.18	
10G5	1.395	1.518	27.34	
AMG1	1.307	1.799	32.77	32.39 ± 0.003
AMG2	1.309	1.801	32.39	
AMG3	1.308	1.803	32.26	
AMG4	1.309	1.805	32.56	

AMG5	1.306	1.810	31.98	

In all colonies of Praia da Alburrica, it was also not possible to verify the visualization of the color change, so it was necessary to analyze the absorbances and the due percentages obtained to see if any type of biodegradation occurred.

In the analysis of the study of the biodegradability of gasoline using the colonies obtained in the sample Praia da Alburrica it is possible to verify that the percentages of biodegradation are lower than those obtained in the sample Mata da Machada, thus proving that Mata da Machada presents microorganisms with greater capacity to degrade the gasoline than Praia da Alburrica. In this case, the colony mixture also presents a higher percentage than the individual action of each colony.

In the study of the biodegradability of Hexane with colonies of type 8, 9, 11 and AMH the absorbances and percentages of biodegradation obtained are shown in table 15. The absorbances obtained from Hexane are the same as previously presented (1.881; 1.891; 1.885; 1.88; 1.189).

Table 15 - Biodegradation using cell suspension from colonies 8, 9, 11, and AMH (Alburrica Mix Hexane) over five days

Praia da Alburrica				
Colony	**Test Tube**	**Tube 1 (Control)**	**Biodegradation (%)**	**Biodegradation (average and standard deviation) (%)**
8H1	1.581	1.901	15.95	16.13 ± 0,004
8H2	1.571	1.901	16.92	
8H3	1.581	1.905	16.13	
8H4	1.578	1.908	16.11	
8H5	1.577	1.900	16.56	
9H1	1.476	1.511	21.53	21.59 ± 0,002
9H2	1.477	1.520	21.89	
9H3	1.478	1.518	21.59	
9H4	1.481	1.528	21.27	
9H5	1.479	1.520	21.75	
11H1	1.379	1.620	26.69	26.85 ± 0,002
11H2	1.38	1.614	27.02	
11H3	1.382	1.613	26.68	
11H4	1.376	1.618	26.85	
11H5	1.379	1.603	27.03	
AMH1	1.255	1.971	33.28	35.11 ± 0,008
AMH2	1.227	1.978	35.11	

AMH3	1.221	1.971	35.23	
AMH4	1.220	1.980	35.14	
AMH5	1.231	1.980	34.87	

In the analysis of this study with Hexane, it appears that the percentages of biodegradation are not very high, proving that the optimization of the process is needed. In comparison with the Mata da Machada sample, the colony mixture of Praia da Alburrica managed to be more efficient in the degradation of Hexane, thus showing that Praia da Alburrica contains microorganisms more likely to degrade this hydrocarbon.

Type 8 and 9 colonies are more efficient at degrading gasoline than hexane.

In the study of toluene's biodegradability with type 12 colony, the absorbances and percentages of biodegradation obtained are shown in table 16. The absorbances obtained from toluene are the same as previously presented (1.951; 1.951; 1.957; 1.961 and 1.960).

Table 16 - Biodegradation using the colony 12 cell suspension

Praia da Alburrica				
Colony	**Test Tube**	**Tube 1 (Control)**	**Biodegradation (%)**	**Biodegradation (average and standard deviation) (%)**
12T1	1.662	1.681	14.81	16.37 ± 0,007
12T2	1.628	1.651	16.56	
12T3	1.641	1.678	16.15	
12T4	1.64	1.688	16.37	
12T5	1.639	1.690	16.38	

In this analysis it appears that although there was only one type of colony, it has higher values compared to Mata da Machada including the mixture of colonies, although the results have been quite low.

In the measurements made in the analysis of the biodegradability of gasoline, it is possible to verify, through standard deviations and error bars (figure 47), that the error and associated uncertainties are minimal, proving that these percentages obtained are acceptable. The highest associated error is represented in the MMG colony.

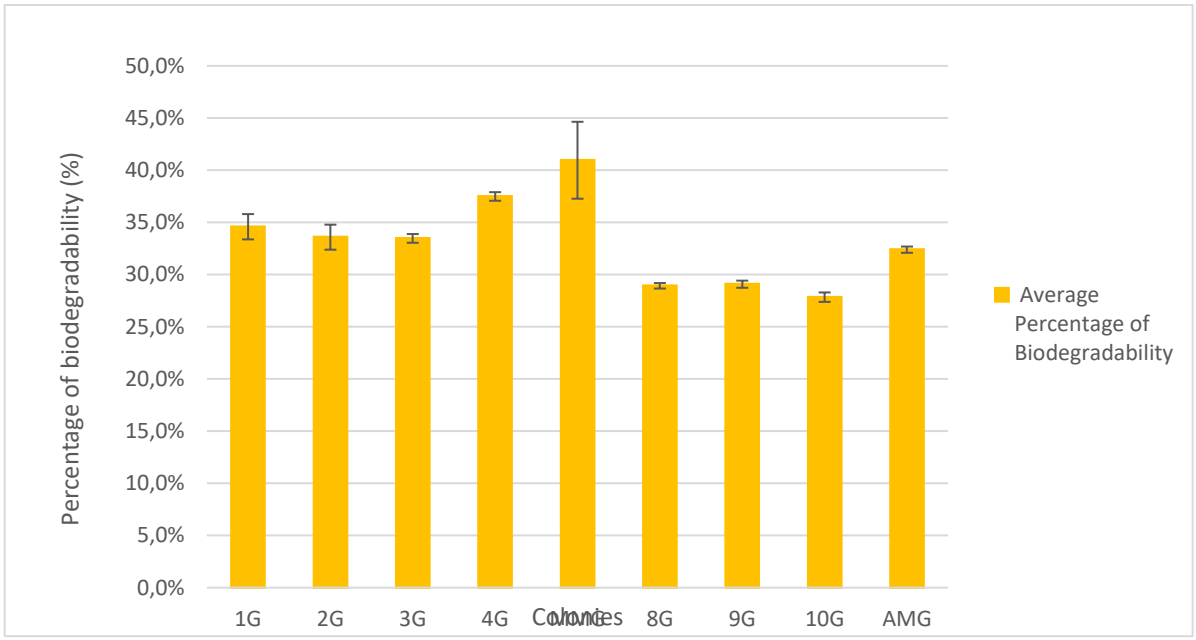

Figure 47 - Comparative study of biodegradability rates of gasoline with error bar analysis.

In the measurements carried out in the analysis of hexane's biodegradability, it is also possible to verify, through standard deviations and error bars (figure 48), that the error and associated uncertainties of all measurements are minimal, thus being able to confirm the viability of the results obtained.

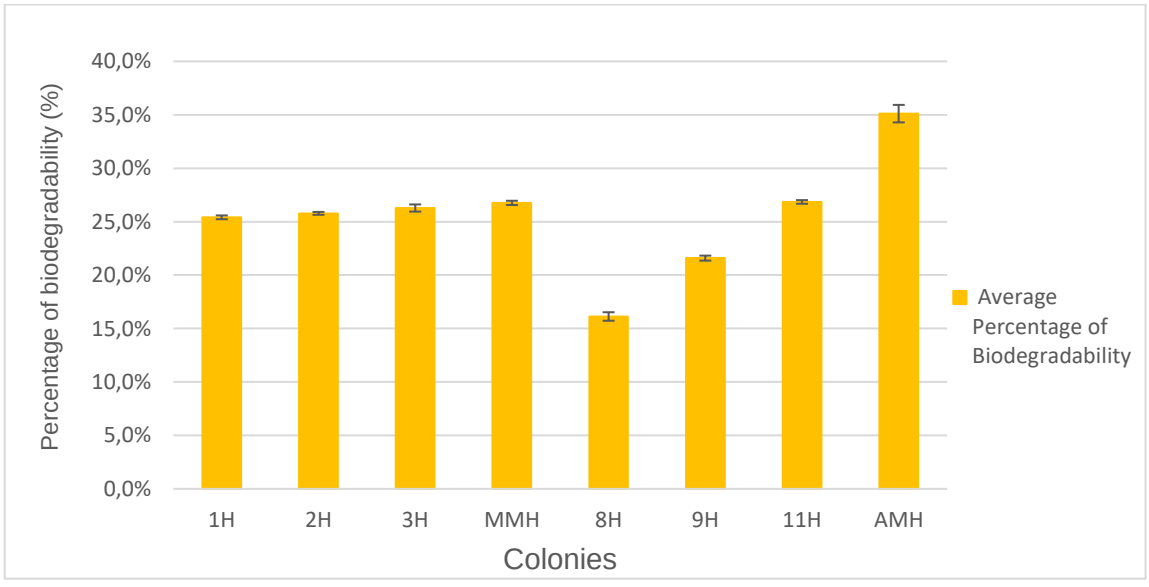

Figure 48 - Comparative study of biodegradability rates of hexane with error bar analysis.

Finally, in the measurements made in the analysis of toluene's biodegradability, as in the previous situations, it appears that the associated errors and uncertainties are minimal, as can

be seen in Figure 49. Therefore, all measurements and percentages obtained in the study of the hydrocarbon biodegradability are feasible.

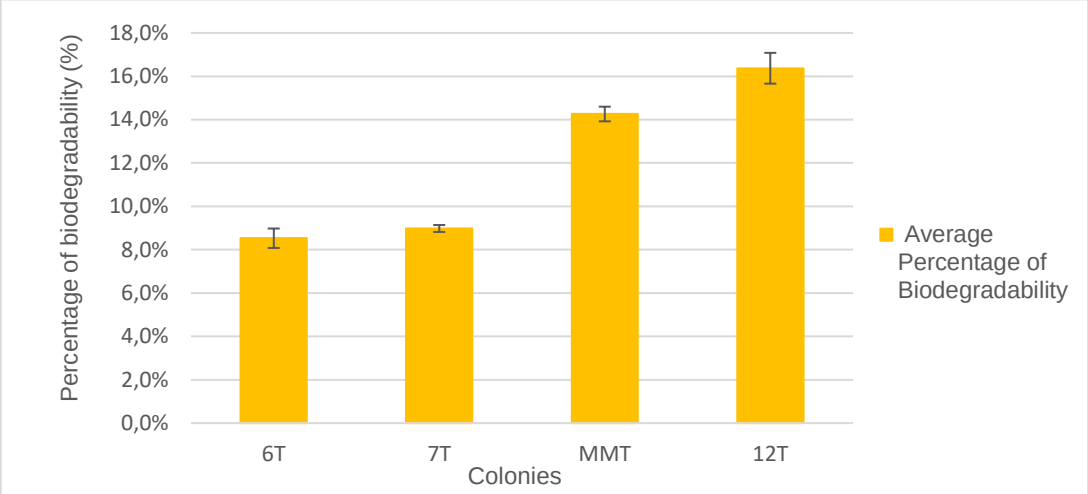

Figure 49 - Comparative study of biodegradability rates of toluene with error bar analysis.

5. Discussion and Conclusion

The main propose of this research work was completely achieved since it was possible to evaluate the hydrocarbon degradation potential of microorganisms present in the soil samples selected. The microorganisms have not only been isolated, but also have also been studied in their morphological and biochemical characteristics. The biodegradation of the hydrocarbons was achieved, although in a small percentage, and the analysis of the results indicated that the synergistic action of microorganisms in mixed colonies leads to higher percentage of biodegradation.

The samples soils chosen were from Praia da Alburrica and Mata da Machada. The choices of these two locations were intended to be collected in a location that was constantly in contact with hydrocarbons, in the case of Praia da Alburrica due to the proximity of the Tagues River zone, and a soil that was protected and away from the possibility of the presence of pollutants, hence the choice of Mata da Machada for being a protected place and local nature reserve. But as it is possible to verify with the developed growths, both obtained microorganisms that degrade hydrocarbons. In the case of Praia da Alburrica, it was expected, but in the case of Mata da Machada it was more likely that there would be no growth or very little because it is supposedly a protected area and even considered the “lung of the Barreiro city”. The possibility of the development of microorganisms in this sample that degrade hydrocarbons may have been due to being very close to the Barreiro highway and to the air pollution caused by vehicles that pass through this road that over the years the soil itself absorbs and develops microorganisms that resist and survive the presence of pollutants. In addition to being very close to this route, the lagoon from which this soil was removed may also have washed away some pollutants due to the movement of water over the years.

The hydrocarbons used to carry out this dissertation were hexane, gasoline and toluene in order to analyze the biodegradability of a simple, complex and aromatic hydrocarbon, respectively.

Through the specific growth rates of the samples with the hydrocarbons, it is possible to conclude that in most cases Mata da Machada obtained higher specific rates, that is, it had more generations formed in an exponentially growing population over time.

In the case of the maximum D.O. obtained in comparison with the growths of both samples, once again, Mata da Machada mostly obtained higher D.O. maximum and earlier than in relation to the growth with Praia da Alburrica, the exponential time also obtained most of them were higher in the growths with the sample Mata da Machada, and in most cases the difference is 1h of exponential growth in relation to the growths with Praia da Alburrica, being able to conclude that the sample of Mata da Machada in most growths had a better adaptation to the environment than the sample from Praia da Alburrica.

When growing with the 600 µL toluene hydrocarbon and the samples did not grow, it can be concluded that this hydrocarbon in this quantity is already very toxic and complex for the microorganisms present in the samples, so they cannot degrade it and consequently do not develop.

In the study of the concentration and type of pollutant in the sample Mata da Machada, 7 different types of colonies were obtained, and in 4 of the types of colonies (type 2, 3, 6 and 7) the presence of two types of microorganisms many different. In the other 3 types of colonies where symbiosis did not occur, the presence of bacilli was obtained (colony 1 – Gram negative bacilli; colony 4 and 5 – Gram positive bacilli). Regarding the number of colonies on each plate, in the case of the presence of 100 µL gasoline, 41 (4.1×10^6 UFC/mL) and 600 µL 169 (1.69×10^7 UFC/mL) gasoline were obtained. In the presence of 100 µL hexane, 129 (1.29×10^7 UFC/mL) and 600 µL 69 (6.9×10^6 UFC/mL) hexane were obtained. Finally, in the presence of toluene, 94 (9.4×10^6 UFC/mL) were obtained and it can be concluded that in the growth with the Mata da Machada sample in the presence of 600µL gasoline, it obtained a higher number of colonies and concentration of colony forming units. Growth with Mata da Machada in the presence of 100 µL gasoline contains more varieties of colony types (4), and in the presence of 600 µL gasoline, 100 µL and 600 µL hexane three different colonies were obtained and in the presence of 100 µL toluene, two different colony types.

In the study of the concentration and type of pollutant in the sample Praia da Alburrica, five different types of colonies were obtained, thus concluding that in the sample Mata da Machada developed more variety of colonies than Praia da Alburrica. Of the five different types of colonies, three of them contained the presence of two different types of microorganisms, thus proving that the symbiosis that occurred both in growth with the sample Praia da Alburrica and Mata da Machada with hydrocarbons is quite common because the environmental pressure that these hydrocarbons do, reinforce the existence of symbiosis for microorganisms to develop and remain active. The two colonies that did not contain two different types of microorganisms had positive bacilli. Regarding the number of colonies on each plate, in the

case of the presence of 100 µL gasoline, 7 (7×10^5 UFC/mL) and 600 µL gasoline were obtained, 104 (1.04×10^5 UFC/mL). In the presence of 100 µL hexane, 7 (7×10^5 UFC/mL) and 600 µL 145 (1.45×10^5 UFC/mL) hexane were obtained. Finally, in the presence of toluene an uncountable value was obtained, thus not being able to determine the number of colony-forming units, and it can be concluded that in the growth with the sample Praia da Alburrica in the presence of toluene 100 µL it obtained a greater number of colonies and concentration of colony-forming units as it contains a few colonies far greater than that of the previously described plates. The growth with Praia da Alburrica in the presence of 100 µL gasoline and 100 µL hexane contains more varieties of colonies (3), and in the presence of 600 µL gasoline two different types of colonies were obtained and in the presence of 600 µL hexane and 100 µL toluene, 1 different type of colony, concluding that the growth with Mata da Machada and hydrocarbons with the quantities of 100 µL and 600 µL obtained more varieties of colonies in each of the growths than those carried out with the sample Praia da Alburrica and hydrocarbons.

After conducting the previous study and identifying the existing colonies in each sample, *Pseudomonas aeruginosa* was investigated. The colonies chosen were the following, 1, 2, 3, 6, 7, 10, 11 and 12, as they had characteristics like the bacteria under study. After the incubation time, there was growth in colonies 1, 2, 3 and 10. The plates were subjected to the action of UV light to confirm the existence of fluorescence. With this research it is possible to conclude that the colonies where growth occurred contain *Pseudomonas aeruginosa*. In colony 1 it specifically presents in its morphology only one type of bacteria, Gram-Negative bacilli, thus being able to conclude that in this colony the type of bacteria that consists of it are *Pseudomonas aeruginosa*.

In the study of biodegradability, the colonies of Mata da Machada and Praia da Alburrica were analyzed by carrying out cell suspensions (growth of 10 mL) to increase the amount of biomass. Only colonies present in growths with hydrocarbons in the amount of 100 µL were analyzed. This decision was made because as there was no growth with 600 µL toluene, we would have an analysis of the colonies developed in the presence of all hydrocarbons. The study was carried out in 2 mL eppendorfs and the necessary volume adjustment was made to obtain the same amounts of components as those present in the reference used. These were placed for 5 days in growth to have the redox indicator DCPIP change from blue to colorless, proving the biodegradation of the hydrocarbon. Unfortunately, this sudden change in color never occurred and the absorbance spectrophotometer was then measured to confirm the rate

of biodegradability. After measurements it was found that there were changes in absorbances confirming some biodegradation. For all eppendorfs, five replicates were performed.

Tube 1, which is represented as a control, must have absorbance values higher than those obtained in the samples, as it is possible to prove that some type of biodegradation has occurred, as this control does not contain the hydrocarbon necessary for it to occur. As soon as all controls had higher values, it is possible to conclude that biodegradation occurred.

The colonies developed from the Mata da Machada sample in the biodegradation of gasoline in the presence of colony 1 obtained an average percentage of 34.59%, in the presence of colony 2 33.59% was obtained, in the presence of colony 3 obtained 33.47%, in the presence of colony 4, 37.49% was obtained, finally in the presence of the colony mixture (MMG) 40.96% was obtained. With the absorbance values obtained, it is possible to conclude that there was some biodegradation of Gasoline, which was not a very high degradation and for that reason the color change did not occur. The highest percentage of biodegradation obtained was in the presence of the colony mixture (MMG), thus it can also be concluded that the symbiosis that occurs between organisms is essential for better biodegradation results.

In the biodegradation of hexane in the presence of colony 1 an average percentage of 25.41% was obtained, in the presence of colony 2 25.78% % was obtained, in the presence of colony 3 26.28% was obtained, lastly in colony mixture (MMH) obtained 26.76%, proving again that although the biodegradation visually did not occur through the absorbances, a percentage of biodegradability was confirmed and, although the difference between the individual action of each colony is not very different, the colony mixture (MMH) shows superior biodegradation. It is also possible to conclude that colonies 1, 2 and 3 are more efficient in degrading Gasoline than Hexane.

In the biodegradation of toluene in the presence of colony 6 an average percentage of 8.53% was obtained, in the presence of colony 7 8.98% was obtained and the colony mixture (MMT) obtained 14.26%. How it is possible to verify and conclude with these obtained values is that despite the low biodegradability with all the analyzed colonies this occurred and that with the mixture of colonies (MMT) the percentage of biodegradation obtained is higher than that which occurred individually, proving, as already said previously, the joint action between microorganisms is very important for the whole process.

In the case of colonies that grew in Praia da Alburrica, the biodegradation of gasoline in the presence of colony 8 obtained an average percentage of 28.93%, in the presence colony 9 obtained 29.08% and in the presence of colony 10 27.84% was obtained. Finally, in

biodegradation with the colony mixture (AMG) an average percentage of 32.89% was obtained. With these percentages obtained, it is possible to conclude that the colonies that developed in the Mata da Machada sample degrade gasoline more effectively than the colonies developed in the Praia da Alburrica sample. In this case too, it is possible to conclude that the action of all the colonies together obtained results superior to those of the individual action of each colony.

In the biodegradation of hexane in the presence of colony 8 an average percentage of 16.13% was obtained, in the presence of colony 9, 21.59% was obtained and in the presence of colony 11, 26.85% was obtained. In the biodegradation with the colony mixture (AMH) an average percentage of 35.11% was obtained. In this analysis and in the previous ones, it is possible to conclude that the percentages are not very high, proving that the use of strategies during the biodegradation process is important for better results. It is also possible to conclude that the mixture of colonies from the sample Praia da Alburrica (AMH) can be more efficient in the degradation of Hexane than the mixture of colonies from the sample Mata da Machada (MMH).

In the biodegradation of toluene in the presence of colony 12, an average percentage of 16.37% was obtained, and it can be concluded that although only one type of colony grew, it presents higher values in comparison with all colonies, including the mixture of colonies, present in the sample Mata da Machada.

In summary of this dissertation, it was proved that in the samples Mata da Machada and Praia da Alburrica there are organisms, in this case only bacteria, which degrade hydrocarbons, even though the obtained biodegradation wiels were very low. The degradation of hydrocarbons occurred more easily through symbiosis and synergy between organisms.

6. Future Perspectives

Over the course of the dissertation, as mentioned above, the percentages of biodegradability of the hydrocarbons obtained were not very high. One of the optimizations that can be carried out in the future to obtain greater biodegradability is the addition of biostimulants, in the performance of growth with microorganisms individually or by mixing for the hydrocarbon biodegradation. Supplementation with one or more limiting nutrients, such as nitrogen, phosphorus, and sulfur, in hydrocarbon-laden places is important, as it helps in accelerating microbial growth and increases the hydrocarbon degradation capacity.

It would also be important, in the future, to study the effect of the pollutant concentration, since for toluene the addition of 600 μL was excessive, for the other contaminants it even led to higher growth rates. Conducting temperature and pH's studies would also be interesting to optimize the process.

In developing this dissertation, it was not possible to perform molecular techniques for obtaining rigorous organisms identification, so the application of these techniques would be important to understand which organisms are present in Mata da Machada and Praia da Alburrica that are able to degrade the studied hydrocarbons and assess the full potential of the bacteria consortium.

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Annex

Annex 1

HIMEDIA®

Technical Data

Bushnell Haas Agar M349

Intended Use:

Recommended for examination of fuels for microbial contamination and for studying hydrocarbon deterioration by microorganisms.

Composition**

Ingredients	Gms / Litre
Magnesium sulphate	0.200
Calcium chloride anhydrous	0.020
Potassium dihydrogen phosphate	1.000
Dipotassium hydrogen phosphate	1.000
Ammonium nitrate	1.000
Ferric chloride	0.050
Agar	20.000
Final pH (at 25°C)	7.0±0.2

**Formula adjusted, standardized to suit performance parameters

Directions

Suspend 23.27 grams in 1000 ml purified/distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri plates. A white precipitate prior to sterilization becoming yellow to orange after sterilization is normal.

Principle And Interpretation

Bushnell Haas Agar is prepared as per the formula of Bushnell and Haas (2) and recommended for the microbiological examination of fuels by the SIM Committee on microbiological deteriorations of fuels (1). These media contain all nutrients except carbon source, necessary for the growth of bacteria. Only those bacteria that are able to decompose hydrocarbon will grow in these media. Specific carbon source i.e. hydrocarbon can be added to this mediumand their utilization by different microorganisms can be studied.

These bacteria can decompose a variety of hydrocarbons like kerosene, mineral oil, paraffin wax and gasoline. For liquid hydrocarbon the hydrocarbon is layered on the surface of inoculated agar. For testing volatile hydrocarbons such as gasoline the Petri-plates containing the medium are inverted and the hydrocarbon is poured into the lid. Magnesium sulphate, calcium chloride and ferric chloride provide trace elements. Ammonium nitrate is a nitrogen source while monopotassium phosphate and potassium phosphate buffers the medium.

Type of specimen

Petroleum industry samples.

Specimen Collection and Handling

After use, contaminated materials must be sterilized by autoclaving before discarding.

Warning and Precautions :

Read the label before opening the container. Wear protective gloves/protective clothing/eye protection/ face protection. Follow good microbiological lab practices while handling specimens and culture. Standard precautions as per established guidelines should be followed while handling specimens. Safety guidelines may be referred in individual safety data sheets.

Limitations :

1. Further biochemical and serological tests must be carried out for further identification.

Performance and Evaluation

Performance of the medium is expected when used as per the direction on the label within the expiry period when stored at recommended temperature.

Please refer disclaimer Overleaf.

Quality Control

Appearance
White to cream homogeneous free flowing powder

Gelling
Firm, comparable with 2.0% agar gel.

Colour and Clarity of prepared medium
Light amber coloured, clear to slightly opalescent gel forms in Petri plates.

Reaction
Reaction of 2.33% w/v aqueous solution at 25°C. pH : 7.0±0.2

pH
6.80-7.20

Cultural Response
Cultural characteristics observed after an incubation at 25-30°C within 1 week.

Organism	Inoculum (CFU)	Growth (Plain)	Growth w/ minerals
Pseudomonas aeruginosa ATCC 27853 (00025*)	50-100	poor	good-luxuriant
Pseudomonas aeruginosa ATCC 9027 (00026*)	50-100	poor	good-luxuriant

Key : *Corresponding WDCM numbers.

Storage and Shelf Life

Store between 10-30°C in a tightly closed container and the prepared medium at 20-30°C. Use before expiry date on the label. On opening, product should be properly stored dry, after tightly capping the bottle in order to prevent lump formation due to the hygroscopic nature of the product. Improper storage of the product may lead to lump formation. Store in dry ventilated area protected from extremes of temperature and sources of ignition. Seal the container tightly after use. Product performance is best if used within stated expiry period.

Disposal

User must ensure safe disposal by autoclaving and/or incineration of used or unusable preparations of this product. Follow established laboratory procedures in disposing of infectious materials and material that comes into contact with sample must be decontaminated and disposed of in accordance with current laboratory techniques (3,4).

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