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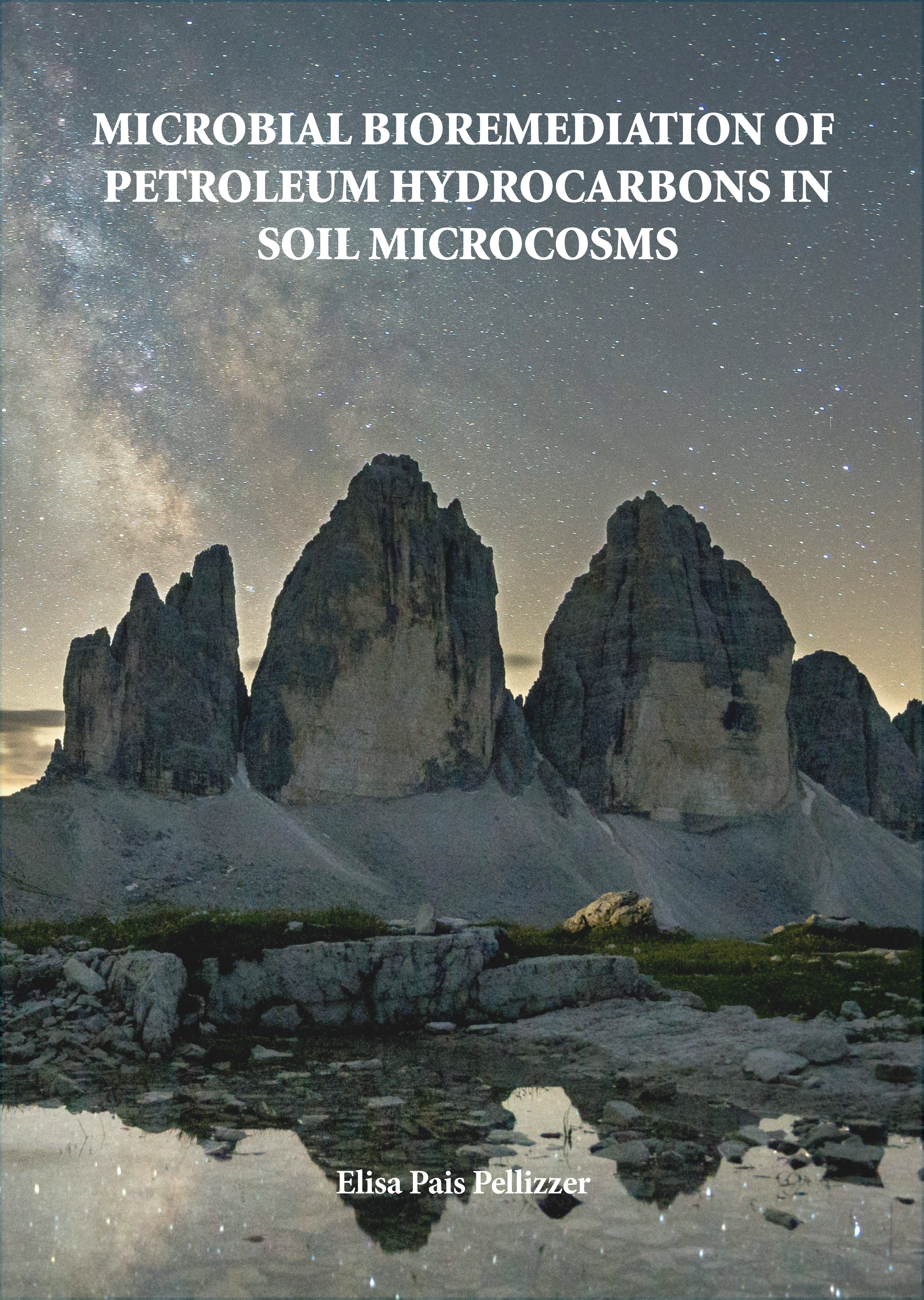
MICROBIAL BIOREMEDIATION OF PETROLEUM HYDROCARBONS IN SOIL MICROCOSMS



Elisa Pais Pellizzer

Microbial bioremediation of petroleum hydrocarbons in soil microcosms

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ERRATA

Correction regarding the acknowledgements in Thesis “Microbial bioremediation of petroleum hydrocarbons in soil microcosms”

The corrected information should be considered as follows on page 122:

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Elisa Pais Pellizzer

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ERRATA

Correção referente aos agradecimentos na tese “Biorremediação microbiana de hidrocarbonetos de petróleo em microcosmos do solo”

A informação correta deve ser considerada a seguinte na página 124:

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Elisa Pais Pellizzer

22 de maio 2025

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PhD thesis

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Dolce è capire
Che non son più solo
Ma che son parte di una immensa vita
Che generosa risplende intorno a me
Dono di Lui, del Suo immenso amore

San Francesco d’Assisi

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Chapter 1

General Introduction and Thesis Outline

General Introduction

1. Characterization and Uses of Petroleum

Petroleum is a complex mixture of various organic compounds (Andrade et al., 2010), mainly composed of four fractions of hydrocarbons: aromatics, saturates, resins, and asphaltenes. Aromatic hydrocarbons contain at least one aromatic ring, with the most well-studied being benzene, toluene, ethylbenzene, and xylene (BTEX), as well as polycyclic aromatic hydrocarbons (PAHs). Saturated hydrocarbons lack double bonds and can be classified based on their chemical structure into paraffins, which consist of aliphatic chain hydrocarbons, and naphthenes, represented by cyclic hydrocarbons with one or more saturated rings (Harayama et al., 1999). Resins and asphaltenes, in turn, include polar compounds in addition to carbon and hydrogen, containing trace amounts of nitrogen, sulfur, and/or oxygen. Asphaltenes are high-molecular-weight compounds that are insoluble in solvents such as n-heptane. In contrast, resins are polar molecules soluble in such solvents and include heterocyclic compounds, acids, and sulfoxides (Harayama et al., 1999).

Since the 19th century, modern industry has extracted and utilized petroleum and its derivatives on a large scale for various purposes. These include, among others, fuel for transportation, electricity generation, heating, plastic production, pharmaceutical applications, fertilizers, pesticides, lubricants, asphalt, cosmetics, and cleaning products. Such uses demonstrate how petroleum has become part of modern society, enabling significant advancements in different socioeconomic sectors. For example, petroleum-derived fuels allow intercontinental travel to be completed in hours rather than days. Sterile plastic materials in hospitals reduce infection risks, while the widespread use of disposable materials in laboratories accelerates analyses and experiments, minimizing contamination risks and contributing to scientific progress (Hess et al., 2011; Landrigan et al., 2023). Additionally, hydrogen derived from methane is used to produce ammonia, a globally significant fertilizer that enables agriculture in previously infertile areas (American Geosciences Institute, 2018).

2. Petroleum Production and Transport: Risks and Environmental Issues

Global petroleum production has been steadily increasing for decades (Figure 1) and reached 102 million barrels per day (Mb/d) in 2023, the highest recorded figure, according to the U.S. Energy Information Administration. Furthermore, Brazil experienced a 12% increase in petroleum production in 2023 compared to the previous year (U.S. Energy Information Administration, 2024). The vast quantity of petroleum extracted must be processed, transported, and distributed to various locations. However, accidents during these processes can occur, posing potential threats to ecosystems in affected regions (Varjani et al., 2017).

One significant incident was the 2010 Deepwater Horizon platform explosion in the Gulf of Mexico, approximately 80 km off the coast of Louisiana, USA, which released 779,000 tons of oil into the sea. This spill reached coastal zones, causing severe social and economic damage (CETESB, 2016). Another notable event was the 2019 contamination of beaches in northeastern Brazil, which is considered the most extensive oil spill in tropical oceans (Soares & Rabelo, 2023). The Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) reported that 159 individual animals were affected by the oil, 112 of which were found dead (IBAMA, 2020). Moreover, polycyclic aromatic hydrocarbons (PAHs) were detected in fish samples from the region, which were subsequently sold for human consumption (MAPA, 2019).

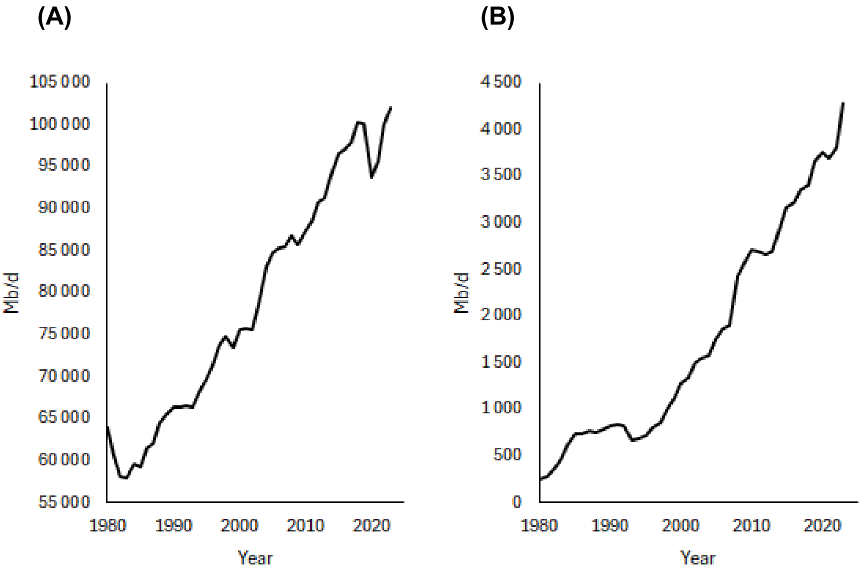


Figure 1. Production of petroleum and other liquids annually (a) worldwide and in (b) Brazil. (Modified from U.S. Energy Information Administration, 2024).

Despite most Brazilian oil being of marine origin, refineries, where petroleum derivatives are obtained, are located onshore (National Petroleum Agency, 2020). Moreover, the transportation and storage of petroleum-derived fuels are predominantly conducted via terrestrial routes. Consequently, the soil becomes highly susceptible to contamination risks from potential accidents, which may lead to groundwater pollution, a critical resource for the survival of plants, animals, and humans (Jagtap et al., 2014). Furthermore, soil contamination by petroleum and its derivatives significantly impacts its physical, chemical, biological, and structural properties, compromising fertility, water conductivity, and microbial diversity (Popoola et al., 2022; Nassar & Said, 2021). Hydrocarbons form hydrophobic films that reduce water retention capacity, disrupt nutrient balance, and promote anaerobic conditions, thereby hindering essential microbiological processes (Sutton et al., 2013; Truskewycz et al., 2019). As a consequence of these contamination events, aquatic and terrestrial ecosystems can be severely affected (Almeida & Corso, 2014; Ugochukwu & Ifeanyi, 2013).

3. Soil Hydrocarbon Remediation

Given the detrimental impacts of petroleum and its derivatives on living organisms and the environment, the remediation of contaminated soils is a crucial issue in environmental management and public health. Different techniques have been developed to mitigate these effects, including physical, chemical, and biological methods (Kwon et al., 2023; Gao et al., 2024). These strategies can be performed either *in situ*, directly at the contaminated site, or *ex-situ*, involving the removal of soil for treatment in another location. The choice of the most appropriate method depends on factors such as the type and structure of the contaminant, soil characteristics, available budget, and future land use objectives (Stepanova et al., 2022).

Among biological methods, bioremediation has been widely studied and validated in cases of oil-contaminated soils. This process primarily utilizes microorganisms, plants, or enzymes to remove contaminants from the soil and other environments (Gouma et al., 2014). Two principal bioremediation techniques are bioaugmentation and biostimulation. Biostimulation involves enhancing the activity of native microbiota by adding nutrients or substrates, thereby promoting their growth and metabolic activity (Boopathy, 2000; De Almeida Andrade et al., 2010; Fuentes et al., 2014). Conversely, bioaugmentation introduces exogenous microorganisms capable of degrading contaminants to accelerate remediation, particularly in areas with high contaminant concentrations or where rapid intervention is required (Boopathy, 2000; Fuentes et al., 2014).

Biostimulation is particularly effective in environments where native microbiota possess the inherent ability to degrade contaminants but are limited by essential nutrients, such as nitrogen and phosphorus, which are often scarce in contaminated sites. Both organic and inorganic fertilizers can effectively boost microbial degradation capacities (Udume et al., 2023; Macci & Doni, 2023). Meanwhile, bioaugmentation faces challenges such as ensuring that introduced exogenous microorganisms adapt and compete successfully with native microbial communities, which often does not occur (Udume et al., 2023). In environments where contaminants are aged and resistant to biological degradation, biostimulation has proven more effective, especially in cases where immediate intervention is unnecessary. Both techniques have advantages and limitations, with the choice depending on factors such as contamination type, remediation urgency, and site characteristics. Combining these approaches can maximize bioremediation outcomes across diverse environmental contexts.

4. Bioremediation Using Microorganisms

Microorganisms are abundant in soil and can be classified based on their energy source. Chemotrophs derive energy from redox reactions, while phototrophs obtain energy from light. Considering the carbon source utilization, they can also be categorized as autotrophs and heterotrophs. The autotrophs utilize inorganic carbon, while the heterotrophs use organic carbon. Chemotrophic heterotrophs, which obtain energy through redox reactions involving organic compounds, are prevalent in soil. These organisms require soil nutrients for growth and reproduction, with nutrient availability depending on nutrient cycling and environmental conditions.

In petroleum-contaminated soil, hydrocarbons become a carbon source for various microorganisms (Xu et al., 2018). Bioremediation's success in treating contaminated soils is often linked to the activity of microorganisms capable of degrading diverse hydrocarbon classes (Ayilara & Babalola, 2023). Many bacterial and fungal species can degrade hydrocarbons into less recalcitrant products (Cerqueira, 2011). Thus, identifying microorganisms involved in petroleum degradation is vital for developing effective bioremediation strategies (Janani Prathiba et al., 2014).

Microbial enzymes are key components of biodegradation processes (Xu et al., 2018). For instance, alkane monooxygenase, alcohol dehydrogenase, cyclohexanol dehydrogenase, methane monooxygenase, and cyclohexane monooxygenase are involved in alkane degradation. Enzymes such as naphthalene dioxygenase, cis-2,3-dihydroxybiphenyl-2,3-diol dehydrogenase, and salicylaldehyde dehydrogenase contribute to naphthalene breakdown. Meanwhile, benzene, toluene, and ethylbenzene dioxygenases target other hydrocarbons (Bacosa et al., 2018).

4.1. Hydrocarbon Degradation by Bacteria

Bacteria metabolize hydrocarbons to fulfill their nutritional needs, mitigating the chronic environmental impacts of these compounds. Numerous studies have identified bacteria in petroleum-contaminated habitats, including spill sites and reservoirs, with bacterial species richness and abundance directly linked to pollutant types and associated ecological variables (Mishra et al., 2023; Chunyan et al., 2023). Common bacterial genera involved in hydrocarbon degradation include Pseudomonas, Acinetobacter, Rhodococcus, Bacillus, and Streptomyces (Chunyan et al., 2023).

Hydrocarbon degradation by bacteria involves specific metabolic pathways and a range of enzymes. For aliphatic hydrocarbons, primary pathways include terminal and subterminal oxidation, where enzymes like alkane monooxygenases and Cytochrome P540 catalyze the initial conversion into alcohols (Figure 2). These intermediates are subsequently transformed into fatty acids and metabolized via β-oxidation or the tricarboxylic acid (TCA) cycle (Chunyan et al., 2023). Degradation efficiency can reach up to 95% for short-chain aliphatics (Grishchenkov et al., 2000).

However, efficiency decreases with increasing molecular weight and structural complexity (Yang et al., 2014).

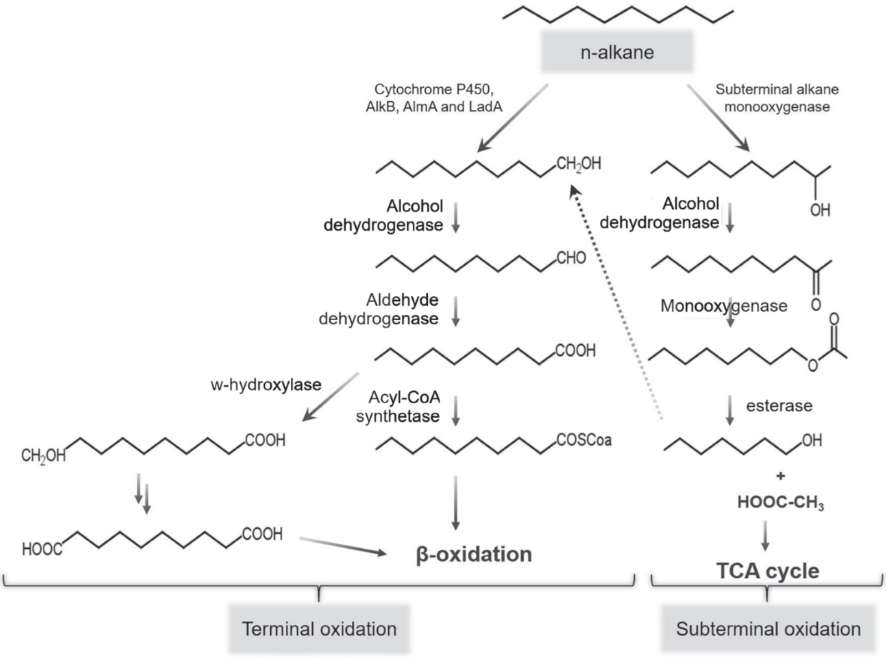


Figure 2. Aerobic pathway for n-alkanes degradation by oxidation (Modified from Cabral et al., 2022).

In the degradation of polycyclic aromatic hydrocarbons (PAHs), such as naphthalene, the process typically involves the action of dioxygenases, dehydrogenases, and other enzymes that convert these compounds into metabolic intermediates. These intermediates subsequently enter the tricarboxylic acid (TCA) cycle, fully mineralizing them into CO_2 and water. Aromatic Ring Hydroxylating Dioxygenases (ARHDs) play a key role in the breakdown of aromatic compounds. These enzymes are encoded by α-ARHD genes, named explicitly according to the substrate they degrade. Examples include genes such as *bphA* (biphenyl), *bnzA* (benzene), *ndoB* (naphthalene), *cbaA* (chlorobenzoate), *xylX* (toluate), *todC1* (toluene), *CumA1* (cumene), *ipbA1* (isopropylbenzene), *edoA* (ethylbenzene), and *ebdA* (alkylbenzene), among others (Seo, Keum, & Li, 2009). Although numerous catabolic genes from cultured microorganisms have been identified, many catabolic pathways remain poorly characterized, necessitating further research for their complete elucidation.

4.2. Hydrocarbon Degradation by Fungi

Similar to bacteria, fungi are essential microorganisms in hydrocarbon degradation, owing to their adaptability and capacity to thrive under adverse environmental conditions (Palmgren & Ivarsson, 2024). In addition to using hydrocarbons as energy sources, many fungi can incorporate these compounds into anabolic processes, synthesizing cellular components such as phospholipids, free fatty acids, sterols, and triglycerides (Napolitano & Juárez, 1997). This metabolic versatility positions fungi as critical agents in the bioremediation of soils and water contaminated with petroleum hydrocarbons (Mekonnen et al., 2024).

Fungi can degrade PAHs via two main pathways: one involving ligninolytic enzymes and the other involving non-ligninolytic enzymes (Figure 3). The primary ligninolytic enzymes involved in this process are lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac) (Bamforth & Singleton, 2005; Niladevi et al., 2008). These extracellular enzymes utilize free-radical-based reactions to oxidize and mineralize PAHs into non-toxic compounds like carbon dioxide and water (Rathankumar et al., 2022).

Among the non-ligninolytic enzymes involved in PAH and alkane degradation, cytochrome P450 monooxygenases stand out. These enzymes, which are also found in bacteria, catalyze oxidation reactions that increase the solubility of hydrocarbons, making them more amenable to subsequent metabolic processes. Cytochrome P450 enzymes have been identified in several fungal species, including *Aspergillus niger*, *Fusarium oxysporum*, and *Penicillium chrysogenum* (Kitazume et al., 2000; Huarte-Bonnet et al., 2018).

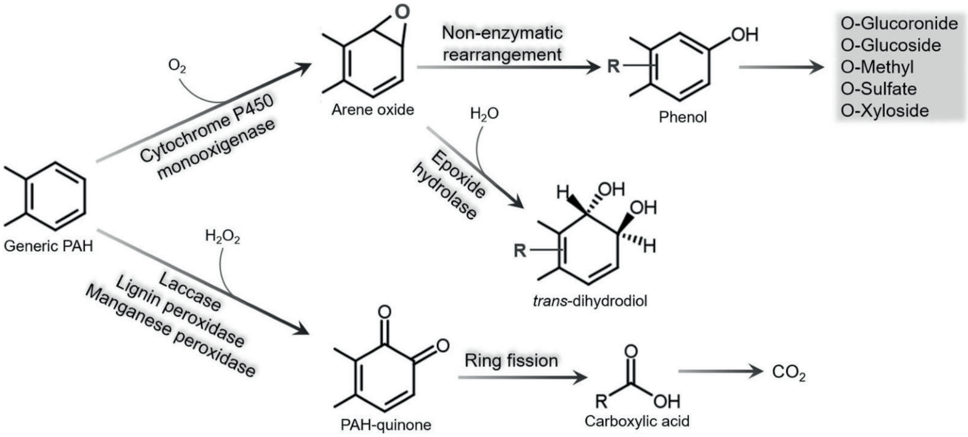


Figure 3. Pathways of aerobic degradation of Polycyclic Aromatic Hydrocarbons (PAHs) (Cabral et al., 2022).

Genes encoding hydrocarbon-degrading enzymes have been extensively studied in fungi, particularly in the CYP52 and CYP53 families, which encode cytochrome P450 enzymes. For instance, studies on fungi such as *Aspergillus niger* and *Penicillium chrysogenum* have associated the CYP52 family with the ability to oxidize alkanes of varying chain lengths (Huarte-Bonnet et al., 2018). Meanwhile, the *P450foxy* gene from the CYP505 family plays a role in subterminal oxidation, a less common pathway in the fungal kingdom (Palmgren & Ivarsson, 2024). Research by Trippe et al. (2014) revealed that fungi capable of metabolizing gaseous alkanes, such as those with C_2–C_4 chain lengths, possess genes that are induced by the presence of these compounds, underscoring their importance in survival and growth in hydrocarbon-contaminated environments.

4.3. Microbial Consortia

Bacteria and fungi's ability to degrade aliphatic and aromatic hydrocarbons is directly linked to their enzymatic production. These enzymes, encoded by specific genes, make microorganisms highly versatile and efficient agents in the bioremediation of contaminated environments. Using multiple organisms in consortium-based bioremediation increases the enzymatic diversity, often leading to more effective hydrocarbon degradation.

Microbial consortia composed of hydrocarbon-degrading microorganisms show greater efficiency in bioremediation than single organisms (Ebadi et al., 2021; Mekonnen et al., 2024). The mineralization of complex hydrocarbon compounds often requires synergistic cooperation between fungi and bacteria (Prenafeta-Boldú et al., 2018; Giovanella et al., 2020). However, the interactions within microbial consortia must be carefully evaluated, as they can either enhance or inhibit the degradation process. Certain microorganisms may inhibit the growth of others, compromising overall efficiency. Antagonism tests are crucial in bioaugmentation strategies to detect such inhibitory effects (Vieira et al., 2021).

On the other hand, positive interactions such as cross-feeding, co-metabolism, and biofilm formation can significantly enhance hydrocarbon degradation. For instance, some bacterial strains can utilize intermediate metabolites produced by the fungal degradation of PAHs, effectively completing the mineralization process (Harms et al., 2011). Such synergistic interactions illustrate why well-selected microbial consortia are often more effective than single strains in complex bioremediation scenarios. Understanding these interactions enables the selection of more effective consortia, optimizing bioremediation performance and mitigating potential inhibition issues (Giovanella et al., 2021).

5. Toxicity Analyses in Bioremediation

The toxicity of hydrocarbons has been widely evaluated using various organisms and cells. Toxicity assays have included plants such as *Allium cepa*, *Eruca sativa*, *Vigna radiata*, *Phaseolus mungo*, *Cucumis sativus*, and *Lycopersicum esculentum*; bacteria such as *Salmonella typhimurium* (Ames test); and human leukocytes (Comet assay) (Cruz et al., 2019; Feretti et al., 2019; Haq & Kalamdhad, 2021; Sharma et al., 2021; Mbadra et al., 2023). Mbadra et al. (2023) assessed the impact of PAHs, including naphthalene, fluoranthene, benzo(a)pyrene, and chrysene, found in soils near highways. Their study highlighted the negative effects of PAHs on seed germination and the growth of *L. esculentum* (tomato). Ames and Comet assays also revealed the mutagenic effects of PAHs and metals in industrial air pollution in northern Italy (Feretti et al., 2018).

Petroleum is a complex mixture of organic compounds; contamination by a single hydrocarbon is rare. However, studies focusing on the toxicity of individual substances or derivatives are essential to identify which petroleum molecules cause cytotoxic, genotoxic, or mutagenic effects. For example, Cabaravdic (2010) demonstrated the cytotoxic and clastogenic effects of varying concentrations of benzo(a)pyrene, a petroleum-derived PAH, on *A. cepa*. Similarly, Cruz et al. (2019) showed phytotoxic and genotoxic effects of diesel-contaminated soil, while crude oil-contaminated soil exhibited even greater toxicity. These findings highlight the challenges of pinpointing specific compounds responsible for observed effects. Additionally, the synergistic toxicity of compound mixtures underscores the importance of testing individual hydrocarbons and their combinations.

Given hydrocarbons' toxicity to various organisms, evaluating toxic effects after bioremediation is crucial. Bahar et al. (2024) observed improved earthworm survival rates in hydrocarbon-contaminated soil treated with peat. Similarly, Sivaram et al. (2018) demonstrated reduced cytotoxic and genotoxic effects in *A. cepa* cells after phytoremediation of PAH-contaminated soil. However, Haq and Kalamdhad (2021) reported that secondary biological treatment alone was insufficient to remove pollutants from oil refinery effluent, as evidenced by residual phytotoxic, cytotoxic, and genotoxic effects in *A. cepa*.

Using a variety of test organisms (bioindicators) complements toxicity analyses. However, some limitations exist, particularly in experiments using petroleum as a pollutant. For instance, using invertebrates like collembolans requires large soil volumes, limiting microcosm studies. Similarly, assays using colorimetric changes, such as resazurin tests with *Escherichia coli* or *Salmonella*, are impeded by petroleum's opacity, which interferes with spectrophotometric readings. This issue also affects luminescent bacteria like *Aliivibrio fischeri*, whose bioluminescence reduction indicates toxicity but cannot be accurately measured in petroleum-contaminated samples.

6. Ecological Analyses in Bioremediation

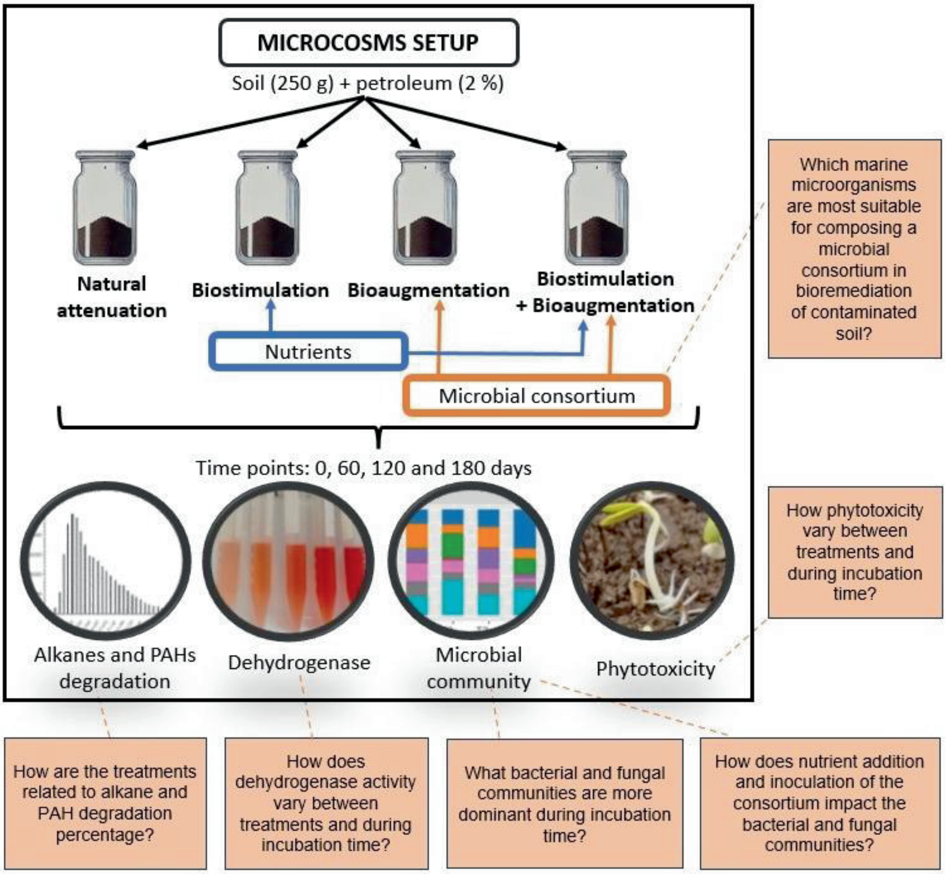
Bioremediation has emerged as an essential tool for mitigating hydrocarbon contamination in terrestrial and aquatic ecosystems. This process relies on the complex interactions between microorganisms and their environment, highlighting the importance of microbial diversity for ecological health. Rare and dominant microorganisms play distinct but crucial roles in hydrocarbon degradation, with their resilience and resistance driving ecosystem recovery. Rare bacterial species are particularly significant in pollutant degradation. For example, rare *Alkanindiges* species in diesel-contaminated soils can proliferate rapidly, contributing to hydrocarbon breakdown (Free et al., 2018).

Environmental factors shape microbial community assembly processes, which can be deterministic or stochastic. Deterministic processes arise when environmental selection favors specific traits, adapting the community to new conditions (Potts et al., 2022). Conversely, stochastic processes, driven by dispersal and drift, dominate in stable environments. Acute pollution events favor determinism, while chronic disturbances lead to stable, adapted states. Historical disturbance memory can facilitate rapid responses to new challenges, underscoring the importance of microorganisms in biodegradation and ecosystem resilience. Understanding the processes that control the assembly of microbial communities can help us predict the success of the bioremediation process.

Aim of the Thesis

This thesis aims to investigate bioremediation methods for petroleum-contaminated soil and understand microbial ecological dynamics of microcosms during incubation time (Figure 4). Specifically, the study seeks to:

1. Develop a microbial consortium based on antagonism tests, previous studies, and growth rate, using *Aspergillus sclerotiorum* CBMAI 849 as a base.
2. Compare the efficacy of bioremediation treatments, including natural attenuation, biostimulation, bioaugmentation, and combined biostimulation/bioaugmentation, by evaluating hydrocarbon degradation percentages, enzymatic activity, microbial communities, and phytotoxicity.
3. Elucidate the ecological dynamics of bacterial and fungal communities during bioremediation, focusing on microbial assembly processes, interactions, and adaptive responses to hydrocarbon contamination.



Which marine microorganisms are most suitable for composing a microbial consortium in bioremediation of contaminated soil?

How phytotoxicity vary between treatments and during incubation time?

How are the treatments related to alkane and PAH degradation percentage?

How does dehydrogenase activity vary between treatments and during incubation time?

What bacterial and fungal communities are more dominant during incubation time?

How does nutrient addition and inoculation of the consortium impact the bacterial and fungal communities?

Figure 4. Diagram of the research questions in this thesis. Questions related to the bioremediation of contaminated soil with petroleum using four different treatments.

Outline of the Thesis

Chapter 2 focuses on developing a microbial consortium for petroleum biodegradation based on the filamentous fungus *Aspergillus sclerotiorum* CBMAI 849, previously studied for its potential in diesel degradation. The consortium was created through *in vitro* antagonism tests with microorganisms derived from marine environments. Additionally, interactions between the fungal consortium and indigenous soil microbiota, including other fungal and bacterial species, could accelerate biodegradation.

Chapter 3 employs four different treatments (natural attenuation, biostimulation, bioaugmentation, and a combination of biostimulation and bioaugmentation) for the bioremediation of soil contaminated with petroleum. The marine fungal consortium composed in **Chapter 2** was used in the bioaugmentation treatments. We compared the effectiveness of these treatments in metabolizing alkanes and polycyclic aromatic hydrocarbons. We also monitored dehydrogenase enzymatic activity and phytotoxicity throughout the incubation time. Lastly, we evaluated bacterial and fungal community dynamics during the bioremediation process.

Chapter 4 delves into the ecological dynamics of soil microbiomes under hydrocarbon contamination, emphasizing the role of microbial community structure and assembly processes. By exploring the effects of biostimulation, bioaugmentation, and their combined application, we seek to understand how these interventions influence microbial interactions and community composition over time. A particular focus is placed on the distinct responses of bacterial and fungal communities and the interplay between deterministic and stochastic processes in shaping bacterial dynamics.

Chapter 5 discusses all results and links the chapters to answer the main questions made in this study. Moreover, it shows the limiting factors and future perspectives for research in bioremediation using microorganisms.

Appendices

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Summary

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Summary

Contamination by petroleum hydrocarbons poses significant environmental and health risks due to their toxicity and persistence in ecosystems. As global petroleum production continues to rise, the risks associated with such contamination remain a pressing concern. This challenge highlights the need for effective soil remediation techniques, particularly microbial bioremediation. By leveraging the enzymatic potential of microorganisms, bioremediation offers a promising approach to mitigating the environmental damage caused by petroleum hydrocarbons. Although numerous studies have investigated microbial bioaugmentation and biostimulation for the remediation of contaminated soils, few have examined hydrocarbon degradation and detoxification over time. Furthermore, most bioremediation studies have been conducted over short experimental periods, with relatively few extending beyond 120 days. Given the highly dynamic nature of microbial communities in petroleum-contaminated environments, there is still a limited understanding of how native and introduced microorganisms interact over extended periods and how these interactions impact bioremediation success. The present study aims to address these gaps by investigating the long-term hydrocarbon degradation and microbial community dynamics in petroleum-contaminated soils, providing new insights into the stability, efficiency, and sustainability of bioremediation approaches.

This thesis aims to (i) develop a microbial consortium for the bioaugmentation of petroleum-contaminated soil microcosms, (ii) apply bioremediation treatments in petroleum-contaminated soil under controlled microcosm conditions, and (iii) investigate the ecological dynamics of bacterial and fungal communities during bioremediation. The study is structured in five chapters: general introduction (Chapter 1), three experimental chapters (Chapters 2, 3, and 4), and a concluding chapter (Chapter 5).

Chapter 2 focuses on developing a microbial consortium for bioaugmentation in petroleum-contaminated soil. This consortium is based on the filamentous fungus *Aspergillus sclerotiorum* CBMAI 849. It was developed through antagonism tests involving microorganisms from marine environments. These tests revealed no antagonism between *A. sclerotiorum* and *Trichoderma harzianum* CBMAI 1229, leading to their selection as a fungal consortium. This chapter highlights the importance of considering microbial interactions when designing effective bioaugmentation strategies for petroleum-contaminated environments.

Chapter 3 evaluates the effectiveness of different bioremediation strategies, including natural attenuation, biostimulation, bioaugmentation, and a combination of biostimulation and bioaugmentation, in microcosm experiments. The marine fungal consortium developed in Chapter 2 was applied in the bioaugmentation treatment. The study monitored the degradation of alkanes and polycyclic aromatic hydrocarbons (PAHs), enzymatic activities, and phytotoxicity over 60, 120, and 180 days. The results

showed that bioaugmentation enhanced alkane degradation, while biostimulation promoted the degradation of PAHs and reduced soil toxicity. The combined biostimulation/bioaugmentation not only facilitated alkane and PAH degradation but also promoted the breakdown of high molecular weight alkanes. Microbial community analyses revealed a succession of hydrocarbon-degrading bacteria and fungi, emphasizing the importance of temporal dynamics in bioremediation.

Chapter 4 explores the ecological dynamics of microbial communities involved in bioremediation, focusing on the structure and drivers of bacterial and fungal populations under hydrocarbon contamination. By examining the effects of biostimulation, bioaugmentation, and their combined application, this chapter aims to elucidate how these interventions influence microbial interactions over time. The study found that bacterial communities tended to converge across treatments, particularly in nutrient-rich conditions, while fungal communities exhibited greater divergence, indicating different adaptive strategies. Rare microbial taxa became dominant during the bioremediation process, suggesting their crucial role in hydrocarbon degradation. Furthermore, bioaugmentation appeared to drive deterministic selection in bacterial communities, while biostimulation primarily influenced the growth of bacteria dependent on available nutrients. This chapter concludes that both biostimulation and bioaugmentation significantly impact microbial dynamics, enhancing hydrocarbon degradation through distinct ecological processes.

Finally, Chapter 5 synthesizes the findings to address the primary research questions, discussing their implications, the limitations encountered, and potential directions for future research in microbial-based bioremediation. This thesis underscores the critical role of microorganisms and their ecological dynamics in the bioremediation of petroleum-contaminated soil.

Nederlanndse Samenvatting

Verontreiniging door aardolieproducten vormt een aanzienlijk risico voor het milieu en de volksgezondheid vanwege hun toxiciteit en persistentie in ecosystemen. Nu de wereldwijde aardolieproductie blijft stijgen, blijven de risico's die met deze verontreiniging gepaard gaan een urgente zorg. Deze uitdaging onderstreept de noodzaak van effectieve bodemsaneringsmethoden, net zo als microbiële bioremediatie. Door gebruik te maken van het enzymatische potentieel van micro-organismen biedt bioremediatie een veelbelovende aanpak om milieuschade veroorzaakt door aardolieproducten te beperken. Hoewel talrijke studies microbiële bioaugmentatie en biostimulatie voor de sanering van verontreinigde bodems hebben onderzocht, hebben slechts weinigen zich gericht op de afbraak en detoxificatie van koolwaterstoffen in de tijd. Bovendien zijn de meeste bioremediatiestudies uitgevoerd over korte experimentele perioden, waarbij relatief weinig studies verder gingen dan 120 dagen. Gezien de sterk dynamische aard van microbiële gemeenschappen in met aardolie verontreinigde omgevingen, is er nog steeds een beperkt inzicht in hoe inheemse en geïntroduceerde micro-organismen op lange termijn met elkaar interageren en hoe deze interacties de effectiviteit van bioremediatie beïnvloeden. Deze studie beoogt deze kennishiaten te overbruggen door de langetermijnafbraak van koolwaterstoffen en de dynamiek van microbiële gemeenschappen in met aardolie verontreinigde bodems te onderzoeken. Dit zal nieuwe inzichten opleveren in de stabiliteit, efficiëntie en duurzaamheid van bioremediatiebenaderingen.

Deze thesis heeft als doel: (i) het ontwikkelen van een microbiële consortium voor bioaugmentatie van met aardolie verontreinigde bodemmicrokosmos, (ii) het toepassen van bioremediatiebehandelingen onder gecontroleerde microkosmosomstandigheden, en (iii) het onderzoeken van de ecologische dynamiek van bacteriële en schimmelgemeenschappen tijdens de bioremediatie. De studie is opgebouwd uit vijf hoofdstukken: een algemene inleiding (Hoofdstuk 1), drie experimentele hoofdstukken (Hoofdstukken 2, 3 en 4) en een concluderend hoofdstuk (Hoofdstuk 5).

Hoofdstuk 2 richt zich op de ontwikkeling van een microbiële consortium voor bioaugmentatie in met aardolie verontreinigde bodem. Dit consortium is gebaseerd op de draadvormige schimmel *Aspergillus sclerotiorum* CBMAI 849 en werd ontwikkeld via antagonismetests met micro-organismen uit mariene omgevingen. Deze tests toonden geen antagonisme aan tussen *A. sclerotiorum* en *Trichoderma harzianum* CBMAI 1229, wat leidde tot hun selectie als schimmelconsortium. Dit hoofdstuk benadrukt het belang van microbiële interacties bij het ontwerpen van effectieve bioaugmentatiestrategieën voor met aardolie verontreinigde omgevingen.

Hoofdstuk 3 evalueert de effectiviteit van verschillende bioremediatiestrategieën, waaronder natuurlijke attenuatie, biostimulatie, bioaugmentatie en een combinatie van biostimulatie en bioaugmentatie, in microkosmosexperimenten. Het in hoofdstuk 2 ontwikkelde mariene schimmelconsortium werd toegepast in de bioaugmentatiebehandelingen. De studie volgde de afbraak van alkanen en polycyclische aromatische koolwaterstoffen (PAK's), enzymatische activiteiten en fytotoxiciteit gedurende 60, 120 en 180 dagen. De resultaten toonden aan dat bioaugmentatie de afbraak van alkanen verbeterde, terwijl biostimulatie de afbraak van PAK's bevorderde en de bodemtoxiciteit verminderde. De gecombineerde toepassing van biostimulatie en bioaugmentatie versnelde niet alleen de afbraak van alkanen en PAK's, maar bevorderde ook de afbraak van alkanen met een hoog molecuulgewicht. Analyses van microbiële gemeenschappen toonden een opeenvolging aan van bacteriën en schimmels die koolwaterstoffen afbreken, wat het belang van temporele dynamiek in bioremediatie benadrukt.

Hoofdstuk 4 onderzoekt de ecologische dynamiek van microbiële gemeenschappen die betrokken zijn bij bioremediatie, met de nadruk op de structuur en drijvende krachten van bacteriële en schimmelpopulaties onder koolwaterstofverontreiniging. Door de effecten van biostimulatie, bioaugmentatie en hun gecombineerde toepassing te analyseren, wil dit hoofdstuk verduidelijken hoe deze interventies microbiële interacties in de tijd beïnvloeden. De studie toonde aan dat bacteriële gemeenschappen de neiging hadden om te convergeren tussen de behandelingen, vooral onder nutriëntenrijke omstandigheden, terwijl schimmelgemeenschappen grotere divergentie vertoonden, wat wijst op verschillende aanpassingsstrategieën. Zeldzame microbiële taxa werden dominant tijdens het bioremediatieproces, wat suggereert dat ze een cruciale rol spelen in de afbraak van koolwaterstoffen. Daarnaast leek bioaugmentatie een deterministische selectie in bacteriële gemeenschappen te stimuleren, terwijl biostimulatie vooral de groei beïnvloedde van bacteriën die afhankelijk zijn van beschikbare nutriënten. Dit hoofdstuk concludeert dat zowel biostimulatie als bioaugmentatie aanzienlijke invloed hebben op microbiële dynamiek en de afbraak van koolwaterstoffen bevorderen via verschillende ecologische processen.

Tot slot synthetiseert hoofdstuk 5 de bevindingen om de centrale onderzoeksvragen te beantwoorden, bespreekt hun implicaties, de ondervonden beperkingen en mogelijke richtingen voor toekomstig onderzoek naar microbiële bioremediatie. Deze thesis benadrukt de cruciale rol van micro-organismen en hun ecologische dynamiek in de sanering van met aardolie verontreinigde bodems.

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