Potential of Native Microorganisms in Tagus Estuary

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Abstract**—** The Tagus estuary is heavily affected by industrial and urban activities, making bioremediation studies crucial for environmental preservation. Fuel contamination in the area can arise from various anthropogenic sources, such as oil spills from shipping, fuel storage and transfer operations, and industrial discharges. These pollutants can cause severe harm to the ecosystem and the organisms, including humans, that inhabit it. Nonetheless, there are always natural organisms with the ability to resist these pollutants and transform them into non-toxic or harmless substances, which defines the process of bioremediation. Exploring the microbial communities existing in soil and their capacity to break down hydrocarbons has the potential to enhance the development of more efficient bioremediation approaches. The aim of this investigation was to explore the existence of hydrocarbonoclastic microorganisms in six locations within the Tagus estuary, three on the north bank: Trancão River, Praia Fluvial do Cais das Colinas and Praia de Algés, and three on the south bank: Praia Fluvial de Alcochete, Praia Fluvial de Alburrica, and Praia da Trafaria. In all studied locations, native microorganisms of the genus *Pseudomonas* were identified. The bioremediation rate of common hydrocarbons like gasoline, hexane, and toluene was assessed using the redox indicator 2,6-dichlorophenolindophenol (DCPIP). Effective hydrocarbon-degrading bacterial strains were identified in all analyzed areas, despite adverse environmental conditions. The highest bioremediation rates were achieved for gasoline (68%) in Alburrica, hexane (65%) in Algés, and toluene (79%) in Algés. Generally, the bacteria demonstrated efficient degradation of hydrocarbons added to the culture medium, with higher rates of aerobic biodegradation of hydrocarbons observed. These findings underscore the necessity for further *in situ* studies to better comprehend the relationship between native microbial communities and the potential for pollutant degradation in soil.

Keywords **—** Biodegradability rate, Hydrocarbonoclastic microorganisms, Soil bioremediation, Tagus estuary.

I. INTRODUCTION

SITUATED near Lisbon and its metropolitan area, the Tagus estuary serves as the convergence point of the international river and the Atlantic Ocean. It holds the distinction of being the largest wetland in Portugal and one of the most significant in Europe, playing a pivotal role in both ecological and economic contexts. However, the presence of heavily urbanized zones and industrial clusters along its banks causes concerning environmental impacts. Although the sources of pollutants and contaminants in the Tagus estuary are varied, and despite global efforts to reduce the use of fossil fuels, petroleum hydrocarbons remain among the most prevalent chemicals contributing to soil and water pollution. Their presence alters the soil ecosystem balance and has far-reaching implications as not only does it lead to land degradation and water contamination, but also xenobiotics enter the food chain where they accumulate in various organisms [1], [2].

The presence of contaminants causes environmental pressure that promotes the growth of microorganism strains capable of metabolizing polluting agents. Many bacteria found in environments contaminated with oil-derived pollutants possess the ability to use hydrocarbons as a metabolic source of carbon and energy. Due to their high mutation rate, bacteria demonstrate greater adaptability and can survive in diverse environmental conditions, including varying levels of salinity, temperature, and oxygen availability. As a result, bacteria are the organisms with the highest potential for breaking down hydrocarbons and contributing to the bioremediation of contaminated locals [3], [4] ,[5], [6].

The five genera of bacteria indicated in the bibliography as having high hydrocarbonoclastic potential are: *Alcanivorax*, *Acinetobacter, Bacillus, Pseudomonas,* and *Rhodococcus* (Table 1).

Increasingly, the bioremediation approach, using living organisms or enzyme-mediated transformation, offers several opportunities to solve problems related to contamination of solid or liquid waste, since more than helping to reduce the volume of hydrocarbon contamination by breaking it down into smaller, less harmful compounds is a cost-effective solution, especially in areas that are difficult to access or where traditional clean-up methods may be impractical. can be carried out with minimal disruption to the environment, as it does not require heavy equipment or excavation [6], [7]. However, it is still a time-consuming process that depends not only on the chemical and physical properties of the pollutants, but also on the degradation capacity of the organisms and, therefore, is dependent on all the limiting factors that affect growth and survival [2].

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TABLE I
GENUS, CONSIDERATIONS, AND REFERENCES OF BACTERIA WITH HIGH HYDROCARBONOCLASTIC POTENTIAL

Genus	Considerations	References
Alcanivorax	Gram-negative and non-spore forming, and have been found in various marine environments, including open ocean water, sediment, and oil-contaminated beaches. They are also capable of utilizing a wide range of hydrocarbons, including alkanes, cycloalkanes, and aromatic compounds, as a source of carbon and energy.	[6],[8], [9], [10],[11]
Acinetobacter	Gram-negative bacteria that are commonly found in soil, water, and hospital environments. Are highly versatile and can be both beneficial and harmful to humans and the environment, depending on the species and context. *A. oleivorans* is known for its ability to degrade hydrocarbons	[3], [9], [10], [12], [13], [11]
Bacillus	Gram-positive bacteria that are commonly found in soil and other environments. These bacteria are rod-shaped and form endospores, which are highly resistant structures that allow them to survive in harsh conditions. Have great potential for bioremediation of environmental contaminants, including various hydrocarbons, such as crude oil, diesel, and gasoline due to their ability to produce a wide range of enzymes and their tolerance to extreme conditions.	[1], [10], [11] [12], [14], [15]
Pseudomonas	Gram-negative, aerobic bacteria that are found in a variety of natural and man-made environments, including soil, water, plants, and animals. Pseudomonas is known for its metabolic versatility, which allows it to use a broad range of substrates, including hydrocarbons, for energy and carbon. Can create biofilms, intricate communities of cells that are protected by a matrix.	[1], [3], [12], [10], [15], [16], [17], [11]
Rhodococcus	Gram-positive bacteria that are found in a wide range of natural and man-made environments, including soil, water, and plants. These bacteria are known for their ability to degrade a variety of organic compounds, including hydrocarbons, pesticides, and polychlorinated biphenyls (PCBs), making them important players in bioremediation. Can create biofilms.	[1], [8], [10], [11] [15], [17]

Studies on bioremediation in the Tagus estuary can lead to the development of more effective and sustainable methods for remediation. By understanding the mechanisms of microbial degradation and identifying the most efficient and robust microbial strains, it is possible to promote a natural, cost-effective, and safe way of cleaning up polluted sites. In addition, research on bioremediation in the Tagus estuary can provide insights into the unique characteristics of the estuarine environment and the factors that affect the efficiency of the process.

II. METHODOLOGY

A. Sampling

Experimental studies were carried out, referring to the verification of the existence and characterization of hydrocarbonoclastic microorganisms in soil samples obtained in six sampling sites, three on the south bank and three on the north bank of the Tagus estuary (Table 2) and Figure 1.

TABLE II
IDENTIFICATION OF SAMPLE COLLECTION SITES

Tagus bank	Code	Address	Coordinates
North	Trancão	Rio Trancão, Praça Mar da Palha, Sacavém, Lisboa	38º47’45’’N9º05 ’29’’W
	Cais das Colinas	Praia Fluvial do Cais das Colinas, Praça do Comércio, Lisboa	38º42’23’’N9º08 ’13’’W
	Algés	Praia de Algés, Carnaxide, Cascais, perto do Caminho Marítimo de Algés	38º41’45’’N9º13 ’52’’W
South	Alochete	Praia Fluvial de Alcochete, Rua da Praia, nº 15, Samouco, Alcochete	38º43’41’’N9º00 ’40’’W
	Alburrica	Praia Fluvial de Alburrica, Alburrica, Barreiro	38º39’26’’N9º05 ’18’’W
	Trafaria	Praia Trafaria, Trafaria, Almada	38º40’26’’N9º14 ’22’’W

In each location, subsamples were taken in ten zones chosen by zig zag technique with 20 cm depth. The soil subsamples collected were mixed and homogenized to obtain a representative single composite sample. Measurements were made of the ambient temperature and the pH and conductivity of the composite sample. The collected samples were stored in a refrigerator at 4°C.

Fig. 1 Map with the location of the six sampling sites of the Tagus estuary.

B. Selection of hydrocarbonoclastic microorganisms

The study aimed to address the variability of petroleum hydrocarbons (PHCs) by selecting three simpler representative compounds: gasoline (a mixture of linear hydrocarbons with 5-10 carbon atoms), hexane (a linear 6 carbon compound), and toluene (an aromatic compound also known as methylbenzene). To select the hydrocarbonoclastic microorganisms, 1 g of each composite sample was placed in 100 mL of freshly prepared Bushnell Haas Mineral Salt medium (BH) and stirred continuously at 180 rpm at 25°C for 5 days. After microbial selection, an enrichment step was performed by transferring the microbial population to Luria–Bertani medium (LB) and allowing for overnight growth at 25°C and 180 rpm before conducting further growth studies. Optical density (OD) readings at 600 nm were taken in a microplate reader (BMG LABTECH) to monitor bacterial growth in BH containing 0.4% (v/v) and 0.6% (v/v) of the corresponding pollutant as the sole carbon source. The initial OD 600nm was adjusted to 0.1 for all assays.

C. Morphological characterization of organisms

Cultivation of bacterial colonies was carried out on BH agar plates supplemented with 0.4% and 0.6% (v/v) of PHCs and incubated at 25 °C for 7 days. To avoid bias, morphological characterization was conducted on plates with 5 to 15 colonies. Gram staining and bioremediation studies were performed using purified strains. *Pseudomonas* identification was done using a selective medium and various test such as Wood's lamp and oxidase test. Colonies showing fluorescent pigment under Wood's lamp and positive oxidase test were confirmed as *Pseudomonas aeruginosa*, while colonies without fluorescent pigment but a positive oxidase test and glucose fermentation were also identified as *Pseudomonas aeruginosa*. Other colonies with a positive oxidase reaction but no glucose fermentation were considered as *Pseudomonas spp*.

D. Determination of the rate of bioremediation

The rates of bioremediation for the three PHCs at bench-scale experiment were established by utilizing the redox indicator 2,6-dichlorophenolindophenol (DCPIP) [18] following a 15-day incubation period at 25°C and 180 rpm. Assays were performed in triplicate, and the rates calculated using Equation 1.

$$\%\ bioremediation = 100 - \left(\frac{Abs\ 15\ day}{Abs\ 0\ day} * 100\right) \quad (1)$$

III. Results

A. Samples Characterization

The composite samples obtained from each location were examined for color. Among them, the samples collected from Cais das Colinas, Algés, Alburrica, and Trafaria exhibited relatively similar color patterns, characterized by a blend of yellow, gray, and brown sand grains with a sandy texture, resulting in a partially homogeneous sand mixture. On the other hand, the samples from Trancão and Alcochete showed a distinct color pattern, displaying shades of gray, black, and brown, with a texture that was both clayey and sandy.

At the sampling sites, the air temperature, the temperature of the composite sample, its pH value and conductivity were determined and are recorded in Table 3.

TABLE III

Characterization of Temperature, pH and Conductivity of Samples

Code	T air (°C)	T sample (°C)	pH	Conductivity (mS/cm)
Trancão	24.6	24.9	7.0	8.00
Cais das Colinas	21.3	21.9	6.5	4.08
Algés	20.0	20.5	6.0	3.03
Alochete	30.4	28.1	6.0	8.54
Alburrica	31.9	30.9	6.0	3.55
Trafaria	30.3	28.5	6.0	3.90

B. Microbial growth curves

The growth curves (Figure 2) show that in all conditions studied, the maximum OD achieved remained below 1 being the stationary phase reached after a long period of time, 18 to 124 hours. The more relevant difference was observed during the microbial growth in the presence of toluene, with longer lag phases. A long lag phase at a microbial growth curve indicates that the microorganisms are taking longer than usual to adapt to their new environment and start dividing rapidly. This can be due to a variety of factors such as a suboptimal growth environment, lack of essential nutrients, or the presence of inhibitory compounds.

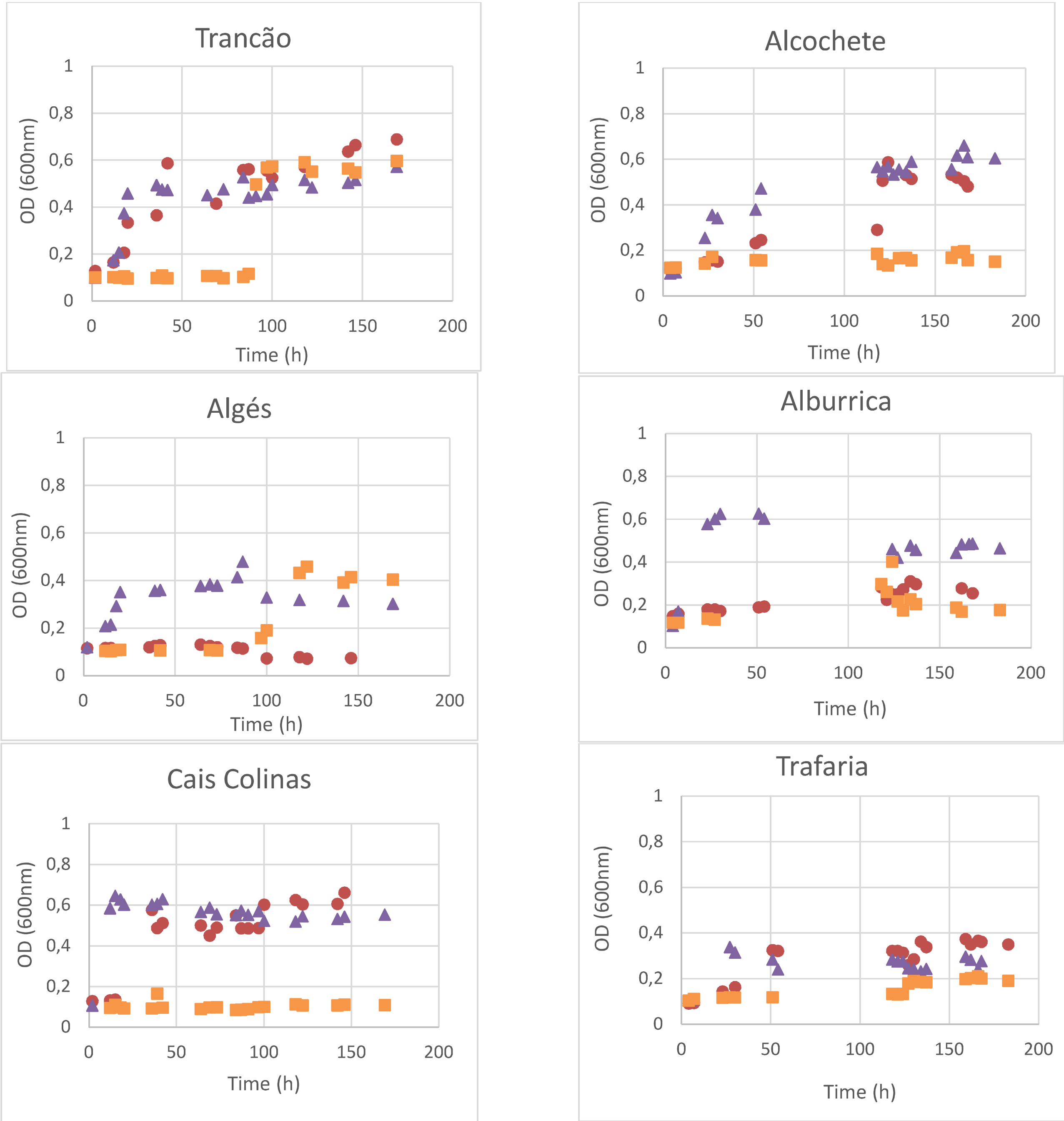

Fig. 2 Growth curves with hydrocarbons (square – toluene, triangle - gasoline, circle - hexane).

TABLE IV

RESULTS ACHIEVED FROM THE MAXIMUM OD, AND THE TIME FOR THE MAXIMUM OD, THE SPECIFIC GROWTH RATES, OF EACH SAMPLE WITH THE VARIOUS HYDROCARBONS

Sites	PHC	Max. OD	Time Max. OD (h)	Specific growth rate - μ (h^{-1})
Trancão	Gasoline	0.687	64	0.0107
	Hexane	0.459	20	0.0374
	Toluene	0.570	97	0.0467
Cais das Colinas	Gasoline	0.512	42	0.0194
	Hexane	0.629	18	0.0335
	Toluene	0.093	36	0.0026
Algés	Gasoline	0.131	64	0.0020
	Hexane	0.480	87	0.0055
	Toluene	0.459	122	0.0122
Alcochete	Gasoline	0.588	124	0.0047
	Hexane	0.566	118	0.0048
	Toluene	0.172	27	0.0064
Alburrica	Gasoline	0.282	118	0.0024
	Hexane	0.601	27	0.0229
	Toluene	0.401	124	0.0032
Trafaria	Gasoline	0.325	51	0.0063
	Hexane	0.749	23	0.0359
	Toluene	0.134	118	0.0012

C. Cultivation and identification of bacterial colonies

A colony morphology, also known as morphotype, refers to a cluster of bacteria originating from a single cell that has been grown on the surface of an agar. These clusters are known as colony forming units (CFUs) and exhibit a distinct colonial pattern. To be included, the morphotypes observed were required to appear in at least 3 out of 5 replicates. All observable colonies exhibited a smooth structure, colour pigmented (white/beige) and opaques, raised position in relation to the culture medium, had completely intact margins. Differentiation of the hydrocarbonoclastic colonies was achieved based on size, and margin.

TABLE V

CHARACTERIZATION, GRAM TEST AND IDENTIFICATION OF *PSEUDOMONAS* SPECIES, OF THE CULTIVABLE BACTERIAL COLONIES FROM THE SIX SAMPLED LOCALS

	Colony Morphology		Microscopic Characteristics		
Type	Size (mm)	Margin	Shape	Gram Test	Identification of *Pseudomonas*
1	Large (> 5)	Wavy	Bacilli	Negat.	*Pseudomonas spp*
2	Medium (2 -5) and small (<2)	Entire	Bacilli	Negat.	*Pseudomonas aeroginosa*

Morphotype 1 colonies were present in every sample, with exclusive presence in Cais das Colinas, Algés, Alburrica, and Trafaria. Meanwhile, morphotype 2 colonies were predominantly found in soil samples from the river Trancão and Alcochete.

D. Bioremediation rates

Relatively high bioremediation rates were observed for the three pollutants, with values ranging from 10% (Cais das Colinas) to 79% (Algés) for the biodegradation of toluene. The degradation rates of gasoline ranged from 31% (Cais das Colinas) to 68% (Alburrica), while the degradation rates of hexane ranged from 47% (Trancão) to 65% (Algés) (Figure 3).

Fig. 3 Bioremediation rates for the three pollutants and respective sampling locals.

IV. DISCUSSION

Both the air temperature and the temperature of the composite sample showed similar values ranging from 20 ^{0}C to 31.9 ^{0}C. The pH values ranged from 6 to 7, while the conductivity ranged between 3.03 and 8.54 mS/cm. There was no correlation found between these physical parameters and the variation in the number of cultivable colonies selected or their morphotype.

The growth rates observed in this study were very low, ranging from 0.0026 h^{-1} (Cais das Colinas) to 0.0467 h^{-1} (Trancão), with long lag phase times indicating a slow adaptation of microorganisms to their new environment before they start dividing rapidly. This could be due to suboptimal growth conditions, lack of essential nutrients, or the presence of inhibitory compounds, but it can also be an indicator of the low abundance of these microorganisms.

All of the cultivable microorganisms selected from the six locations belonged to the genus *Pseudomonas*, and there was no difference in morphotypes between the north and south banks of the Tagus River. These results are consistent with others studies [19], [20] that identified *Pseudomonas aeruginosa* in samples from a freshwater lake, which was responsible for 87-100% of the biodegradation of n-alkanes derived from petroleum with carbon chain lengths between C-13 and C-15.

Despite the low levels and limited variation of the hydrocarbonoclastic microorganisms, the bioremediation rates were remarkably high. The biodegradation rates for toluene ranged from 10% to 79%. Similarly, the bioremediation rates for hexane consistently reached or exceeded 50%. The bioremediation rates for gasoline were also greater than 50%, except for the location of Cais das Colinas, where a substantially lower rate of 31% was recorded.

V. Conclusion

Although laboratory-based biodegradation tests simulate natural processes, it is impossible to precisely replicate natural biodegradation due to the variation of multiple environmental factors, such as soil physicochemical properties, environmental conditions, and microbial populations involved in biodegradation. However, utilizing autochthonous microorganisms in these studies has an advantage since they are better adapted not only to the presence of pollutants but also to the abiotic and biotic parameters of the environment. Nevertheless, the research helped to enhance our understanding of indigenous microorganisms in various locations within the Tagus estuary. Furthermore, the study concludes that the low levels and limited variability of these bioindicators do not suggest elevated levels of pollution.

Bioremediation studies in the Tagus estuary are crucial for addressing the environmental challenges caused by human activities in the region. By developing effective and sustainable remediation methods, it is possible to restore the ecological balance of the estuary, protect the health of the organisms and humans that depend on it, and promote the sustainable use of its resources.

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