UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

KELLY JOHANNA HIDALGO MARTINEZ

ASSESSMENT OF THE EFFECT OF DIFFERENT BIOREMEDIATION APPROACHES ON THE SOIL MICROBIOME OF BIOFUEL-AFFECTED AREAS

AVALIAÇÃO DO EFEITO DE DIFERENTES ABORDAGENS DE BIORREMEDIAÇÃO SOBRE O MICROBIOMA DE SOLOS DE ÁREAS CONTAMINADAS COM BIOCOMBUSTÍVEIS

CAMPINAS

2023

KELLY JOHANNA HIDALGO MARTINEZ

ASSESSMENT OF THE EFFECT OF DIFFERENT BIOREMEDIATION APPROACHES ON THE SOIL MICROBIOME OF BIOFUEL-AFFECTED AREAS

AVALIAÇÃO DO EFEITO DE DIFERENTES ABORDAGENS DE BIORREMEDIAÇÃO SOBRE O MICROBIOMA DE SOLOS DE ÁREAS CONTAMINADAS COM BIOCOMBUSTÍVEIS

Thesis presented to the Institute of Biology of the University of Campinas in partial fulfilment of the requirements for the degree of Doctor in Genetics and Molecular Biology in GENETICS OF MICROORGANISMS

Tese apresentada ao Instituto de Biologia da Universidade de Campinas para a obtenção do título de Doutora em Genética e Biologia Molecular na área de GENÉTICA DE MICRORGANISMOS

Orientador: *DRA. VALÉRIA MAIA MERZEL*

ESTE ARQUIVO DIGITAL CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA KELLY JOHANNA HIDALGO MARTINEZ E ORIENTADA PELA DRA. VALÉRIA MAIA MERZEL.

CAMPINAS

2023

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca do Instituto de Biologia
Mara Janaina de Oliveira - CRB 8/6972

H53a

Hidalgo Martinez, Kelly Johanna, 1985-
Assessment of the effect of different bioremediation approaches on the soil microbiome of biofuel-affected areas / Kelly Johanna Hidalgo Martinez. – Campinas, SP : [s.n.], 2023.

Orientador: Valéria Maia Merzel.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Biodiesel. 2. Gasohol. 3. Hidrocarbonetos - Biodegradação. 4. Biorremediação. 5. Solos - Poluição. I. Oliveira, Valeria Maia de, 1966-. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

Informações Complementares

Título em outro idioma: Avaliação do efeito de diferentes abordagens de biorremediação sobre o microbioma de solos de áreas contaminadas com biocombustíveis
Palavras-chave em inglês:
Biodiesel fuels
Gasohol
Hydrocarbons - Biodegradation
Bioremediation
Soil pollution
Área de concentração: Genética de Microorganismos
Titulação: Doutora em Genética e Biologia Molecular
Banca examinadora:
Valéria Maia Merzel [Orientador]
Juliana Velasco de Castro Oliveira
Marcelo Falsarella Carazzolle
Cynthia Canedo da Silva
Gileno Vieira Lacerda Junior
Data de defesa: 01-09-2023
Programa de Pós-Graduação: Genética e Biologia Molecular

Identificação e informações acadêmicas do(a) aluno(a)
- ORCID do autor: https://orcid.org/0000-0003-4607-3750
- Currículo Lattes do autor: http://lattes.cnpq.br/1712165372716579

Campinas, 01 de setembro de 2023

COMISSÃO EXAMINADORA

Profa. Dra. Valéria Maia Merzel (Orientadora)

Profa. Dra. Juliana Velasco de Castro Oliveira

Prof. Dr. Marcelo Falsarella Carazzolle

Profa. Dra. Cynthia Canedo da Silva

Prof. Dr. Gileno Viera Lacerda Junior

Os membros da Comissão Examinadora acima assinaram a Ata de Defesa, que se encontra no processo de vida acadêmica do aluno.

A Ata da defesa com as respectivas assinaturas dos membros encontra-se no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa (inserir o nome do programa de pós) da Unidade (inserir o nome da faculdade/instituto).

To my parents, Maria Fernanda and John for their unconditional love and support and for being my example of struggle and family unity;

To my sisters Jenny and Camila for always being there and because each one from their essence makes my soul happy;

To my grandfather for being my fan number one;

Thank you all for supporting me from a distance and believing in my potential.

Marcela for being my stronghold every day, supporting me in everything and walking by my side day by day.

I love you all so much

Para os meus pais, Maria Fernanda e John pelo amor e apoio incondicional e por serem meu exemplo de luta e união de família;

Às minhas irmãs Jenny e Camila por sempre estarem presentes e porque cada uma desde sua essência me alegram a alma.

Ao meu avô por ser o meu fã número um.

Obrigada a todos por me apoiar na distância e acreditar no meu potencial.

A Marcela por ser meu bastião todo dia, me apoiando em tudo e caminhando do meu lado dia a dia.

Eu amo muito todos vocês

A mis padres, María Fernanda y John por su amor y apoyo incondicional y por ser mi ejemplo de lucha y unidad familiar;

A mis hermanas Jenny y Camila por estar siempre ahí y porque cada una desde su esencia me alegra el alma;

A mi abuelo por ser mi fan número uno;

Gracias a todos por apoyarme desde la distancia y creer en mi potencial.

Marcela por ser mi fortaleza todos los días, apoyarme en todo y caminar a mi lado día a día.

Los amo mucho a todos!

“No se rinde el que nació donde por todo hay que luchar”

Gloria Martínez

Acknowledgments

Wow *que viagem*! It's been four intense years, full of learning, lessons, and unprecedented situations in everyone's lives, like a pandemic that forced us to rethink and innovate to continue with our lives through the new challenges imposed. In any case, these facts made my doctorate a special stage in my life that I will never forget.

First, I want to give a very special thanks to my supervisor, Dr. Valeria Maia Merzel. Your guidance and unconditional support certainly made all the difference in my work. Thank you for always inspiring me and supporting me to try because it's always possible!

Super special thanks, to my life partner and my future wife, Marcela Goulart, for being my biggest support during this time, believing in me and helping me in everything. Thank you to help me to create my Brazilian identity, and to show me that Minas Gerais is the best of Brazil. Thank you for building a family with me and for make me happy! I love you!

Special thanks to my dear Viví for being my support in the countless attempts of DNA extraction, always with words of encouragement to don't give up. And beyond the DNA world, for your friendship, always willing to listen and advise. To Túlio for always being himself, that's how we love you, *fazer o que*! And to Giba for the long amusing conversations.

To my lab colleagues, Leandro, Jessica, William, Melissa, Alejandra and Mavi, for the partnership at work time and the *bagunça* time too! A special thanks to Leandro for his help and collaboration work.

A mis dos chicos, Gabis y Chris, por las incontables horas de diversión viviendo juntos. Los karaokes y *rumbas* bailando *salsa* y por la *hermandad* que creamos. También por amar tanto a Meg. Los llevaré para siempre a donde quiera que vaya! Amo vocês!

I also want to thank the funding institutions. To the Colombian Government and Colombian Department of Science – COLCIENCIAS for my PhD scholarship. Thanks to Petrobras

time Adriana, Baessa and Luiz Fernando and Professor Admir and Marcio from the Federal University of Santa Catarina, for allowing me to be part of the Project and for always helping to carry out the analyzes.

A mis amigas del alma y sus bendiciones. En Colombia: Silvi, Nana y mi sobrina Mapiz, Jennifer y mi sobrino Nicolás, Marcela y mis ahijados Juan José y Juan Martin. En Chile, Eliana y mi sobrino Emiliano. En Francia, Churca. En Australia, Cindy y mi sobrina Lunita. A todos gracias por su apoyo incondicional siempre. Amistades para siempre!

Gracias a mi familia por apoyarme en esta jornada y especialmente por aceptar mi ausencia durante todos estos años. También por siempre dejarme ser lo que yo quisiera ser, siempre alentándome a ser cada día mejor. Esto es para y por ustedes.

Finalmente quero agradecer ao Brasil, a UNICAMP, e especialmente à ciência brasileira pela ENORME oportunidade de me tornar cientista.

OBRIGADA!

Este trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

Resumo

Em decorrência das crises climáticas e energéticas, bem como das implicações ambientais do consumo de combustíveis fósseis, o uso de biocombustíveis tem se expandido nas últimas décadas. No Brasil, o biodiesel é usado em mistura com o diesel (também conhecido como B12 – 12% Biodiesel / 88% Diesel) e o etanol em mistura com gasolina (também conhecido como E27 – 27% etanol / 63% gasolina). O uso crescente de tais misturas aumenta o risco de contaminação de solos, águas superficiais e subterrâneas com biocombustíveis. Estudos prévios sugerem que a presença de biocombustíveis afeta as taxas e a dinâmica de biodegradação dos hidrocarbonetos do petróleo. No entanto, pouco se sabe sobre a composição, estrutura e potencial metabólico do microbioma de solos contaminados com essas misturas quando são submetidos a tratamentos de biorremediação. Este trabalho teve como objetivo caracterizar o microbioma de quatro áreas de solo contaminadas com diferentes misturas de biocombustíveis e combustíveis fósseis (p.e., biodiesel/ diesel ou etanol/ gasolina) e submetidas a diferentes tratamentos de biorremediação usando técnicas multi-ômicas como metataxonomia e metagenômica (abordagem centrada em genes e centrada em genomas). O estudo revelou claras diferenças entre a composição da comunidade microbiana, padrões de co-occurrência e perfis funcionais dependendo da mistura, bem como, antes e depois do tratamento de biorremediação. Os microbiomas associados aos solos contaminados com biodiesel se mostraram mais complexos e diversos. Os solos não submetidos a qualquer tratamento de biorremediação por um longe período de tempo foram mais resilientes às perturbações. Nossos resultados mostraram que as espécies-chave mais abundantes são degradadoras de hidrocarbonetos bem documentadas na literatura. Por outro lado, os resultados mostraram claramente o aumento da abundância dos genes de degradação de hidrocarbonetos após o tratamento de biorremediação. No entanto, se observou que os mesmos genes de degradação de hidrocarbonetos foram enriquecidos após o tratamento de biorremediação nas diferentes áreas, embora tenham sido afiliados a diferentes táxons. Em conjunto, os resultados obtidos contribuíram para expandir nossa compreensão dos efeitos das abordagens de biorremediação sobre o microbioma de solos impactados por biocombustíveis / combustíveis fósseis, fornecendo

subsídios para definir o tratamento mais adequado e eficiente de acordo com o tipo de contaminação.
Keywords: biodiesel, gasohol, hidrocarbonetos – biodegradação, bioremediation, poluição solos

Abstract

As a result of climate and energetic crises, as well as the environmental implications of fossil fuel consumption, the use of biofuels has expanded in recent decades. In Brazil, biodiesel is used in blends with diesel (also known as B12 – 12% Biodiesel / 88% Diesel) and ethanol in blends with gasoline also named gasohol (also known as E27 – 27% ethanol / 63% gasoline). The growing use of such blends increases the risk of biofuel contamination in soil, surface water, and groundwater. Previous studies suggest that the presence of biofuels affects petroleum hydrocarbon degradation rates and dynamics. However, little is known about the microbial community composition, structure, and function in microbiomes of soil polluted with fuel blends followed by bioremediation treatments. This work aimed to characterize the microbiome of four soil areas contaminated with different blends of biofuels and fossil fuels (e.g., biodiesel/ diesel or ethanol/ gasoline) and submitted to different bioremediation treatments using multi-omics techniques such as metataxonomics and metagenomics (gene-centric and genome-centric approaches). The study revealed clear differences between the microbial community composition, co-occurrence patterns and functional profiles depending on the blend, as well as before and after the bioremediation treatment. The microbiome associated to soil contaminated with biodiesel was shown to be more complex and diverse. Soils not submitted to any bioremediation treatment for a long period of time were more resilient to perturbations. Our results showed that the more abundant keystone species are well known hydrocarbon degraders. On the other hand, the results clearly showed the increase of the abundance of hydrocarbon degradation genes after the bioremediation treatments. Nevertheless, it was observed that the same hydrocarbon degradation and associated genes were enriched after the bioremediation treatments in the different areas, although they were affiliated to different taxa. Altogether, these findings contribute to expand our understanding of the effects of bioremediation approaches over the microbiome in biofuel/ fossil fuel impacted soils, helping to define the most appropriate and efficient treatment according to the type of contamination.

Keywords: biodiesel fuels, gasohol, hydrocarbons – biodegradation, bioremediation, soil pollution

Contents

INTRODUCTION

Spills of oil and/or its derivatives usually occur as a result of leakage from underground storage tanks, rupture of pipelines and transport accidents (Islam et al., 2013), delivering into nature compounds that can cause major impacts to the environment and human health for its toxicity, mutagenicity and carcinogenicity (Tahhan and Abu-Ateih, 2009). Due to the environmental impacts associated with the use of fossil fuels, their replacement by renewable and clean energy sources is urgent and a major challenge for the industry. In this sense, the search for renewable fuels from raw materials of plant origin, such as sugar cane, soybean, corn, among others, to produce ethanol or biodiesel, has been stimulated. In Brazil, ethanol was introduced in the energy matrix in the 1990s, in blends of 20 to 25% of ethanol in combination with gasoline (Ramos, 2013). In 2005, soybean biodiesel began to be introduced in Brazil as B2 (Biodiesel 2%/ Diesel 98%) (Law 11.097). In the Resolution 16 of 2018, a new regulation was created to promote the gradual increase of the biodiesel proportion up to 15% until 2023. However, due to the current price and high demand of soy oil, the proportion was reduced to 10% (ANP, 2020; Ramos, 2013). Brazil is considered the world pioneer in the production and use of biofuels (Serbent, 2012).

With the increase in the use of biodiesel/diesel and gasohol blends, and based on future projections (OECD/FAO, 2023), and raise in events of biofuel pollution in soils, surface and underground waters is expected, due to possible accidents of spills or leaks during the different stages of the fuel supply chain. Given the different dynamics in the biodegradation of petroleum hydrocarbons when ethanol or biodiesel is added (Chen et al., 2008; Corseuil et al., 2011; Costa et al., 2009; da Silva and Corseuil, 2012; Rama et al., 2019; Ramos et al., 2014; Steiner et al., 2018), there is an urgent need to develop specific alternatives for the remediation of environments impacted by these mixtures. Since bioremediation treatments are well known as a cost-effective solution to treat hydrocarbon-polluted areas (Baniasadi and Mousavi, 2018; Ng et al., 2015; Zhao et al., 2011), studies addressing biodegradation of biofuel/petrofuel pollution have grown (Alvarez and Hunt, 2002; Chen et al., 2008; Corseuil et al., 1998; Cyplik et al., 2011; Da Silva et al., 2005; Da Silva and Alvarez, 2004b; Heermann and Powers, 1998; Ng et al.,

2015; Rama et al., 2019; Ramos, 2012; Satapanajaru et al., 2017). However, a considerable number of previous studies have focused only on the assessment of the effect of biofuels on petroleum hydrocarbon degradation dynamics, rate, and efficiency (Alvarez and Hunt, 2002; Cyplik et al., 2011; Rama et al., 2019; Ramos, 2012). Despite the importance of microbial metabolism in the removal of pollutants, the behavior of degrading microbial communities has only been studied in only a few cases (Luisa et al., 2015; Müller et al., 2017; Satapanajaru et al., 2017).

Currently, several technologies can be applied for the remediation of environments impacted with hydrocarbons, such as natural attenuation, bioaugmentation and biostimulation. Natural attenuation, or intrinsic bioremediation, uses microorganisms indigenous to the contaminated environment to transform toxic organic compounds into others of lower toxicity or even inert (Neuhauser et al., 2009; Varjani and Upasani, 2012; Zhao et al., 2011). Biostimulation comprises the addition of nutrients or electron acceptors, or even air injection named specifically biosparging. While the bioaugmentation encompasses the addition of microbial degraders or enzymes (Koshlaf and Ball, 2017; Okoh et al., 2020). The use of these techniques helps modulate the microbial community to increase the degradation rates. There has already extensive research on the application of these bioremediation approaches in environments affected by fossil fuels (Baniasadi and Mousavi, 2018; Ng et al., 2015; Zhao et al., 2011). In the case of areas impacted by fuel blends, there has been growing interest in studying and implementing biodegradation processes of these mixtures (Alvarez and Hunt, 2002; Chen et al., 2008; Corseuil et al., 1998; Cyplik et al., 2011; Da Silva and Alvarez, 2004a; Da Silva et al., 2005; Heermann and Powers, 1998; Ng et al., 2015; Rama et al., 2019; Ramos, 2012; Satapanajaru et al., 2017).

Soil microorganisms have important roles in biogeochemical processes such as the carbon, nitrogen, sulfur and phosphorus cycles (Falkowski et al., 2008), forming complex co-occurrence networks through indirect and direct interactions (Hallam and McCutcheon, 2015). Despite the relevance of microorganisms in nature’s overall processes, there are no reports in literature on the assessment of the ecological processes (i.e., deterministic or stochastic) driving microbial community assembly,

complexity, diversity and dynamics, as well as on the metabolic potential of microbes in areas affected by biofuel blends and submitted to bioremediation treatments.

The biodegradability of hydrocarbons and, therefore, their degree of persistence in natural environments are influenced by several factors, such as the concentration, solubility and chemical structure of hydrocarbons, the microbial degradation ability and specific environmental conditions (pH, concentration of oxygen, temperature, salinity, etc.) required for microbial metabolism (Chikere et al., 2011; Popp et al., 2006; Sheng et al., 2016). Aerobic degradation of hydrocarbons has been well studied for a long time (Abbasian et al., 2015; Baboshin and Golovleva, 2012; Fuchs et al., 2011; Vaillancourt et al., 2006). However, aquifers are often anaerobic, especially due to the rapid depletion of oxygen in the contaminated environment. Degradation of aromatic hydrocarbon via anaerobic metabolism has been intensively assessed in recent years, with the description of different steps in the transformation of the compounds (Abu Laban et al., 2010; Carmona et al., 2009; Fuchs et al., 2011; Hidalgo et al., 2019; Jeon and Madsen, 2013; Ladino-Orjuela et al., 2016; Meckenstock et al., 2016; Meckenstock and Mouttaki, 2011; von Netzer et al., 2016; Weelink et al., 2010).

Great advances in the combined use of the omics techniques such as genomics, metagenomics, metatranscriptomics and metaproteomics, and bioremediation strategies have contributed to the improvement of biodegradation processes (Abbai and Pillay, 2013; Brennerova et al., 2009; Duarte et al., 2017; El Amrani et al., 2015; Hidalgo et al., 2019; Loviso et al., 2015; Ma et al., 2015; Mason et al., 2014; Muangchinda et al., 2018; Nyyssönen et al., 2009; Techtmann and Hazen, 2016; Tiralerdpanich et al., 2018; Uhlik et al., 2013; Wilhelm et al., 2018; Xu et al., 2018; Xu et al., 2014; Zafra et al., 2016). The use of metagenomics to assess impacted environments can provide information about the ecology of the indigenous microbial communities, which will help identifying who are the dominant and rare members and their functional potential for the transformation of pollutants (Ghosal et al., 2016). Metatranscriptomics, which is based on the large scale sequencing of total mRNA, allows us to identify which genes are being expressed in a sample at a given moment (Simon and Daniel, 2011). The use of molecular biology and bioinformatics has allowed one to expand the knowledge of the biological systems found

in these polluted environments, providing subsidies for the optimization of strategies to mitigate environmental pollution (Hazen et al., 2016; Techtmann and Hazen, 2016). Thus, it is expected that these modern molecular biology techniques combined with bioinformatics analysis can transform bioremediation into an efficient and widely used application.

This work conducted a broad investigation of the soils polluted with different biofuel/petrofuel blends and submitted to different bioremediation treatments using an omics approach (e.g., metataxonomics and metagenomics). Results altogether contribute to clarify the effect of diverse blend proportions and bioremediation techniques on the taxonomic and functional profiles of soil microbiome in impacted areas. The main focus were microorganisms able to degrade petroleum hydrocarbons such as BTEX (benzene, toluene, ethylbenzene and xylenes), alkanes and poly aromatics hydrocarbons (PAHs). These data allowed us to understand the main ecological processes driving the microbial community assembly, diversity and dynamics. In addition, network analysis data enabled to identify the key microbial players and interactions supporting a microbial community able to efficiently cope with petroleum hydrocarbons in soils affected by biofuel and fossil fuel blends.

References

Abbai NS, Pillay B. Analysis of Hydrocarbon-Contaminated Groundwater Metagenomes as Revealed by High-Throughput Sequencing. Molecular Biotechnology 2013; 54: 900-912.

Abbasian F, Lockington R, Mallavarapu M, Naidu R. A Comprehensive Review of Aliphatic Hydrocarbon Biodegradation by Bacteria. Applied Biochemistry and Biotechnology 2015; 176: 670-699.

Abu Laban N, Selesi D, Rattei T, Tischler P, Meckenstock RU. Identification of enzymes involved in anaerobic benzene degradation by a strictly anaerobic iron-reducing enrichment culture. Environmental Microbiology 2010; 12: 2783-2796.

Alvarez PJ, Hunt CS. The effect of fuel alcohol on monoaromatic hydrocarbon biodegradation and natural attenuation. Revista Latinoamericana de Microbiologia 2002; 44: 83.

ANP. Agência Nacional do Petróleo, Gás Natural e Biocombustíveis. Biocombustíveis, 2020.

Baboshin M, Golovleva L. Aerobic bacterial degradation of polycyclic aromatic hydrocarbons (PAHs) and its kinetic aspects. Microbiology 2012; 81: 639-650.

Baniasadi M, Mousavi SM. A Comprehensive Review on the Bioremediation of Oil Spills. In: Kumar V, Kumar M, Prasad R, editors. Microbial Action on Hydrocarbons. Springer Singapore, Singapore, 2018, pp. 223-254.

Brennerova MV, Josefiova J, Brenner V, Pieper DH, Junca H. Metagenomics reveals diversity and abundance of meta-cleavage pathways in microbial communities from soil highly contaminated with jet fuel under air-sparging bioremediation. Environmental Microbiology 2009; 11: 2216-2227.

Carmona M, Zamarro MT, Blázquez B, Durante-Rodríguez G, Juárez JF, Valderrama JA, et al. Anaerobic Catabolism of Aromatic Compounds: a Genetic and Genomic View. Microbiology and Molecular Biology Reviews 2009; 73: 71-133.

Chen YD, Barker JF, Gui L. A strategy for aromatic hydrocarbon bioremediation under anaerobic conditions and the impacts of ethanol: A microcosm study. Journal of Contaminant Hydrology 2008; 96: 17-31.

Chikere C, Okpokwasili G, Chikere B. Monitoring of microbial hydrocarbon remediation in the soil. 3 Biotech 2011; 1: 117-138.

Corseuil HX, Hunt CS, Dos Santos RCF, Alvarez PJ. The influence of the gasoline oxygenate ethanol on aerobic and anaerobic BTX biodegradation. Water Research 1998; 32: 2065-2072.

Corseuil HX, Monier AL, Gomes AP, Chiaranda HS, do Rosario M, Alvarez PJ. Biodegradation of soybean and castor oil biodiesel: implications on the natural attenuation of monoaromatic hydrocarbons in groundwater. Groundwater Monitoring & Remediation 2011; 31: 111-118.

Costa AHR, Nunes CC, Corseuil HX. Biorremediação de águas subterrâneas impactadas por gasolina e etanol com o uso de nitrato. Engenharia Sanitária e Ambiental 2009; 14: 265-274.

Cyplik P, Schmidt M, Szulc A, Marecik R, Lisiecki P, Heipieper HJ, et al. Relative quantitative PCR to assess bacterial community dynamics during biodegradation of diesel and biodiesel fuels under various aeration conditions. Bioresource Technology 2011; 102: 4347-4352.

Da Silva ML, Alvarez PJ. Enhanced anaerobic biodegradation of benzene-toluene-ethylbenzene-xylene-ethanol mixtures in bioaugmented aquifer columns. Applied and Environmental Microbiology 2004a; 70: 4720-4726.

Da Silva ML, Ruiz-Aguilar GM, Alvarez PJ. Enhanced anaerobic biodegradation of BTEX-ethanol mixtures in aquifer columns amended with sulfate, chelated ferric iron or nitrate. Biodegradation 2005; 16: 105-114.

Da Silva MLB, Alvarez PJJ. Enhanced Anaerobic Biodegradation of Benzene-Toluene-Ethylbenzene-Xylene-Ethanol Mixtures in Bioaugmented Aquifer Columns. Applied and Environmental Microbiology 2004b; 70: 4720.

da Silva MLB, Corseuil HX. Groundwater microbial analysis to assess enhanced BTEX biodegradation by nitrate injection at a gasohol-contaminated site. International Biodeterioration & Biodegradation 2012; 67: 21-27.

Duarte M, Nielsen A, Camarinha-Silva A, Vilchez-Vargas R, Bruls T, Wos-Oxley ML, et al. Functional soil metagenomics: elucidation of polycyclic aromatic hydrocarbon degradation potential following 12 years of in situ bioremediation. Environmental Microbiology 2017; 19: 2992-3011.

El Amrani A, Dumas A-S, Wick LY, Yergeau E, Berthomé R. “Omics” Insights into PAH Degradation toward Improved Green Remediation Biotechnologies. Environmental Science & Technology 2015; 49: 11281-11291.

Falkowski PG, Fenchel T, Delong EF. The microbial engines that drive Earth's biogeochemical cycles. science 2008; 320: 1034-1039.

Fuchs G, Boll M, Heider J. Microbial degradation of aromatic compounds--from one strategy to four. Nature reviews. Microbiology 2011; 9: 803.

Ghosal D, Ghosh S, Dutta TK, Ahn Y. Current State of Knowledge in Microbial Degradation of Polycyclic Aromatic Hydrocarbons (PAHs): A Review. Frontiers in Microbiology 2016; 7.

Hallam SJ, McCutcheon JP. Microbes don't play solitaire: how cooperation trumps isolation in the microbial world. Environmental microbiology reports 2015; 7: 26-28.

Hazen TC, Prince RC, Mahmoudi N. Marine oil biodegradation. ACS Publications, 2016.

Heermann SE, Powers SE. Modeling the partitioning of BTEX in water-reformulated gasoline systems containing ethanol. Journal of Contaminant Hydrology 1998; 34: 315-341.

Hidalgo KJ, Teramoto EH, Soriano AU, Valoni E, Baessa MP, Richnow HH, et al. Taxonomic and functional diversity of the microbiome in a jet fuel contaminated site as revealed by combined application of in situ microcosms with metagenomic analysis. Science of The Total Environment 2019: 135152.

Islam TH, Ghosh B, Magnet MMH, Fatema K, Akter S, Khan MAR, et al. Isolation and identification of petroleum degrading bacteria from oil contaminated soil & water and assessment of their potentiality in bioremediation. Journal of Environmental Science, toxocology and Food Technology 2013; 5: 55 - 58.

Jeon CO, Madsen EL. In situ microbial metabolism of aromatic-hydrocarbon environmental pollutants. Current Opinion in Biotechnology 2013; 24: 474-481.

Koshlaf E, Ball AS. Soil bioremediation approaches for petroleum hydrocarbon polluted environments. AIMS microbiology 2017; 3: 25.

Ladino-Orjuela G, Gomes E, da Silva R, Salt C, Parsons JR. Metabolic Pathways for Degradation of Aromatic Hydrocarbons by Bacteria. In: de Voogt WP, editor. Reviews of Environmental Contamination and Toxicology Volume 237. Springer International Publishing, Cham, 2016, pp. 105-121.

Loviso CL, Lozada M, Guibert LM, Musumeci MA, Sarango Cardenas S, Kuin RV, et al. Metagenomics reveals the high polycyclic aromatic hydrocarbon-degradation potential of abundant uncultured bacteria from chronically polluted subantarctic and temperate coastal marine environments. Journal of Applied Microbiology 2015; 119: 411-424.

Luisa W, Letícia T, Francielle B, Raquel D, Patricia DdQ, Kateryna Z, et al. Culture-independent analysis of bacterial diversity during bioremediation of soil contaminated with a diesel-biodiesel blend (B10) S. Journal of Bioremediation and Biodegradation 2015; 6.

Ma B, Lyu X-F, Zha T, Gong J, He Y, Xu J-M. Reconstructed metagenomes reveal changes of microbial functional profiling during PAHs degradation along a rice (Oryza sativa) rhizosphere gradient. Journal of Applied Microbiology 2015; 118: 890-900.

Mason OU, Scott NM, Gonzalez A, Robbins-Pianka A, Bælum J, Kimbrel J, et al. Metagenomics reveals sediment microbial community response to Deepwater Horizon oil spill. The ISME Journal 2014; 8: 1464-1475.

Meckenstock RU, Boll M, Mouttaki H, Koelschbach JS, Cunha Tarouco P, Weyrauch P, et al. Anaerobic Degradation of Benzene and Polycyclic Aromatic Hydrocarbons. Journal of Molecular Microbiology and Biotechnology 2016; 26: 92-118.

Meckenstock RU, Mouttaki H. Anaerobic degradation of non-substituted aromatic hydrocarbons. Current Opinion in Biotechnology 2011; 22: 406-414.

Muangchinda C, Rungsihiranrut A, Prombutara P, Soonglerdsongpha S, Pinyakong O. 16S metagenomic analysis reveals adaptability of a mixed-PAH-degrading consortium isolated from crude oil-contaminated seawater to changing environmental conditions. Journal of Hazardous Materials 2018; 357: 119-127.

Müller JB, Ramos DT, Larose C, Fernandes M, Lazzarin HSC, Vogel TM, et al. Combined iron and sulfate reduction biostimulation as a novel approach to enhance BTEX and PAH source-zone biodegradation in biodiesel blend-contaminated groundwater. Journal of hazardous materials 2017; 326: 229-236.

Neuhauser EF, Ripp JA, Azzolina NA, Madsen EL, Mauro DM, Taylor T. Monitored natural attenuation of manufactured gas plant tar mono-and polycyclic aromatic hydrocarbons in ground water: a 14-year field study. Groundwater Monitoring & Remediation 2009; 29: 66-76.

Ng YF, Ge L, Chan WK, Tan SN, Yong JWH, Tan TTY. An environmentally friendly approach to treat oil spill: Investigating the biodegradation of petrodiesel in the presence of different biodiesels. Fuel 2015; 139: 523-528.

Nyyssönen M, Kapanen A, Piskonen R, Lukkari T, Itävaara M. Functional genes reveal the intrinsic PAH biodegradation potential in creosote-contaminated groundwater following in situ biostimulation. Applied Microbiology and Biotechnology 2009; 84: 169-182.

OECD/FAO. Agricultural Outlook. 2023.

Okoh E, Yelebe ZR, Oruabena B, Nelson ES, Indiamaowei OP. Clean-up of crude oil-contaminated soils: bioremediation option. International Journal of Environmental Science and Technology 2020; 17: 1185-1198.

Popp N, Schlömann M, Mau M. Bacterial diversity in the active stage of a bioremediation system for mineral oil hydrocarbon-contaminated soils. Microbiology 2006; 152: 3291-3304.

Rama F, Ramos DT, Müller JB, Corseuil HX, Miotliński K. Flow field dynamics and high ethanol content in gasohol blends enhance BTEX migration and biodegradation in groundwater. Journal of contaminant hydrology 2019; 222: 17-30.

Ramos DT. Bioestimulação de processos metanogênicos com acetato de amônio para degradação acelerada de hidrocarbonetos de petróleo em águas subterrâneas contaminadas com diesel B20. Engenharia Ambiental. Doutor. Universidade Federal de Santa Catarina, Florianóplis, 2013.

Ramos DT, da Silva MLB, Nossa CW, Alvarez PJJ, Corseuil HX. Assessment of microbial communities associated with fermentative–methanogenic biodegradation of aromatic hydrocarbons in groundwater contaminated with a biodiesel blend (B20). Biodegradation 2014; 25: 681-691.

Ramos SM. Análise comparativa da influência do etanol em diferentes processos de remediação de águas subterrâneas impactadas por gasolina. 2012.

Satapanajaru T, Chokejaroenrat C, Sakulthaew C, Yoo-lam M. Remediation and restoration of petroleum hydrocarbon containing alcohol-contaminated soil by persulfate oxidation activated with soil minerals. Water, Air, & Soil Pollution 2017; 228: 1-15.

Serbent MP. Influência do etanol e do sulfato na biodegradação de compostos aromáticos em águas subterrâneas contaminadas com misturas de combustíveis. Departamento de Engenharia Sanitária e Ambiental. Mestrado. Universidade Federal de Santa Catarina, Florianópolis, SC, 2012, pp. 192.

Sheng Y, Wang G, Hao C, Xie Q, Zhang Q. Microbial Community Structures in Petroleum Contaminated Soils at an Oil Field, Hebei, China. CLEAN – Soil, Air, Water 2016; 44: 829-839.

Simon C, Daniel R. Metagenomic analyses: past and future trends. Appl. Environ. Microbiol. 2011; 77: 1153-1161.

Steiner LV, Ramos DT, Liedke AMR, Serbent MP, Corseuil HX. Ethanol content in different gasohol blend spills influences the decision-making on remediation technologies. Journal of environmental management 2018; 212: 8-16.

Tahhan RA, Abu-Ateih RY. Biodegradation of petroleum industry oily-sludge using Jordanian oil refinery contaminated soil. International Biodeterioration & Biodegradation 2009; 63: 1054-1060.

Techtmann SM, Hazen TC. Metagenomic applications in environmental monitoring and bioremediation. Journal of Industrial Microbiology & Biotechnology 2016; 43: 1345-1354.

Tiralerdpanich P, Sonthiphand P, Luepromchai E, Pinyakong O, Pokethitiyook P. Potential microbial consortium involved in the biodegradation of diesel, hexadecane and phenanthrene in mangrove sediment explored by metagenomics analysis. Marine Pollution Bulletin 2018; 133: 595-605.

Uhlik O, Leewis M-C, Strejcek M, Musilova L, Mackova M, Leigh MB, et al. Stable isotope probing in the metagenomics era: A bridge towards improved bioremediation. Biotechnology Advances 2013; 31: 154-165.

Vaillancourt FH, Bolin JT, Eltis LD. The ins and outs of ring-cleaving dioxygenases. Critical Reviews in Biochemistry and Molecular Biology 2006; 41: 241-267.

Varjani S, Upasani V. Characterization of hydrocarbon utilizing Pseudomonas strains from crude oil contaminated samples. Int. J. of Scientific Computing 2012; 6: 120-127.

von Netzer F, Kuntze K, Vogt C, Richnow HH, Boll M, Lueders T. Functional Gene Markers for Fumarate-Adding and Dearomatizing Key Enzymes in Anaerobic Aromatic Hydrocarbon Degradation in Terrestrial Environments. Journal of Molecular Microbiology and Biotechnology 2016; 26: 180-194.

Weelink SAB, van Eekert MHA, Stams AJM. Degradation of BTEX by anaerobic bacteria: physiology and application. Reviews in Environmental Science and Bio/Technology 2010; 9: 359-385.

Wilhelm RC, Hanson BT, Chandra S, Madsen E. Community dynamics and functional characteristics of naphthalene-degrading populations in contaminated surface sediments and hypoxic/anoxic groundwater. Environmental Microbiology 2018; 20: 3543-3559.

Xu W, Zhang Y, Cao H, Sheng Y, Li H, Li Y, et al. Metagenomic insights into the microbiota profiles and bioaugmentation mechanism of organics removal in coal gasification wastewater in an anaerobic/anoxic/oxic system by methanol. Bioresource Technology 2018; 264: 106-115.

Xu Z, Hansen MA, Hansen LH, Jacquiod S, Sørensen SJ. Bioinformatic Approaches Reveal Metagenomic Characterization of Soil Microbial Community. PLOS ONE 2014; 9: e93445.

Zafra G, Taylor TD, Absalón AE, Cortés-Espinosa DV. Comparative metagenomic analysis of PAH degradation in soil by a mixed microbial consortium. Journal of Hazardous Materials 2016; 318: 702-710.

Zhao D, Liu C, Liu L, Zhang Y, Liu Q, Wu W-M. Selection of functional consortium for crude oil-contaminated soil remediation. International Biodeterioration & Biodegradation 2011; 65: 1244-1248.

General Objective

The main aim of this work was to assess the effect of different bioremediation approaches in polluted areas with blends of biodiesel/diesel or gasohol on the microbial community composition, structure, and metabolic potential, using *microbiomics* (metataxonomics and metagenomics) and focusing on the specific genes involved in hydrocarbon degradation (alkanes, BTEX and PAHs).

Proposal hypotheses

1. The microbiome composition and dynamics vary according to the different biofuel/ fossil fuel blends (Chapter II);

2. The bioremediation treatments influence the microbial metabolism, resulting in shifts in microbial community structure and composition (Chapter II);

3. The microbiome co-occurrence patterns, and keystone species vary according to the different blends and bioremediation treatments, reflecting the level of complexity and strength of the interactions among microbial members depending on the substrate, nutrients and electron acceptor availability (Chapter II);

4. The specific functional profiles (hydrocarbon degradation and related metabolisms) are influenced by the type of biofuels (Chapter III);

5. Different BTEX degradation genes and related metabolism are enriched depending on the bioremediation treatment (Chapter III);

6. The taxonomic groups involved in BTEX degradation are keystone species supporting microbial networks and community homeostasis in the polluted soils (Chapter III)

Organization of the thesis

This thesis is divided into chapters that contain the information of the original manuscripts already submitted or in preparation for publication in scientific journals. The first chapter includes two reviews of the literature and state of the art describing the key concepts and background information on bioremediation of biofuel blends, required to understand the subsequent chapters. The first one is about mechanisms for biodegradation of polycyclic aromatic hydrocarbons across all the oil supply chain, including accidental spills in soil and/or water (https://doi.org/10.3389/fmicb.2020.561506). The second review is about the state of the art of studies of biodegradation of environments contaminated with biofuel and fossil fuel blends. Chapter II covers the assessment of the soil microbial community structure, composition and co-occurrence patterns of the areas impacted with blends of fossil fuels and biofuels submitted to different bioremediation treatments. In this chapter, the changes in the microbial community profiles before, one and two years after the bioremediation treatments were evaluated. Also, co-occurrence network analyses were carried out in order to unravel the interactions between the members of the community (manuscript submitted to Environmental Pollution, currently is under revision). Chapter III presents an in-depth metagenomic (gene-centered and genome-centered) characterization of the soil microbiome functional diversity in the impacted areas before and after the decontamination actions, focusing on the diversity of hydrocarbon degradation genes and pathways. In this chapter, genomes from the most abundant microorganisms were recovered from the metagenomes through the *binning* approach and their metabolisms were analyzed in detail. Finally, chapter IV presents the Petroleum-associated Genome Database (PaGeD), created based on genomic and metagenomic data derived from our research group works and from public databases. PaGeD database aims to offer a catalog of compiled genomes and genes related with any step of the oil and gas supply chain, as well as of the associated information on the beneficial and harmful microorganisms and their potential metabolisms.

Chapter I: Literature review and state of the art

Manuscript 1 – Review 1: Metagenomic insights into the mechanisms for biodegradation of polycyclic aromatic hydrocarbons in the oil supply chain

frontiers in Microbiology

REVIEW
published: 18 September 2020
doi: 10.3389/fmicb.2020.561506

Metagenomic Insights Into the Mechanisms for Biodegradation of Polycyclic Aromatic Hydrocarbons in the Oil Supply Chain

Kelly J. Hidalgo[1,2], Isabel N. Sierra-Garcia[1,3], Bruna M. Dellagnezze[1] and Valéria Maia de Oliveira[1]*

[1] Microbial Resources Division, Research Center for Chemistry, Biology and Agriculture (CPQBA), University of Campinas (UNICAMP), Paulínia, Brazil, [2] Graduate Program in Genetics and Molecular Biology, Institute of Biology, University of Campinas (UNICAMP), Campinas, Brazil, [3] Biology Department & Centre for Environmental and Marine Studies (CESAM), University of Aveiro, Aveiro, Portugal

OPEN ACCESS

Edited by:
Ayush Pandey,
Assam University, India

Reviewed by:
Naresh Singhal,
The University of Auckland, New Zealand
Paulo Costa Lemos,
LAQV Network of Chemistry and Technology, Portugal

****Correspondence:***
Kelly J. Hidalgo
khidalgo@javeriana.edu.co

Specialty section:
This article was submitted to Microbiotechnology, a section of the journal Frontiers in Microbiology

Received: *12 May 2020*
Accepted: *24 August 2020*
Published: *18 September 2020*

Citation:
Hidalgo KJ, Sierra-Garcia IN, Dellagnezze BM and de Oliveira VM (2020) Metagenomic Insights Into the Mechanisms for Biodegradation of Polycyclic Aromatic Hydrocarbons in the Oil Supply Chain. Front. Microbiol. 11:561506. doi: 10.3389/fmicb.2020.561506

Petroleum is a very complex and diverse organic mixture. Its composition depends on reservoir location and *in situ* conditions and changes once crude oil is spilled into the environment, making the characteristics associated with every spill unique. Polycyclic aromatic hydrocarbons (PAHs) are common components of the crude oil and constitute a group of persistent organic pollutants. Due to their highly hydrophobic, and their low solubility tend to accumulate in soil and sediment. The process by which oil is sourced and made available for use is referred to as the oil supply chain and involves three parts: (1) upstream, (2) midstream and (3) downstream activities. As consequence from oil supply chain activities, crude oils are subjected to biodeterioration, acidification and souring, and oil spills are frequently reported affecting not only the environment, but also the economy and human resources. Different bioremediation techniques based on microbial metabolism, such as natural attenuation, bioaugmentation, biostimulation are promising approaches to minimize the environmental impact of oil spills. The rate and efficiency of this process depend on multiple factors, like pH, oxygen content, temperature, availability and concentration of the pollutants and diversity and structure of the microbial community present in the affected (contaminated) area. Emerging approaches, such as (meta-)taxonomics and (meta-)genomics bring new insights into the molecular mechanisms of PAH microbial degradation at both single species and community levels in oil reservoirs and groundwater/seawater spills. We have scrutinized the microbiological aspects of biodegradation of PAHs naturally occurring in oil upstream activities (exploration and production), and crude oil and/or by-products spills in midstream (transport and storage) and downstream (refining and distribution) activities. This work addresses PAH biodegradation in different stages of oil supply chain affecting diverse environments (groundwater, seawater, oil reservoir) focusing on genes and pathways as well as key players involved in this process. In depth understanding of the biodegradation process will provide/improve knowledge for optimizing and monitoring bioremediation in oil spills cases and/or to impair the degradation in reservoirs avoiding deterioration of crude oil quality.

Keywords: oils reservoirs, sea water, groundwater, bioremediation, PAH

INTRODUCTION

The petroleum industry is one of the largest global industries and covers a vast range of activities across the world. All the activities and processes involved in the petroleum industry are referred to as oil supply chain activities and are divided in three parts (**Figure 1**): (1) upstream, (2) midstream and (3) downstream. Upstream refers to the origin of the oil: petroleum exploration and extraction. Midstream refers to transportation of raw crude oil through pipelines, rail car and/or tanker to refineries. Downstream describes the refining processes to produce different usable products, like gasoline, diesel, jet fuel and other petrochemicals. One barrel of crude oil (42 gallons) as input can produce 44% of gasoline, 24% of diesel and heating oil, 4% of jet fuel, and 22% of assorted products including petrochemicals, lubricants and asphalt. This manufacturing, refining and petrochemical activities are part of the downstream stage (Big Data and Analytics, 2020). During oil supply chain stages, crude oils are subjected to biodegradation, biodeterioration, acidification and souring. Also, oil or by-products spills are frequently reported affecting not only the environment, but also the economy and human resources.

Petroleum is naturally formed by a process of deposition of algae in marine sediments that for millions of years were subjected to high temperatures and pressures (Hazen et al., 2016). It is a complex mixture of hydrocarbons, many of which are classified as mutagenic, carcinogenic and teratogenic (Liao et al., 2014; Schwarz et al., 2019). Oil hydrocarbons can be classified in four groups according with their solubility in organic solvents and water (Han et al., 2018). These groups are described as: (i) saturated hydrocarbons (or alkanes, or aliphatic), including all the n- and branched alkanes and cycloparaffins, (ii) aromatic hydrocarbons, including monoaromatics such as BTEX (Benzene, Toluene, Ethylbenzene, and Xylenes) and polycyclic aromatic compounds (PAHs), (iii) resins, are compounds that contains sulfur, oxygen and nitrogen and that are dissolved in oil such as quinolines, pyridines, amides and sulfoxides, and (iv) asphaltenes, consisting of aggregates of molecules with naphthenic rings and condensed aromatic connected by paraffin chains (Gentili et al., 2006; Costa et al., 2012; Wu et al., 2013; Abdel-Shafy and Mansour, 2016; Varjani, 2017; Shahsavari et al., 2019). PAHs are one of the major components of crude oil and their by-products are released into the environment during incomplete combustion or by accidental spills over the oil supply chain (Johansson and Van Bavel, 2003). PAHs are organic compounds compose by at least two fused benzene or aromatic rings in linear, angular, or cluster arrangements, resulting in diverse structural configurations (Bamforth and Singleton, 2005; Sharma, 2014; Ghosal et al., 2016). There are two kinds of PAHs, low-molecular-weight (LMW) PAHs, that contain up to two or three rings (naphthalene, acenaphthene, acenaphthylene, fluorene, anthracene, and phenanthrene) and high-molecular-weight (HMW) PAHs, with more than three rings (fluoranthene, pyrene, benzo[a]pyrene, perylene, etc.). HMW-PAHs are more toxic and structurally more stable than the light PAHs (Kuppusamy et al., 2016; Li et al., 2016). The increase in the hydrophobicity, electrochemical stability and resistance toward biodegradation and carcinogenic index, occurs with the increase in the number of rings (Zander, 1983; Harvey, 1998; Mackay and Callcott, 1998; Marston et al., 2001). Due to their complex structure, low water solubility and high hydrophobicity, PAHs tend to be recalcitrant compounds, resulting in their accumulation in the ecosystems and limited availability to biodegradation (Lawal, 2017). In the last decades, due to the increase of the amount of PAHs from natural and anthropogenic resources, there has been an increase of PAHs concentration in the ecosystems (Juhasz and Naidu, 2000). PAHs are largely present as pollutants in diverse ecosystems, such as air, soils, sediments, surfaces and groundwater (Ghosal et al., 2016). The ubiquitous distribution, combined to their toxic, genotoxic, mutagenic and carcinogenic properties, led PAHs to be considered as priority pollutants (Ghosal et al., 2016). The United States Environmental Protection Agency (US EPA) enlisted 16 PAHs as priority environmental pollutants based on their toxicity and abundance (**Table 1**; U.S. Department of Health and Human Services, 1999; Liu et al., 2001; Zhang et al., 2011; Abdel-Shafy and Mansour, 2016; Lamichhane et al., 2016; Varjani, 2017). Thus, research aimed at removing these PAHs from the environment has gained substantial increase (Ghosal et al., 2016).

The physicochemical transformations of PAHs in the environment include adsorption, volatilization, photolysis and chemical oxidation. However, microbial degradation is still the most important environmental process which affects the fate of PAHs in contaminated aquatic and terrestrial ecosystems (Lu et al., 2011). Aiming at a remediation approach, PAHs can be transformed by different physical and chemical treatment techniques, like UV oxidation, incineration, solvent extraction and base-catalyzed dechlorination (Gan et al., 2009). However, these techniques have several disadvantages such as cost, complexity, regulation, etc. Additionally, these methods, in many cases, are not efficient enough to completely destroy PAHs molecules, and may instead transform them in intermediates even more toxic (Ghosal et al., 2016). An alternative strategy is bioremediation, that involves the use of the potential of microorganisms to degrade organic pollutants to inoffensive molecules, such a carbon dioxide and water (Zhao et al., 2011; Varjani and Upasani, 2012). This approach appears as an eco-friendly alternative to solve some of the disadvantages of traditional methods. However, the effectiveness of bioremediation processes depends on several factors including the type of contaminant, its bioavailability and the microbial capacity of degradation (Adetutu et al., 2012). Different bioremediation techniques based on microbial metabolism, such as natural attenuation, biostimulation and bioaugmentation are promising approaches to minimize the environmental impact of polluted areas. Microbial hydrocarbon degradation can be performed via different pathways such as phototrophic, anoxygenic, and aerobic or anaerobic chemotrophs pathway (Varjani, 2017) and are limited by multiple factors, like nutrient availability, pH, oxygen content, temperature, PAHs concentration and chemical properties and the type and abundance of microorganisms present in the affected area. Diverse bacterial and fungal species have the potential

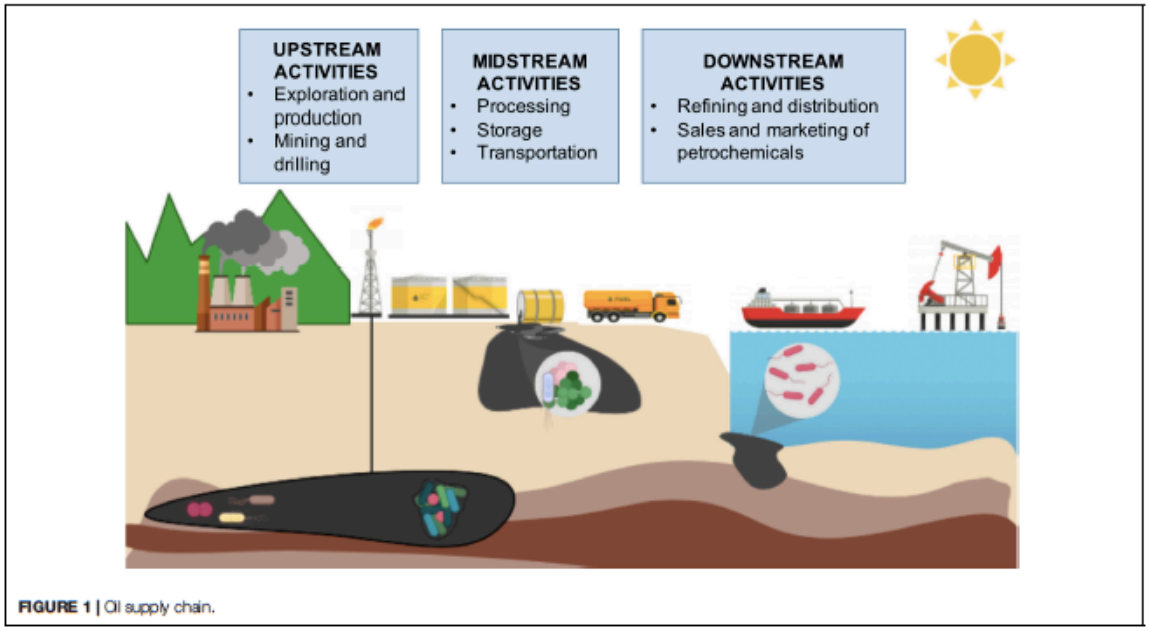


FIGURE 1 | Oil supply chain.

TABLE 1 | Sixteen PAHs priority pollutants by United States EPA.

Naphthalene $C_{10}H_8$	Acenaphthene $C_{12}H_{10}$	Acenaphthylene $C_{12}H_8$	Anthracene $C_{14}H_{10}$
Phenanthrene $C_{14}H_{10}$	Fluorene $C_{13}H_{10}$	Fluoranthene $C_{16}H_{10}$	Benzo[a]anthracene $C_{18}H_{12}$
Chrysene $C_{18}H_{12}$	Pyrene $C_{16}H_{10}$	Benzo[a]pyrene $C_{20}H_{12}$	Benzo[b]fluoranthene $C_{20}H_{12}$
Benzo[k]fluoranthene $C_{20}H_{12}$	Dibenzo[a,h]anthracene $C_{22}H_{14}$	Benzo[g,h,i]pyrene $C_{22}H_{12}$	Indeno[1,2,3-c,d]pyrene $C_{22}H_{12}$

to degrade/transform PAHs. Microbial communities, metabolic pathways, genes, enzymes and genetic regulation involved in the PAHs degradation have been the focus in PAHs research over the last few decades and have been explored in a great extent (Ghosal et al., 2016). Emerging approaches, such as (meta-) taxonomics and (meta-)genomics can be used to scrutinize and monitoring the diversity and microbial structure, providing access to the taxonomic and functional genes (Thomas et al., 2012). Several studies on hydrocarbon-polluted environments have efficiently used high throughput sequencing techniques to monitor bioremediation processes (Dos Santos et al., 2011; Simon and Daniel, 2011; Coulon et al., 2012; Yergeau et al., 2012a,b; Ma Q. et al., 2015; Wang et al., 2016). Thus, with this modern approaches of molecular biology, the current knowledge about non-cultured microorganisms have been increased (Urgun-Demirtas et al., 2006) and brought new insights on microbial communities populations, metabolic profiles and specific enzymes involved in PAH biodegradation at both single species and community levels in oil reservoirs and groundwater/seawater spills.

The present review provides a global perspective of the current knowledge on PAH biodegradation taking place in diverse environments (groundwater, seawater, oil reservoir) at different stages of oil supply chain, not necessarily in linear order, with emphasis on genes and pathways as well as key players involved in this process.

MICROBIAL BIODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS

Microorganisms are able to aerobically and/or anaerobically degrade many hydrocarbons (Head et al., 2006; Meckenstock et al., 2016). There are 175 prokaryotic genera belonging to different phyla of Bacteria and Archaea described, and also almost equal number of fungal genera, able to use hydrocarbons as their carbon source (Hazen et al., 2016). Microorganisms able to degrade PAHs are widely distributed in many environments, even in pristine areas, representing up to 0.1% of the microbiota (Margesin et al., 2003; Ghazali et al., 2004; Brooijmans et al., 2009; Uad et al., 2010; Souza et al., 2014; Shen et al., 2015; Varjani et al., 2015; Lamichhane et al., 2016). Nevertheless, in oil-contaminated ecosystems hydrocarbon degraders can dominate the microbial community (Leahy and Colwell, 1990; Greenwood et al., 2009; Varjani and Upasani, 2016). The most affected (polluted) sites are surrounding areas (water and/or soils) of oil refineries, gas plants, air bases, chemical manufacturing facilities and petrol stations (Juhasz et al., 2005; Seo et al., 2009). The development and improvement of the omics approaches have brought a broader understanding of the diversity, distribution and dynamics of microorganisms, as well as of their specific genes and proteins, involved in hydrocarbon degradation in polluted ecosystems (Pieper and Reineke, 2000; Varjani and Upasani, 2013; Ron and Rosenberg, 2014; Varjani, 2014). In the literature there are many reviews about the degradation pathways of several compounds like phenol, BTEX (monoaromatics) and naphthalene, pyrene, phenanthrene and anthracene (PAHs) at the enzyme level (Heider, 2007; Fuchs et al., 2011; Boll et al., 2014; Waigi et al., 2015; Meckenstock et al., 2016; Wilkes et al., 2016).

PAH degradation can occur by oxygen-dependent or independent pathways (**Figure 2**). The aerobic degradation is mainly *via* oxygenase enzymes. The first reaction is an hydroxylation usually catalyzed by a multicomponent dioxygenase enzyme (or ring-hydroxylation dioxygenase-RHD) yielding *cis*-dihydrodiol (Albaigés et al., 1983; Cerniglia, 1993; Saito et al., 1999; Juhasz and Naidu, 2000; Bongiorni et al., 2005; Ghosal et al., 2016). Then, the aromatic ring is rearomatized by the enzyme *cis*-dihydriol dehydrogenase forming dihydroxylated intermediates, which is oxidized to form catechol, the main intermediate of aerobic aromatic hydrocarbon degradation (Shahsavari et al., 2019). The next step depends on the position of the hydroxyl (OH) group in the dihydroxylated intermediates. If the intermediate has the OH group in the *ortho*-position, so an intradiol cleaving dioxygenase will act between the two OH groups yielding *cis*-muconic acid (Juhasz and Naidu, 2000). If the intermediate is in the *meta*-position, cleavage occurs by an extradiol cleaving dioxygenase forming 2-hydroxymuconic semi-aldehyde (Cerniglia, 1993; Shahsavari et al., 2015; Ghosal et al., 2016). Once this process occurs in the first aromatic ring, the second ring is transformed in the same way and so on (Atlas, 1998). At last, transformation of all rings in the PAH molecule results in the production of tricarboxylic acid cycle (TCA) intermediates that can enter in the bacterial central metabolism for further use in the synthesis of cellular constituents and energy (Habe and Omori, 2003). The final products are carbon dioxide and water. The HMW-PAH dioxygenases are encoded by *nid* and *pdo* genes in gram positive bacteria (Gupta et al., 2015). However, several studies demonstrated that PAH-ring hydroxylating dioxygenase genes *pah-rdh*? were more effective than *nidA* gene for detecting and quantifying pyrene-degrading bacteria (Bacosa and Inoue, 2015). The LMW-PAH dioxygenases are encoded by *nah* (naphthalene dioxygenase) genes (Gupta et al., 2015) (**Figure 2**). These genes have been well described and studied in the gram negative *Pseudomonas* (Habe and Omori, 2003; Zhou et al., 2006). The metabolism of LMW-PAHs degradation is carried out in two main stages: a) upper and b) lower pathways (**Figure 2**). In the upper pathway, the enzymes are encoded by the *nah*AaAbAcAdBCDEF genes and in the lower pathway by the *nah*GTHINLOMKJ genes (Habe and Omori, 2003; Zhou et al., 2006; Gupta et al., 2015). Also, *pah* and *phn* genes have been found in other gram-negative bacteria, such as in the naphthalene and phenanthrene degrader *Comamonas testosteroni* and in the naphthalene, phenanthrene and anthracene degrader *Burkholderia* sp., respectively (Gupta et al., 2015).

In the anaerobic degradation, compounds other than oxygen act as final electron acceptors, such as nitrate and/or sulfate. The anaerobic degradation of PAHs differs substantially from monoaromatic degradation and has so far been studied only for the diaromatics like naphthalene as a model, mainly due to the fact that this compound is intermediate in the degradation pathway of several PAHs with more than two rings (Von Netzer et al., 2016; **Figure 2**). PAH anaerobic degradation pathway begins with an initial activation attack. For naphthalene, two main activation mechanisms have been proposed: (i) carboxylation to naphthoic acid (Zhang and Young, 1997), and (ii) methylation to 1-methylnaphthalene followed by fumarate addition to naphthyl-2-methylsuccinate, catalyzed by the naphthylmethylsuccinate synthase (NMS) (Meckenstock et al., 2000; Safinowski and Meckenstock, 2006; Musat et al., 2009). Although some authors have also suggested the hydroxylation as an activation mechanism due to the presence of naphthol in a sulfate-reducing naphthalene-degrading culture (Bedessem et al., 1997), this mechanism has not yet been demonstrated in the anaerobic degradation of naphthalene. The subsequent degradation pathway involves different aryl-CoA reductases (Eberlein et al., 2013a,b). The first two activation mechanisms converge in the central intermediate 2-naphthoyl-CoA and thereafter the aromatic ring is reduced by beta-oxidation-like reaction (Zhang et al., 2000; Annweiler et al., 2002; Phelps et al., 2002). The non-activated ring is dearomatized by the 2-naphthoyl-CoA reductase (NCR), which is a flavoprotein with a flavin mononucleotide cofactor (Von Netzer et al., 2016).

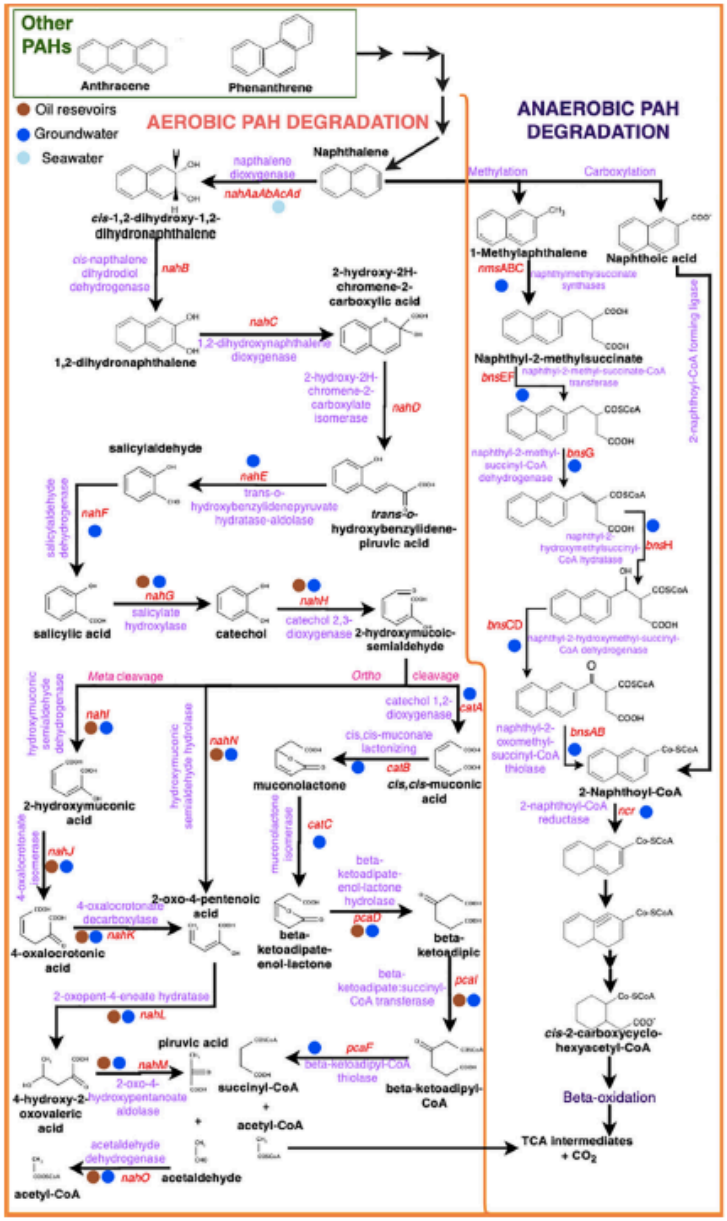


FIGURE 2 | PAH aerobic (left) and anaerobic (right) microbial degradation pathways with intermediate molecular structures (black), enzyme names (purple) and gene names (red). Colored circles represent the presence of the genes in the different environments (brown – oil reservoirs / blue – groundwater / light blue – seawater).

Analogous to naphthalene, the activation mechanisms of phenanthrene may be carboxylation, or methylation followed by fumarate addition and oxidation to phenanthroic acid (Von Netzer et al., 2016). Some studies have reported both phenanthrene and naphthalene degradation (Coates et al., 1997; Zhang and Young, 1997; Rockne and Strand, 2001).

As hydrocarbon degradation has been demonstrated under aerobic and anaerobic conditions resulting in mitigation of contamination (Wiedemeier et al., 1999; Yu et al., 2005; Vasconcellos et al., 2010; Shin et al., 2019; Bianco et al., 2020), it is mandatory to improve our knowledge on microbial diversity in hydrocarbon-polluted environments, since the microbial composition and metabolic potential is one of limiting factors of the degradation fate of pollutants in contaminated sites (Lovley, 2003). Currently, the genetic basis of the catabolic pathways of the various PAHs remains poorly understood. Thus, the use of recent approaches as meta-taxonomics and metagenomics offers great potential to contribute with information to elucidate PAH degradation pathways (Brennerova et al., 2009; Nyyssönen et al., 2009; Abbai and Pillay, 2013; Uhlik et al., 2013; Mason et al., 2014; Xu et al., 2014; El Amrani et al., 2015; Loviso et al., 2015; Ma B. et al., 2015; Techtmann and Hazen, 2016; Zafra et al., 2016; Duarte et al., 2017; Muangchinda et al., 2018; Tiralerdpanich et al., 2018; Wilhelm et al., 2018; Xu et al., 2018; Hidalgo et al., 2019). From now on, we will present the information available from several studies carried out to explore PAH biodegradation in natural or impacted environments rich in hydrocarbons. We will focus on microbial community approaches when available or in isolated microorganisms from hydrocarbon impacted environments. Most of these studies focus on naphthalene degradation since, as previously stated, it is a centralized intermediate in the degradation pathway of several PAHs with more than two rings.

METAGENOMICS AND CATABOLIC GENES

"Omics" approach has been used to deepen knowledge about the diversity and distribution of PAH degraders as well as their genes and metabolic pathways related to hydrocarbon degradation in several marine environments, such as sediments of the ocean floor, water surfaces, beaches, deep sub-surfaces, as well as in terrestrial environments, as groundwater and soils.

Marine microbial metagenomics can provide an increase of data in marine ecology and oceanography, and several works based on different approaches (metagenomic fosmid/cosmid libraries, Sanger and Next generation sequencing shotgun metagenomics) have been described (Gilbert and Dupont, 2011; Nikolaivits et al., 2017).

The explosion of the Deepwater Horizon (DWH) well from British Petroleum (BP) company, in 2010 in Mississippi, United States, was considered the largest oil spill in history, with nearly five million barrels spilled and spread along 1100 km of the American coast (Atlas and Hazen, 2011). Over time, several studies were performed in order to understand the biological effects of the oil release and its microbial community impact. In one of these studies, Hazen et al. (2010) evaluated the microbial diversity from several oil plume samples (water column) formed in the surroundings of the well and in uncontaminated seawater, employing different molecular, chemical and physiological approaches. The authors observed that most OTUs in oil contaminated seawater were associated to the order *Oceanospirillales*, belonging to Y-Proteobacteria, largely composed of known psychrophilic hydrocarbon degraders including *Oleispira antarctica*, *Oleiphilus messinensi*, and *Thalassolituus oleivorans*. Moreover, they found hydrocarbon degradation genes significantly increased in oil plume samples, such as *phd*CI gene encoding carboxylate isomerase for naphthalene degradation (Hazen et al., 2010). Later, a complementary work focused on the functional role of *Oceanospirillales* and other bacteria, using shotgun metagenomics and metatranscriptomics. The authors could corroborate not only the massive abundance of this group in oil contaminated samples, but also their role as active members in the plume. However, genes involved in degradation of aromatic compounds showed low abundance and low levels of expression when compare to those involved in alkane degradation. This could be explained by the fact that recalcitrant compounds were not actively degraded at the time sampling was performed (Mason et al., 2012). Other works involving the DWH accident based on metagenomics were later carried out also focusing on contaminated sediments, corroborating the microbial shift toward the high abundance of hydrocarbon degraders as Alpha- and Gammaproteobacteria, associated to members of taxa known to degrade hydrocarbons, such as *Rhodobacteraceae*, *Alteromonadaceae*, and *Pseudomonadaceae*, and genes and pathways related to PAH, n-alkane and toluene degradation (Lamendella et al., 2014; Mason et al., 2014). Also, high levels of PAH compounds (above 24,000 mg/kg) were discovered in deep sediments in the area near the wellhead. Metagenomic analysis and functional gene assays of these deep-sea sediments closer to the well (3 km) showed high abundance of deltaproteobacteria and genes related to the anaerobic degradation of aromatic and aliphatic hydrocarbons, like *ass*A and *bss*A, both encoding subunits of glycyl radical enzymes: ASS, linked to alkane degradation (addition of fumarate to alkane), and BSS, related to the addition of fumarate to aromatic hydrocarbons to yield benzylsuccinic acids and benzylsuccinate derivatives, respectively. The detection of benzylsuccinate metabolites provided indication for anaerobic biodegradation of alkylbenzenes *in situ* (Kimes et al., 2014).

Basins in tropical seas have also been studied, such as Campos Basin, an area susceptible to oil contamination in South Atlantic Ocean (Appolinario et al., 2019). The authors investigated the biodegradation potential of marine microorganisms in three different depths: (i) surface (5 m); (ii) intermediate (chlorophyll maximum layer, 80 m); and (iii) near the bottom (1,200 m), using seawater samples supplemented with crude oil and incubated during 52 days. Metagenomics was used to characterize taxonomic and functional microbial diversity and infer microbial abundance related to oil degradation. The genera *Alteromonas* (~10%) and *Marinobacter* (~13%) were more abundant in surface and chlorophyll maximum (80 m) samples, respectively, whereas *Colwellia* (~24%) was the most abundant in bottom samples. The genus *Colwellia* has been reported as alkane and aromatic degrader (Mason et al., 2014; Campeão et al., 2019) and results found by Appolinario et al. (2019) could suggest a higher oil-degrading potential of such bacteria in deep seawater. Moreover, genes associated with metabolism of

aromatic compounds, as naphthalene and phenanthrene, were also monitored and reached a peak at the end of 52 days of experiment (Appolinario et al., 2019). In a previous work from our group, a consortium consisting of metagenomic clones and a *Bacillus* strain, both recovered from petroleum reservoir, were employed in bioaugmentation experiments using artificially petroleum contaminated seawater from Messina harbor (Italy) in mesocosms scale (3000 L). After 30 days, aromatic degradation was more effective in the bioaugmentation treatment compared to control, with biodegradation rates ranging from 70 to 99%. Autochthonous community dynamics varied between treatments throughout the experimental days, and although the microorganisms added to the bioaugmentation treatment were not detected in high abundances, the consortium was shown to contribute to a significant increase in aromatic hydrocarbon degradation (Dellagnezze et al., 2016). Metagenomics and cultivation-based studies from polluted environments reporting genes associated to PAH degradation have been extensively described in literature (**Table 2**).

Oil supply chain associated activities are extensive to some polar regions, making them susceptible to petroleum hydrocarbon contamination. Thus, studies focusing on these environments are highly relevant in order to improve/optimize bioremediation strategies. Nonetheless, the management in these regions encounters some challenges due to intrinsic harsh conditions. In 2008, a survey was carried out aiming at bioprospecting marker genes of PAH degradation by using Aromatic Ring-Hydroxylating Dioxygenase (ARHD) gene libraries obtained from sediments of the Patagonia Coast, Argentina. The authors could find eight different ARHD gene types, with five of them showing no close relatives in the databases. The remaining three were associated to *nah*Ac-like and *phn*Ac-like genes, related to naphthalene and phenanthrene degradation, respectively, as described in *Alcaligenes faecalis* AFK2, and to *phn*A1-like genes from marine bacteria belonging to *Cycloclasticus* genus (Lozada et al., 2008). Later, the same authors investigated functional targets for PAH degradation in chronically polluted subantarctic marine sediments, in Ushuaya Bay, Argentina. Based on the use of primers designed for the gram negative bacterial dioxygenase genes, the authors identified 14 different groups of genes, most of them significantly related to dioxygenases from gram positive bacteria belonging to genera *Bacillus, Rhodococcus, Mycobacterium, Nocardioides*, and *Terrabacter* (Marcos et al., 2009).

In the Arctic Ocean, a study evaluated the presence of PAHs and their bioattenuation in the open sea. Samples from 19 sediment cores (deep sea sediments) were collected from Canada Basin, the Chukchi Plateau, Alpha Ridge and Makarov Basin and evaluated by 16S rRNA gene large scale sequencing to determine the diversity of bacteria involved in PAH degradation *in situ*. The potential degrading groups observed were members of the genera *Pseudomonas, Pseudoalteromonas, Cycloclasticus, Halomonas, Bacillus, Colwellia, Marinomomas, Salinisphaera, Shewanella, Alcanivorax, Dietzia*, and *Acinetobacter*, being the genus *Dietzia* widespread in all sediment samples and the most abundant group (Dong et al., 2015). In another study, Mcfarlin et al. (2014) evaluated the biodegradation of crude oil carried out at −1°C using seawater mesocosm from Chukchi Sea, Alaska, simulating natural water column. Surfactant Corexit 9500 was added along with oil in order to biostimulate degradation. By the end of experiment, the indigenous microbial community was capable to biodegrade chrysene, a four-ringed PAH. Nevertheless, the degradation rates were higher for the lower molecular weight compounds, such as naphthalene and phenanthrene, demonstrating that the indigenous microbiota from Arctic seawater is capable of performing extensive biodegradation at low environmental temperature (Mcfarlin et al., 2014).

PAH BIODEGRADATION IN UPSTREAM OPERATIONS: PETROLEUM RESERVOIRS

Both traditional microbiological methods and *omics* approaches have been widely used to assess microbial community degraders and hydrocarbon biodegradation potential in petroleum and gas industry-associated environments. These scientific efforts have started long time ago aiming at a deeper understanding of the role of microorganisms in petroleum deterioration, as well as at bioprospecting specific properties of indigenous microorganisms for improving/optimizing biotechnological processes, such as Microbial Enhanced Oil Recovery (MEOR) (Röling et al., 2003; Youssef et al., 2009; Wentzel et al., 2013; Pannekens et al., 2019).

In oil industry, upstream operations include exploring, drilling and bringing to the surface oil resources from petroleum reservoirs. Equipment in upstream oil industry operations such as pipelines, vessels and others, integrate a broad environment were microorganisms predominantly anaerobes thrive. The existence of microorganisms in petroleum reservoirs and associated facilities is well known for those working in oil industry and petroleum microbiologist. In fact, the study of microbiology in petroleum systems worldwide has been encouraged by the operational and economic consequences of microbial activities in the petroleum systems.

Biodegradation of petroleum hydrocarbons is one of the microbial activities in oil reservoirs that has major implications in the properties and quality of oil and, consequently, its production and value. In the *in reservoir* microbial degradation process, lighter fractions of petroleum hydrocarbons such as saturated hydrocarbons and light aromatic hydrocarbons are consumed, leading to an increase of the proportion of branched and cyclic hydrocarbons, heavier aromatic hydrocarbons and polar fractions of oils such as resins and asphaltenes (Head, 2017). There is also an increase in the concentration of recalcitrant compounds, commonly referred to as "unresolved complex mixture" (UCM). The resulting heavy oils have physical and chemical properties that make them more difficult and costly to be produced and refined (Head et al., 2010; Head, 2017).

The microbial degradation of lighter hydrocarbons in worldwide petroleum reservoirs has led to the transformation of conventional oils to heavy -unconventional- oils which are characterized by high viscosity and low API gravity. Heavy oils are distinguished from light oils mainly by the API gravity values. Definition of heavy oils is often applied inconsistently to crude

TABLE 2 | Genes related to PAH degradation in oil reservoirs, groundwater and marine environments.

Gene	Microbial origin	Source	References
*nah*AaAbAcAd cluster, *nah*EFGHIJKLMNO *cat*AB *pcs*DFI	Metagenome	Jet-fuel contaminated aquifer (Brazil)	Hidalgo et al., 2019
*nms*ABC *bns*ABCDEFGH *ncr*	Metagenome	Creosote-polluted groundwater	Nyyssönen et al., 2009; Meckenstock et al., 2016
bss (benzylsuccinate synthase)	Metagenome	Deep sea sediments (Gulf of Mexico)	Kimes et al., 2013
bss (benzylsuccinate synthase)	Metagenome Assembled Genomes (MAGs)	Alaska North Slope oil fields	Hu et al., 2016
bss (benzylsuccinate synthase)	Metagenome Assembled Genomes (MAGs)	Petroleum reservoirs Brazil	Sierra-Garcia et al., 2020
*pah*A1-4 gene cluster (dioxygenase)	*Cycloclasticus* sp. 78-ME	Mediterranean Sea (Italy)	Messina et al., 2016
*nah*Ac/NDO (naphtalene dioxygenase)	*Alteromonas* sp. strain SN2	Contaminated sea (South Korea)	Jin et al., 2012
*cat*E (catechol-2,3-dioxygenase)	*Limnobacter* sp. Metagenome Metagenome	Baltic Sea (Estonia/Finland) Deep sea (Australia) Bohai Sea, China	Vedler et al., 2013; He et al., 2016; Van De Kamp et al., 2019
*phn*Ac (phenanthrene dioxygenase)	Metagenome	Ushuaya Bay (Argentina) Zhoushan Archipelago, China	Marcos et al., 2009; Peng et al., 2020
*car*ABC (carbazole 1,9a-dioxygenase)	*Neptunibacter* sp. strain CAR-SF	Isolate from seawater Japan	Nagashima et al., 2010
LmPH (multicomponent phenol hydroxylase)	*Limnobacter* sp.	Baltic Sea (Estonia/Finland)	Vedler et al., 2013

oil, sometimes it is applied to API gravity of less than 20, but other definitions embrace gravities less than 22 or less than 25 (Speight, 2015). In general, the exploration and extraction of petroleum resources is preferably conducted from better quality and accessible resources like conventional oils, before progressing to lower quality, lower API and less accessible resources that require more efforts and higher economic and environmental costs (unconventional oil resources) (Nduagu and Gates, 2015). Nevertheless, heavy oils dominate the reserves of petroleum around the world, estimated to approach 5.6 trillion barrels (bbl), predominantly located in the western hemisphere (Hein et al., 2013; Head et al., 2014).

Petroleum reservoir microbiology and reservoir fluid chemistry indicate that oil reservoirs are primarily anoxic environments (Head, 2017). Therefore, the current understanding is that biodegradation of oil and formation of heavy oils is the result of anaerobic metabolism of microorganisms living in the subsurface environments (Wentzel et al., 2013; Li et al., 2017; Sierra-Garcia et al., 2017). However, current knowledge of the pathways for anaerobic hydrocarbon degradation in petroleum reservoirs is scarce. In fact, aerobic heterotrophs (or in some cases facultative) organisms are continuously reported in literature, such as *Bacillus* spp., *Acinetobacter* spp., and *Pseudomonas* spp., being detected or isolated from subsurface hydrocarbon-rich reservoir environments (Orphan et al., 2000; Da Cruz et al., 2011; Berdugo-Clavijo et al., 2012; Li et al., 2012; An et al., 2013; Meslé et al., 2013; Gieg et al., 2014). Previous studies from Da Cruz et al. (2011) have proposed that oil degradation could be a combined accomplishment of both aerobic and anaerobic bacteria living in consortia. Similarly, a metagenomic approach applied by An et al. (2013) revealed that aerobic hydrocarbon-degrading related genes and bacteria are highly abundant in a wide range of hydrocarbon-rich environments (coal beds, tailing ponds, waters from oil reservoirs, etc.). In addition, a functional-based metagenomic study from reservoir samples showed that hydrocarbon degradation activities expressed by metagenomic fosmid clones were coded by fragmented gene clusters from aerobic and anaerobic degradation pathways occurring in the same fosmid inserts (Sierra-García et al., 2014).

Specific literature on the *in reservoir*-degradation of larger and recalcitrant compounds such as PAH and its influence on oil viscosity is limited (Xia et al., 2016). Conversely, for saturated hydrocarbons such as alkanes, biodegradation is now widely accepted to occur anaerobically by syntrophic n-alkane degradation and methanogenesis. In many studies, the ability of anaerobic enrichment cultures derived from oil fields to degrade saturated hydrocarbons has been reported (Zengler et al., 1999; Siddique et al., 2006; Jones et al., 2008; Gieg et al., 2010; Mbadinga et al., 2011; Wang et al., 2011; Zhou et al., 2012; Tan et al., 2013). This is not the case for PAH degradation, which is scarcely reported in samples originating from oil reservoirs. An early study observed that microbial community structure changes during the progressive degradation of oil and the removal of n-alkanes (Hallmann et al., 2008). In this case, parallel to the removal of n-alkanes, bacterial biomass increases, and diversity differs from that of the alkane degrading community. Therefore, degradation of recalcitrant compounds in

oil reservoirs is thought to involve hydrolytic and fermentative bacteria that carry a wider range of metabolic capabilities (Röling et al., 2003; Hallmann et al., 2008).

Considering that biodegraded oils have higher content of cyclic and heavier aromatic hydrocarbons compared to undegraded oils, the study of biodegraded oil reservoirs may offer fundamental insights into the aerobic and/or anaerobic nature of the polycyclic hydrocarbon biodegradation in such environments. Metabolites indicative of anaerobic degradation of naphthalene and polyaromatic hydrocarbons have been detected in biodegraded oils (Aitken et al., 2004) and production fluids from oil reservoirs (Bian et al., 2015). Particular microbial community analyses in highly biodegraded oil fields, have shown the dominance of specific bacterial groups like Epsilonproteobacteria (Voordouw et al., 1996; Grabowski et al., 2005; Hubert et al., 2012). Some previous comparative studies between degraded and non-degraded oils apparently did not show significant differences in microbial communities between those reservoirs using ARDRA analysis (Sette et al., 2007). However, more recent research, based on higher resolution techniques, has shown differences between biodegraded and non-degraded petroleum samples where biodegraded oils contain higher microbial diversity (Hallmann et al., 2008; Silva et al., 2013; Sierra-Garcia et al., 2017). This is in accordance with the fact that it is expected that the microbial populations able of degradation of light oils, rich in saturated hydrocarbons like n-alkanes, are different from the ones associated with heavy oils, containing high levels of aromatic hydrocarbons (Head et al., 2014).

Recently, the microbiome associated with biodegraded and non-biodegraded oil reservoirs was analyzed in a more comprehensive and thorough study using whole shotgun metagenomic approach (Sierra-Garcia et al., 2020). The analysis showed that the biodegraded oil encompassed equal abundance of bacteria and archaea together with higher proportion of genes corresponding to anaerobic hydrocarbon degradation. On the other hand, the reservoir containing non-degraded oil was dominated by bacteria mainly associated with *Marinobacter* and less proportion of archaea together with lower frequency of genes of anaerobic hydrocarbon degradation. These results reinforced the important role of syntrophic interactions between bacteria and archaea to perform biodegradation of petroleum components in oil reservoirs through the processes of anaerobic metabolism of hydrocarbons and methanogenesis (Zengler et al., 1999; Gieg et al., 2014; Sierra-Garcia et al., 2020).

Although diversity and physiology of microorganisms inhabiting oil fields have been compiled and reviewed worldwide (Wentzel et al., 2013; Li et al., 2017; Sierra-Garcia et al., 2017), determining if the microorganisms recovered are indigenous to the subsurface environment and not from contamination sources is a thorny issue in petroleum microbiology. The reason is because several of the microbial community studies in petroleum reservoirs have analyzed produced waters and thus, there are some drawbacks to consider when interpreting petroleum microbiology. Firstly, because of the nature of the produced waters and water injection practices, some of the microorganisms detected in such are considered to be contaminants (Head et al., 2014). Secondly, there is an increasing evidence that microbial life can exist in the oil itself and even that the microbial communities associated with the different components of the oil fluids (e.g., crude oil or production or formation waters) could be different (Kryachko et al., 2012; Meckenstock et al., 2014; Wang L.-Y. et al., 2014; Cai et al., 2015). From these studies, it seems that bacterial communities and functional genes are more abundant and diverse in the oil phase than in the water phase (Kobayashi et al., 2012; Wang L.-Y. et al., 2014; Cai et al., 2015; Sierra-Garcia et al., 2020).

Regardless of the origin of the microbial life in petroleum reservoirs, the *in-situ* activities of indigenous microorganisms have significant consequences for petroleum systems. Before drilling or during oil production, microorganisms are interacting and responding to the availability of carbon sources, nutrients, electron donors and other biotic and abiotic conditions (Head, 2017). For example, when sulfate is abundant, sulfate reduction is a significant driver of the petroleum biosphere. But the abundance of sulfate reducing bacteria (SRB) is considered an artifact of seawater injection in petroleum reservoirs. SRB mostly depend on small organic substrates such as volatile fatty acids and hydrogen, as electron donors (Hallmann et al., 2008). In petroleum reservoirs where no seawater injection for secondary recovery has been practiced, the microbiome is mainly associated with fermentation reactions carried out by syntrophic bacteria that deliver hydrogen, carbon dioxide and acetate to methanogens (Head, 2017).

Sierra-Garcia et al. (2020) compared the microbiome of crude oils in two production wells, one that been had been exposed to water flooding for secondary recovery and the other one considered pristine (no water flooded). In this study, evidences for the ongoing oil biodegradation of the pristine reservoir were found in the taxonomic and functional composition of the reservoir microbial community, where fermentative bacteria of the genera *Syntrophus* and *Syntrophomonas* and members of the phyla Synergistia and Candidatus Atribacteria carried the genes for hydrocarbon degradation in close association with methanogenic bacteria. At the same time, the microbiome of the water flooded oil was dominated by *Marinobacter*-like organisms with a wider heterotrophic metabolism including genes for oxygen reduction, and for sulfate and nitrogen metabolism. In this reservoir, the anthropogenic perturbation with seawater injection was assumed to alter the indigenous microbial community toward fast-growing opportunists that had little effect on the hydrocarbon degradation due to the likely use of other easily available carbon sources or due to the combination of high salinity and temperature limiting *in situ* conditions (Sierra-Garcia et al., 2020). Generally, water injection practices lower temperatures, increase salinity (in seawater injections), and promote the enrichment with exogenous chemicals/nutrients and/or microorganisms, stimulating changes in indigenous microbial communities (Pannekens et al., 2019). Therefore, besides the well-known factors that limit life (nutrient availability, metabolic products, temperature and salinity), indigenous microbial communities in oil reservoirs are affected by the anthropogenic factor, that for a long time has exploited the deep geological resources leading to changes in microbial ecology

and activity, ultimately resulting in the modulation of beneficial and/or detrimental microbial processes (Pannekens et al., 2019).

PAH BIODEGRADATION IN MIDSTREAM ACTIVITIES: CONSEQUENCES ON MARINE ENVIRONMENT

Independently of the petroleum extraction method (primary or secondary recovery), and of the oil type (light or heavy), after drilling, the oil is transported through pipes to tankers or to oil terminals and then to refineries. During transport, accidental release of crude oil or its derivatives can occur, including tanker spills, explosions and ruptures of pipelines, which can lead to the spill of large volumes of pollutants on the sea surfaces or sub-surfaces. Such oil contamination events result in PAH acute pollution of the marine environment (Duran and Cravo-Laureau, 2016; Nelson and Grubesic, 2018).

Oil pollution brings several consequences, not only environmental impact with contamination of the whole food chain, from phytoplankton to large mammals, but also economic and social impacts in the affected region (Perelo, 2010; Taleghani and Tyagi, 2017). Depending on the dimension of the oil spill, several human activities may be affected as fisheries and mariculture, tourism and recreational facilities, shipping and salt production (ITOPF, 2011; Court et al., 2017). Some of these accidents and their consequences have been vastly reported, such as Exxon Valddez, Alaska, in 1989 (Atlas and Hazen, 2011), Amoco Cadiz, France in 1978 (Ward et al., 1980) and Deepwater Horizon, United States in 2010 (Kimes et al., 2014; Lamendella et al., 2014).

In marine environments, petroleum derivatives undergo a process called weathering, i.e., a series of biological and physicochemical transformations (Mcgenity et al., 2012). Physicochemical processes include evaporation, dissolution, dispersion, emulsification, photo oxidation, adsorption and sedimentation. Biological weathering occurs through microbial metabolism and intake by other organisms (Hassanshahian and Cappello, 2013). The more complex the structure of the hydrocarbon molecule, the more difficult its degradation, hence, saturated hydrocarbon degrades faster compared to aromatics and asphaltenes (Xue et al., 2015).

The restoration of the impacted area involves the attenuation of toxic compounds to reach bearable level and lays on several biotic and abiotic factors, including availability of colonizing microorganisms, biological and climate, among others (Kingston, 2002; Chen and Denison, 2011).

In order to identify possible microbial degraders and unravel their metabolic potential, studies on the characterization of marine bacterial communities have been reported (Atlas and Hazen, 2011; Neethu et al., 2019). These studies have greatly contributed to the identification of key organisms capable to degrade contaminants mainly for applying new *in situ* bioremediation approaches (Hassanshahian and Cappello, 2013).

The release of oil and its derivatives into seawater can cause changes in autochthonous marine microbial communities leading to successive blooms of some bacterial groups observed at low or undetectable levels before the polluting event. These groups composed by particularly specialized obligate hydrocarbon utilizers are defined as "obligate hydrocarbonoclastic bacteria (OHCB)" or "specialized hydrocarbonoclastic bacteria (SHCB) (Yakimov et al., 2007; Teramoto et al., 2013). *Alcanivorax* spp., described as alkane degraders; *Cycloclasticus* spp., as degrading PAHs; *Marinobacter* and *Thalassolituus*, also present in cold environments, are some of these specific hydrocarbon degrading genera. When an oil spill event occurs, approximately 90% of the microbial community are compounded by obligate hydrocarbon-degrading bacteria, extremely relevant in the natural attenuation of oil-polluted marine environments (Yakimov et al., 2007; Mcgenity et al., 2012). The OHCB are broadly distributed; however, some of the species, as *Oleispira antarctica*, have only been detected in cold ocean. Moreover, the type of hydrocarbon contamination can influence microbial shifts, selecting specific genera, such as aliphatic-degrading *Alcanivorax* and aromatic- degrading *Cycloclasticus* (Berthe-Corti and Nachtkamp, 2010).

Nevertheless, other bacteria not associated to obligate or specific hydrocarbon degrading groups have been related to hydrocarbon degradation in marine environments, such as *Bacillus* (Zhuang et al., 2002; Hentati et al., 2016), *Pseudomonas*, *Acinetobacter* (Tarhriz et al., 2019), *Alcanivorax*, *Marinobacter* and *Rhodococcus* (Wang W. et al., 2014; Catania et al., 2015) *Dietzia* and *Rhodococcus* (Wang W. et al., 2014; Yang et al., 2017).

As previously described, some parameters are taken into account as modulators of PAH degradation. Temperature is a key factor, especially in polar and temperate regions. Low temperature influence oil weathering processes and metabolic activity of microorganisms, hindering the biodegradation process or reducing it at extremely low rates (Naseri et al., 2014). This can be a great concern for the delineation of bioremediation strategies in cold oil polluted areas as Arctic regions, rich in hydrocarbon resources and exploited since 1970 by offshore drilling activities.

PAH BIODEGRADATION IN DOWNSTREAM ACTIVITIES: GROUNDWATER SPILLS

The risk of petroleum spills during downstream activities (refining and distribution), including leakages of underground tanks, represents a major factor of groundwater contamination. These pollutants can migrate and reach the groundwater causing serious contamination of water resources. In some cases, released hydrocarbons are distributed as light non-aqueous phase liquid (LNAPL). Although hydrocarbons are known as "immiscible", they have a very low miscibility in concentrations at the order of micrograms up to a few grams per liter. Thus, the LNAPL can continuously release hydrocarbons to the aqueous phase, leading to a rapid dissolved phase plume formation with high potential toxicity (Coates et al., 2002; Chakraborty and Coates, 2004).

Commonly, due to the rapid oxygen depletion by aerobic hydrocarbon degraders, the hydrocarbon-polluted aquifers are anaerobic. As previously described, in the anaerobic degradation,

microorganisms use alternative final electron acceptors (e.g., iron, sulfate or nitrate), frequently in a syntrophic relationship with other bacteria and methanogenic archaea (Van Hamme et al., 2003; Keller et al., 2018).

The -omics approaches have been used by several authors for monitoring/improving bioremediation process. The taxonomic and functional microbial diversity have been characterized in aquifer sediments collected in the saturated zone and in *in situ* microcosms enriched with some mono- and di-aromatic hydrocarbons (toluene, benzene and naphthalene) from jet fuel, revealing the key microorganisms involved in the natural attenuation of these compounds. In *in situ* microcosms, b-diversity analyses showed a clear difference between the microbiome of the microcosms enriched with monoaromatics and diaromatics, confirming the hydrocarbon-degrading microorganisms have preference for each class of molecule. Families like SR-FBR-L83 (Ignavibacteriales order), Syntrophaceae and Spirochaetaceae were almost exclusive in the *in situ* microcosm amended with naphthalene. On the other hand, Geobacteraceae and Peptococcaceae were the most relevant families in the toluene and benzene amended microcosms (Hidalgo et al., 2019). Syntrophaceae and Spirochaetaceae have been reported as naphthalene degraders under sulfate conditions (Kümmel et al., 2015). Assessment of the functional profiles revealed that genes related with all stages of monoaromatic anaerobic degradation were found, including genes involved with carboxylation and fumarate addition for the initial activation mechanism. In aquifer sediments, genes of the later stages of naphthalene aerobic degradation pathway (catechol conversion) (**Figure 2** and **Table 2**), mainly *via* dioxygenases (*ortho* cleavage; *nah*F, *nah*G, *nah*I, *nah*J, *nah*K, *nah*N, *nah*L, *nah*M, *nah*O, *cat*A, *cat*B, *cat*C, *pca*D, *pca*I, *pca*F) were found, especially in the border of the contamination plume, where oxygen concentration was higher. These genes were mostly affiliated to *Acinetobacter* genus. However, genes for anaerobic degradation of naphthalene were not found. Based on the results, the authors concluded that the affected area was able to decontaminate monoaromatic hydrocarbons by natural attenuation due to the presence of degrading taxa and genes related with degradation of the contaminant. On the other hand, for naphthalene decontamination, other bioremediation approaches, such as biostimulation with electron acceptors like nitrate and/or sulfate for anaerobic microbiota or oxygen for aerobic microbes, should be necessary (Hidalgo et al., 2019).

Ramos et al. (2014) investigated microbial communities related to aromatic hydrocarbons bioremediation by methanogenic syntrophic biodegradation in groundwater polluted with biodiesel mixture (diesel/biodiesel) through controlled field experiment. They used ammonium acetate to biostimulate the methanogenic metabolism and compared the microbial community shifts using 16S rRNA gene pyrosequencing. They observed an increase in the relative abundance of *Desulfitobacterium* and *Geobacter* spp., which are known as anaerobic hydrocarbon degraders, demonstrating the importance of enriching hydrocarbon degrading microorganisms by a syntrophic process with methanogenic archaea to enhance the biodiesel biodegradation (Ramos et al., 2014). In the same polluted site, Müller et al. (2017) used ammonium acetate and a low-cost sustainable product recovered from acid mine drainage treatment to stimulate iron and sulfate reduction metabolisms. Amplicon 16S rRNA sequencing analyses showed a shift in the community after 3 months of treatment and almost 100% of the population were affiliated to *Geobacter* genus in 7.4 months. This genus has been described as dominant member in microbial communities with high iron concentration (Botton et al., 2007; Lin et al., 2009) and they can degrade organic compounds coupled to iron reduction (Anderson et al., 1998; Botton et al., 2007; Maithreepala and Doong, 2009; Zhang et al., 2012; Li et al., 2014). These results were in accordance with the high iron (II) concentration and the acetate depletion observed. Thirteen months later, almost 60% of the sequences were assigned to GOUTA19 genus (family Thermodesulfovibrionaceae). GOUTA19 genus was also observed before in soil irrigated with water contaminated with acid mine drainage (Sun et al., 2015), oil storage tanks (Watanabe et al., 2002) and in monochlorobenzene-contaminated water (Alfreider et al., 2002). Some members of the Thermodesufovibrionaceae family have been related with sulfate reduction process (Rabus et al., 2006; Bhatnagar et al., 2015; Sun et al., 2015). The lower concentration of benzene and naphthalene compared with the control (without stimulation) over the whole experiment supports the key role of the genera *Geobacter* and GOUTA19 in the degradation of these aromatic hydrocarbons by iron and sulfate reduction metabolisms, respectively (Müller et al., 2017).

The efficacy of the bioaugmentation approach was investigated in a large-scale treatment of petroleum contaminated groundwater in a petroleum facility. The bioaugmentation consortium composed by 22 aerobic bacterial strains was obtained from a biofilter of a wastewater treatment plant located in the petroleum facility. The treatment was performed on a modified aerated ISO tank (18 m^3 of capacity). The contaminated groundwater was pumped into the ISO tank and 16 L of the microbial consortium was added. Total petroleum hydrocarbons (TPH) concentration decreased from 1,563 $mg.L^{-1}$ to 89 $mg.L^{-1}$. The microbial diversity and composition were assessed by 16S rRNA sequencing, which showed a first shift in the microbial profile when the consortium was inoculated but after 18 days of treatment a stable microbial community was observed. The results suggested that indigenous bacteria growth, overtaken the survival rate of some strains of the consortium (e.g., bacilli). This might have played an important role in the continuous degradation of TPH. At the beginning of the treatment, the genera *Cloacibacterium*, *Sediminibacterium*, *Brevundimonas* and *Curvibacter* were the most abundant. After the 18th day, the predominant genus was *Flavobacterium* followed by *Sediminibacteium* and *Limnobacter*. At the end of the treatment *Flavobacterium* was the dominant genus accounting for almost 41% of the community (Poi et al., 2018). This results supply evidence for the use of bioaugmentation as an option for the treatment of large volumes of hydrocarbon-polluted groundwater and corroborates previous findings on the metabolic potential for PAH degradation of the consortia members (Poi et al., 2018). Previously, *Cloacibacterum* spp. have been reported in fresh water microcosms contaminated with

naphthalene (Aburto et al., 2009), whereas in aerobic reactors for treatment of PAH-contaminated soil, *Sediminibacterium* members was observed in aerobic reactors treating PAH-contaminated soil (Aburto et al., 2009). The increase of the PAH degradation potential and activity have been related with the presence of the genera *Brevundimonas* and *Pseudomonas* (Chen et al., 2010). Yet, other species of *Brevundimonas* have also been reported as naphthalene oxidizers (El Fantroussi and Agathos, 2005; Rodríguez-Martínez et al., 2006).

High throughput techniques in combination with other approaches, like stable isotope probing (SIP), are very useful for tracing the fate of pollutant compounds. SIP is based on the principle that microorganisms are able to assimilate stable isotope-labeled substrates and incorporate them into the different microbial components (DNA, RNA, proteins, phospholipid fatty acids), which can be further detected by molecular techniques (Neufeld et al., 2007). A long-term coal-tar contaminated groundwater called "Site 24" located in the state of New York, is one of the best characterized fields in terms of PAH contamination and has served as a model for natural attenuation during almost three decades (Madsen et al., 1991; Bakermans et al., 2002; Yagi et al., 2010). Wilhelm et al. (2018) executed a study in "Site 24" to evaluate the natural attenuation process in the affected area. They used a polyphasic approach based on SIP with 13C-napthalene, 16S rRNA gene and shotgun metagenomic sequencing to identify genes and taxa specialized in the naphthalene degradation under oxic (DO < 2.0 mgL^{-1}) and hypoxic/suboxic conditions (DO < 0.2 mgL^{-1}). The authors observed a naphthalene-degrading bacteria dominance in the microbial community by the decrease in the species richness and Shannon's diversity index. Due to the increase of about three-fold in the relative abundance of 16S rRNA gene in the metagenomes with 13C-labeled naphtalhene, members of six bacterial genera (*Cupriavidus, Ralstonia, Methylobacterium, Sphingomonas, Stenotrophomonas,* and *Rhodococcus)* were appeared to be responsible for the mineralization and assimilation of 13C-napthalene. However, key genes for the anaerobic conversion of naphthalene by sulfate-reducing bacteria, such as naphthalene carboxylase and naphthoate-CoA, were not present in the metagenomes. On the other hand, genes related in more general anaerobic aromatic degradation pathways were detected, like phenylphosphate carboxylase, benzoate-CoA ligase and succinylbenzoate-CoA ligase. In the search for the genetic basis for alternative redox coupling degradation, the authors found genes related with the dissimilatory nitrate (*narG*) and nitrite (*nirB*) reduction in a metagenome-assembled genome (MAG) affiliated to the *Burkholderiaceae* family. Genes for sulfate and iron reduction were not found in any MAG. Two ring-cleaving dioxygenases (2,3-dihydroxybiphenyl 1,2 dioxygenase and 3-hydrixyanthranilate 3,4-dioxygenase) were present in some MAGs (Wilhelm et al., 2018). The study contributed to gain information about undescribed taxa and metabolic potential associated to the natural attenuation process in the polluted subsurface environment (Wilhelm et al., 2018).

Literature data raised in this review show that there are several genera able to aerobically and/or anaerobically degrade PAHs in the main environments associated to the oil supply chain. A Venn diagram was plotted to show the genera that are exclusive to and those shared between oil reservoirs, seawater and groundwater (**Figure 3**). Genera like *Acinetobacter* and *Pseudomonas* are common to the three environments reviewed, demonstrating that some of the microorganisms may be considered as generalists, able to grow under different conditions. *Bacillus, Dietzia, and Marinobacter* were reported in seawater samples and oil reservoirs, being the latter specifically found in reservoirs subjected to seawater injection for oil recovery. Genus *Syntrophus* was found in reservoirs and groundwater, showing that syntrophic microorganisms are important to hydrocarbon degradation in these environments. Many exclusive genera like *Cycloclasticus, Halomonas, Colwellia,* among others, were reported in the seawater environment. While *Brevundimonas, Cupriavidus, Methylobacterium, Ralstonia, Rhodococcus, Sphingomonas, Stenotrophomonas,* and *Spirochaeta* are exclusive of the groundwater.

OMICS APPROACHES AND PAH BIOREMEDIATION

Due to the high environmental contamination caused by the oily supply chain with diverse classes of hydrocarbons, including recalcitrant PAHS, and the urgent need to recover the affected ecosystems, bioremediation has become a topic of intensive research. The main challenges are to make bioremediation processes as efficient as possible and more cost-effective. Considering that the key players in the bioremediation processes are the microorganisms, the great challenge is to improve our knowledge about the biochemical pathways, physiology, ecology, biochemistry and metabolism regulatory mechanisms of the microorganisms responsible for biodegradation, as well as limiting factors affecting petroleum hydrocarbon degradation (Varjani and Upasani, 2017). Anaerobic degradation of PAHs has not yet been well understood so far. However, considering the global extensive use of oil and/or its by-products, and the consequent increase of PAHs polluted environments, it is necessary to pursuit successful clean-up strategies for these contaminants.

Many culturable hydrocarbon degrading bacteria are being isolated based on their capacity to use hydrocarbons as their unique energy and carbon sources, and with genome sequencing it is possible to identify all genes of the degradation pathways. There are many complete or almost complete sequenced genomes of cultured microorganisms which have functional potential metabolism for hydrocarbon degradation (Ghosal et al., 2016). Nevertheless, scientists have long known that only about 1% of the total microbial communities has been cultivated (Hugenholtz and Pace, 1996; Hugenholtz et al., 1998) and advances in the use of -omics techniques, like genomics, proteomics and metabolomics, in bioremediation studies have helped to improve the understanding of biodegradation processes (Brennerova et al., 2009; Nyyssönen et al., 2009; Abbai and Pillay, 2013; Uhlik et al., 2013; Mason et al., 2014; Xu et al., 2014, 2018; El Amrani et al., 2015; Loviso et al., 2015; Ma B. et al., 2015; Techtmann and

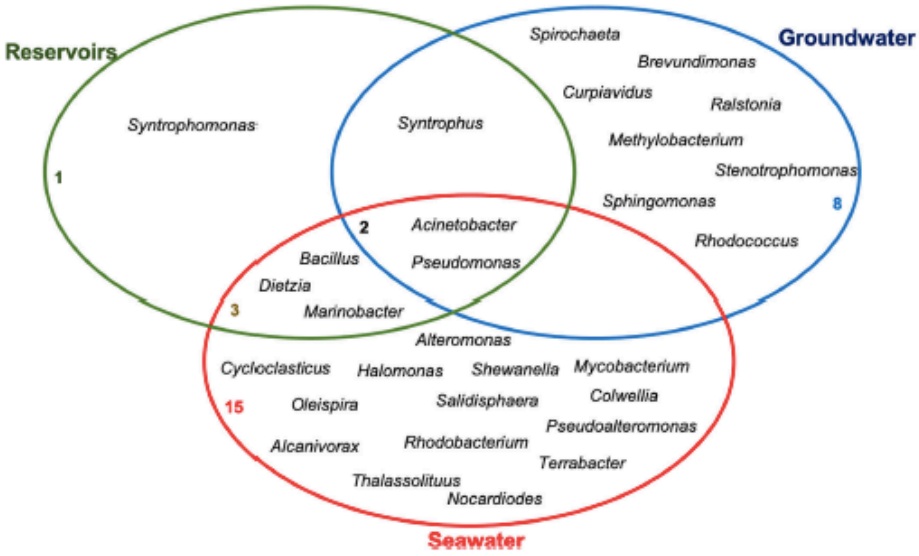


FIGURE 3 | Venn diagram depicting microbial genera that are shared or unique to each environment.

Hazen, 2016; Zafra et al., 2016; Duarte et al., 2017; Muangchinda et al., 2018; Tiralerdpanich et al., 2018; Wilhelm et al., 2018; Hidalgo et al., 2019). Sequencing metagenomes from diverse contaminated environments (soils, aquifers, seawater) can help to providing insights into the ecology of the dominant and rare members and their functional potential for transformation of pollutant molecules such as PAHs and other hydrocarbons (Blow, 2008; Ghosal et al., 2016). Also, the fast improvements in genome sequencing technology revolutionized the bioremediation application, allowing one to investigate the physiology and ecology of hydrocarbon degrading microorganisms in more detail (Buermans and Den Dunnen, 2014).

Taking into account that environmental conditions can stimulate or inhibit microbial growth, many studies have used microbial community members as bioindicators to detect different kinds of contamination. Based on the microbial community diversity, a model able to quantitatively predict the presence of contaminants in non- polluted and polluted samples was reported (Smith et al., 2015). This predictive model, based on statistical analysis of 16S rRNA biomarker, was initially evaluated in samples from a nuclear waste site and could accurately identify environmental contaminants, as uranium and nitrate. However, the authors extended the model to oil contaminated sites, such as samples from *Deep Horizon* oil spill, in order to explore whether this approach could be applied to other types of pollution. For that, samples collected before and after the oil spill in diverse sampling spots were analyzed. A computational model was developed based on several previously gathered data, allowing to distinguish polluted and unpolluted sites with almost perfect accuracy (98%). They showed that according to the results, the bacteria that showed more accuracy for detecting oil (and uranium) are related with these substrates metabolism, suggesting that the statistical approach was robust and uncover ecological interactions (Smith et al., 2015).

As previously mentioned, the accident involving the *Deepwater Horizon* drilling rig was pioneer oil spill investigated through metagenomics (Crone and Tolstoy, 2010; Reddy et al., 2012; King et al., 2015). Metagenomics was used to improve the comprehension about the fate of oil and the dynamics of biodegradation in the sea water (Techtmann and Hazen, 2016). This spill was the largest one in history and has unique characteristics, due to a remaining oil portion in the deep sediments as a deep water plume of oil (Camilli et al., 2010; Hazen et al., 2010). By using target metagenomics it was possible to investigate the differences in microbial structure and dynamics between deep and surface water (Redmond and Valentine, 2012; Gutierrez et al., 2013). This approach revealed that in the deep-water plume were enrich genes for hydrocarbon degradation and chemotaxis compared to uncontaminated deep water. The use of -omics approaches in the *Deepwater Horizon* oil spill allowed to expand the knowledge of the microbial community reaction to oil spills in the seawater environment as well as the identification of cold-adapted degraders and their role in bioremediation of oil pollution in cold environments (Techtmann and Hazen, 2016). Bioremediation potential, with focus on natural attenuation, is also investigated in marine coastal areas (Catania et al., 2015) and polar soils (Yergeau et al., 2012b). A recent work (Duarte et al., 2017) described a survey about the microbial catabolome for aerobic PAH degradation of a contaminated soil expose to 12 years of *in situ* bioremediation in Czech Republic. The results showed a complex microbial network leading to the discovery of microorganisms similar to those newly identified as *Rugosibacter aromaticivorans* and *Immundisolibacter cernigliae* involved in PAH degradation. Other strategies as bioaugmentation, based

on the application of designed microbial consortia, are widely reported in contaminated soils (Zafra et al., 2016; Poi et al., 2018; Haleyur et al., 2019; Wolf et al., 2019) and seawater (Nikolopoulou et al., 2013; Pereira et al., 2019; Shi et al., 2020).

The combination of all these modern molecular biology approaches as metagenomics, metatranscriptomics and metabolomics associated with bioinformatics tools have provided deep insights into the microbial response to PAH pollution, leading to the detection of new metabolic pathways and active degrading microorganisms and contributing to support bioremediation strategies as useful and ecofriendly tools.

CONCLUSION

The advances in high throughput sequencing approaches and bioinformatic tools allowed us to improve the understanding of the catabolism of hydrocarbons in several hydrocarbon-rich environments or contaminated sites. These approaches have enabled scientists to characterize and explore microbial communities from different types of affected environments (i.e., groundwater, oil reservoirs, seawater), where the metabolism of PAHs can be differently influenced by various factors (i.e., oxygen concentration, pH, salinity, temperature, nutrient availability, etc.). Although in many studies these strategies have contributed to the identification of key microbial players, genes and mechanisms for PAH degradation, in many other difficult to access environments, the processes remain unidentified. Also, there is currently scarce information about genes and enzymes in anaerobic environments, making the understanding of PAH anaerobic degradation processes, which is prevalent in polluted areas where oxygen is poorly available, even more difficult. Therefore, further innovative and well-designed research is still necessary to completely unveil aspects of PAH biodegradation that remain unknown.

AUTHOR CONTRIBUTIONS

KJH, IS-G, and BD: conceptualization, investigation, writing – original draft, and visualization. VO: conceptualization, resources, writing – review and editing, supervision, and funding acquisition. All authors contributed to the article and approved the submitted version.

FUNDING

The authors are grateful to São Paulo Research Foundation for the Master scholarship of KJH (process number 2017/16611-0) and doctoral scholarships of IS-G (process number 2011/14501-6) and of BD (process number 2010/52247-1) also, the authors are thankful to Coordenação de Aperfeiçonamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001, for the doctoral scholarship of KJH (process numer 88887.373972/2019-00) and to National Council for Scientific and Technological Development (CNPq) for the Productivity in research grant of VO.

REFERENCES

Abbai, N. S., and Pillay, B. (2013). Analysis of hydrocarbon-contaminated groundwater metagenomes as revealed by high-throughput sequencing. *Mol. Biotechnol.* 54, 900–912. doi: 10.1007/s12033-012-9639-z

Abdel-Shafy, H. I., and Mansour, M. S. M. (2016). A review on polycyclic aromatic hydrocarbons: source, environmental impact, effect on human health and remediation. *Egypt. J. Pet.* 25, 107–123. doi: 10.1016/j.ejpe.2015.03.011

Aburto, A., Fahy, A., Coulon, F., Lethbridge, G., Timmis, K., Ball, A., et al. (2009). Mixed aerobic and anaerobic microbial communities in benzene-contaminated groundwater. *J. Appl. Microbiol.* 106, 317–328. doi: 10.1111/j.1365-2672.2008.04005.x

Adetutu, E. M., Ball, A. S., Weber, J., Aleer, S., Dandie, C. E., and Juhasz, A. L. (2012). Impact of bacterial and fungal processes on 14C-hexadecane mineralisation in weathered hydrocarbon contaminated soil. *Sci. Total Environ.* 414, 585–591. doi: 10.1016/j.scitotenv.2011.11.044

Aitken, C. M., Jones, D. M., and Larter, S. R. (2004). Anaerobic hydrocarbon biodegradation in deep subsurface oil reservoirs. *Nature* 431, 291–294.

Albaigés, J., Frei, R. W., and Merian, E. (1983). *Chemistry and Analysis of Hydrocarbons in the Environment*. New York, NY: Gordon and Breach Science Publishers.

Alfreider, A., Vogt, C., and Babel, W. (2002). Microbial diversity in an in situ reactor system treating monochlorobenzene contaminated groundwater as revealed by 16S ribosomal DNA analysis. *Syst. Appl. Microbiol.* 25, 232–240. doi: 10.1078/0723-2020-00111

An, D., Caffrey, S. M., Soh, J., Agrawal, A., Brown, D., Budwill, K., et al. (2013). Metagenomics of hydrocarbon resource environments indicates aerobic taxa and genes to be unexpectedly common. *Environ. Sci. Technol.* 47, 10708–10717. doi: 10.1021/es402018d

Anderson, R. T., Rooney-Varga, J. N., Gaw, C. V., and Lovley, D. R. (1998). Anaerobic benzene oxidation in the Fe(III) reduction zone of petroleum-contaminated aquifers. *Environ. Sci. Technol.* 32, 1222–1229. doi: 10.1021/es9704949

Annweiler, E., Michaelis, W., and Meckenstock, R. U. (2002). Identical ring cleavage products during anaerobic degradation of naphthalene, 2-methylnaphthalene, and Tetralin Indicate a New Metabolic Pathway. *Appl. Environ. Microbiol.* 68, 852–858. doi: 10.1128/aem.68.2.852-858.2002

Appolinario, L. R., Tschoeke, D., Paixão, R. V., Veras, T., Calegario, G., Leomil, L., et al. (2019). Metagenomics sheds light on the metabolic repertoire of oil-biodegrading microbes of the South Atlantic Ocean. *Environ. Pollut.* 249, 295–304. doi: 10.1016/j.envpol.2019.03.007

Atlas, R. M. (1998). *Microbial Ecology: Fundamentals and Applications*. San Francisco, CA: Pearson Education India.

Atlas, R. M., and Hazen, T. C. (2011). Oil biodegradation and bioremediation: a tale of the two worst spills in US history. *Environ. Sci. Technol.* 45, 6709–6715. doi: 10.1021/es2013227

Bacosa, H. P., and Inoue, C. (2015). Polycyclic aromatic hydrocarbons (PAHs) biodegradation potential and diversity of microbial consortia enriched from tsunami sediments in Miyagi, Japan. *J. Hazard. Mater.* 283, 689–697. doi: 10.1016/j.jhazmat.2014.09.068

Bakermans, C., Hohnstock-Ashe, A., Padmanabhan, S., Padmanabhan, P., and Madsen, E. (2002). Geochemical and physiological evidence for mixed aerobic and anaerobic field biodegradation of coal tar waste by subsurface microbial communities. *Microb. Ecol.* 44, 107–117. doi: 10.1007/s00248-002-3011-y

Bamforth, S. M., and Singleton, I. (2005). Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. *J. Chem. Technol. Biotechnol.* 80, 723–736. doi: 10.1002/jctb.1276

Bedessem, M. E., Swoboda-Colberg, N. G., and Colberg, P. J. S. (1997). Naphthalene mineralization coupled to sulfate reduction in aquifer-derived enrichments. *FEMS Microbiol. Lett.* 152, 213–218. doi: 10.1111/j.1574-6968.1997.tb10430.x

Berdugo-Clavijo, C., Dong, X., Soh, J., Sensen, C. W., and Gieg, L. M. (2012). Methanogenic biodegradation of two-ringed polycyclic aromatic hydrocarbons. *FEMS Microbiol. Lett.* 81, 124–133. doi: 10.1111/j.1574-6941.2012.01328.x

Berthe-Corti, L., and Nachtkamp, M. (2010). "Bacterial communities in hydrocarbon-contaminated marine coastal environments," in *Handbook of Hydrocarbon and Lipid Microbiology*, ed. K. N. Timmis (Heidelberg: Springer-Verlag), 2350–2359.

Bhatnagar, S., Badger, J. H., Madupu, R., Khouri, H. M., O'connor, E. M., Robb, F. T., et al. (2015). Genome sequence of the sulfate-reducing thermophilic bacterium *Thermodesulfovibrio yellowstonii* strain DSM 11347T (phylum Nitrospirae). *Genome Announc.* 3:e01489-14.

Bian, X., Maurice Mbadinga, S., Liu, Y., Yang, S., Liu, J., Ye, R., et al. (2015). Insights into the anaerobic biodegradation pathway of *n*-Alkanes in oil reservoirs by detection of signature metabolites. *Sci. Rep.* 5:9801.

Bianco, F., Race, M., Papirio, S., and Esposito, G. (2020). Removal of polycyclic aromatic hydrocarbons during anaerobic biostimulation of marine sediments. *Sci. Total Environ.* 709, 136141. doi: 10.1016/j.scitotenv.2019.136141

Big Data and Analytics (2020). *Energy Analysis: Oil. Oil. 2020*. Washington, DC: Big Data and Analytics.

Blow, N. (2008). Exploring unseen communities. *Nature* 453, 687–689. doi: 10.1038/453687a

Boll, M., Löffler, C., Morris, B. E. L., and Kung, J. W. (2014). Anaerobic degradation of homocyclic aromatic compounds via arylcarboxyl-coenzyme A esters: organisms, strategies and key enzymes. *Environ. Microbiol.* 16, 612–627. doi: 10.1111/1462-2920.12328

Bongiorni, L., Magagnini, M., Armeni, M., Noble, R., and Danovaro, R. (2005). Viral production, decay rates, and life strategies along a trophic gradient in the North Adriatic Sea. *Appl. Environ. Microbiol.* 71, 6644–6650. doi: 10.1128/aem.71.11.6644-6650.2005

Botton, S., Harmelen, M. V., Braster, M., Parsons, J. R., and Röling, W. F. M. (2007). Dominance of Geobacteraceae in BTX-degrading enrichments from an iron-reducing aquifer. *FEMS Microbiol. Lett.* 62, 118–130. doi: 10.1111/j.1574-6941.2007.00371.x

Brennerova, M. V., Josefiova, J., Brenner, V., Pieper, D. H., and Junca, H. (2009). Metagenomics reveals diversity and abundance of meta-cleavage pathways in microbial communities from soil highly contaminated with jet fuel under air-sparging bioremediation. *Environ. Microbiol.* 11, 2216–2227. doi: 10.1111/j.1462-2920.2009.01943.x

Brooijmans, R. J. W., Pastink, M. I., and Siezen, R. J. (2009). Hydrocarbon-degrading bacteria: the oil-spill clean-up crew. *Microb. Biotechnol.* 2, 587–594. doi: 10.1111/j.1751-7915.2009.00151.x

Buermans, H., and Den Dunnen, J. (2014). Next generation sequencing technology: advances and applications. *Biochim. Biophys. Acta* 1842, 1932–1941.

Cai, M., Nie, Y., Chi, C.-Q., Tang, Y.-Q., Li, Y., Wang, X.-B., et al. (2015). Crude oil as a microbial seed bank with unexpected functional potentials. *Sci. Rep.* 5:16057.

Camilli, R., Reddy, C. M., Yoerger, D. R., Van Mooy, B. A., Jakuba, M. V., Kinsey, J. C., et al. (2010). Tracking hydrocarbon plume transport and biodegradation at Deepwater Horizon. *Science* 330, 201–204. doi: 10.1126/science.1195223

Campeão, M. E., Swings, J., Silva, B. S., Otsuki, K., Thompson, F. L., and Thompson, C. C. (2019). "*Candidatus* Colwellia aromaticivorans" sp. nov.,"*Candidatus* Halocyntiibacter alkanivorans" sp. nov., and "*Candidatus* Ulvibacter alkanivorans" sp. nov. Genome Sequences. *Microbiol. Resour. Announc.* 8:e00086-19.

Catania, V., Santisi, S., Signa, G., Vizzini, S., Mazzola, A., Cappello, S., et al. (2015). Intrinsic bioremediation potential of a chronically polluted marine coastal area. *Mar. Pollut. Bull.* 99, 138–149. doi: 10.1016/j.marpolbul.2015.07.042

Cerniglia, C. E. (1993). Biodegradation of polycyclic aromatic hydrocarbons. *Curr. Opin. Biotechnol.* 4, 331–338. doi: 10.1201/b17587-16

Chakraborty, R., and Coates, J. (2004). Anaerobic degradation of monoaromatic hydrocarbons. *Appl. Microbiol. Biotechnol.* 64, 437–446. doi: 10.1007/s00253-003-1526-x

Chen, J., and Denison, M. S. (2011). The Deepwater Horizon oil spill: environmental fate of the oil and the toxicological effects on marine organisms. *J. Young Invest.* 21, 84–95.

Chen, K.-F., Kao, C.-M., Chen, C.-W., Surampalli, R. Y., and Lee, M. S. (2010). Control of petroleum-hydrocarbon contaminated groundwater by intrinsic and enhanced bioremediation. *J. Environ. Sci.* 22, 864–871. doi: 10.1016/s1001-0742(09)60190-x

Coates, J. D., Chakraborty, R., and Mcinerney, M. J. (2002). Anaerobic benzene biodegradation—a new era. *Res. Microbiol.* 153, 621–628. doi: 10.1016/s0923-2508(02)01378-5

Coates, J. D., Woodward, J., Allen, J., Philp, P., and Lovley, D. R. (1997). Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. *Appl. Environ. Microbiol.* 63, 3589–3593. doi: 10.1128/aem.63.9.3589-3593.1997

Costa, A. S., Romão, L. P. C., Araújo, B. R., Lucas, S. C. O., Maciel, S. T. A., Wisniewski, A., et al. (2012). Environmental strategies to remove volatile aromatic fractions (BTEX) from petroleum industry wastewater using biomass. *Bioresour. Technol.* 105, 31–39. doi: 10.1016/j.biortech.2011.11.096

Coulon, F., Chronopoulou, P.-M., Fahy, A., Païssé, S., Goñi-Urriza, M., Peperzak, L., et al. (2012). Central role of dynamic tidal biofilms dominated by aerobic hydrocarbonoclastic bacteria and diatoms in the biodegradation of hydrocarbons in coastal mudflats. *Appl. Environ. Microbiol.* 78, 3638–3648. doi: 10.1128/aem.00072-12

Court, C. D., Hodges, A. W., Clouser, R. L., and Larkin, S. L. (2017). Economic impacts of cancelled recreational trips to Northwest Florida after the Deepwater Horizon oil spill. *Reg. Sci. Policy Pract.* 9, 143–164. doi: 10.1111/rsp3.1209

Crone, T. J., and Tolstoy, M. (2010). Magnitude of the 2010 Gulf of Mexico oil leak. *Science* 330, 634–634. doi: 10.1126/science.1195840

Da Cruz, G. F., De Vasconcellos, S. P., Angolini, C. F., Dellagnezze, B. M., Garcia, I. N., De Oliveira, V. M., et al. (2011). Could petroleum biodegradation be a joint achievement of aerobic and anaerobic microorganisms in deep sea reservoirs? *AMB Express* 1:47. doi: 10.1186/2191-0855-1-47

Dellagnezze, B., Vasconcellos, S., Angelim, A., Melo, V., Santisi, S., Cappello, S., et al. (2016). Bioaugmentation strategy employing a microbial consortium immobilized in chitosan beads for oil degradation in mesocosm scale. *Mar. Pollut. Bull.* 107, 107–117. doi: 10.1016/j.marpolbul.2016.04.011

Dong, C., Bai, X., Sheng, H., Jiao, L., Zhou, H., and Shao, Z. (2015). Distribution of PAHs and the PAH-degrading bacteria in the deep-sea sediments of the high-latitude Arctic Ocean. *Biogeosci. Discuss.* 12, 2163–2177. doi: 10.5194/bg-12-2163-2015

Dos Santos, H. F., Cury, J. C., Do Carmo, F. L., Dos Santos, A. L., Tiedje, J., Van Elsas, J. D., et al. (2011). Mangrove bacterial diversity and the impact of oil contamination revealed by pyrosequencing: bacterial proxies for oil pollution. *PLoS One* 6:e16943. doi: 0.1371/journal.pone.0016943

Duarte, M., Nielsen, A., Camarinha-Silva, A., Vilchez-Vargas, R., Bruls, T., Wos-Oxley, M. L., et al. (2017). Functional soil metagenomics: elucidation of polycyclic aromatic hydrocarbon degradation potential following 12 years of in situ bioremediation. *Environ. Microbiol.* 19, 2992–3011. doi: 10.1111/1462-2920.13756

Duran, R., and Cravo-Laureau, C. (2016). Role of environmental factors and microorganisms in determining the fate of polycyclic aromatic hydrocarbons in the marine environment. *FEMS Microbiol. Rev.* 40, 814–830. doi: 10.1093/femsre/fuw031

Eberlein, C., Estelmann, S., Seifert, J., Von Bergen, M., Müller, M., Meckenstock, R. U., et al. (2013a). Identification and characterization of 2-naphthoyl-coenzyme A reductase, the prototype of a novel class of dearomatizing reductases. *Mol. Microbiol.* 88, 1032–1039. doi: 10.1111/mmi.12238

Eberlein, C., Johannes, J., Mouttaki, H., Sadeghi, M., Golding, B. T., Boll, M., et al. (2013b). ATP-dependent/-independent enzymatic ring reductions involved in the anaerobic catabolism of naphthalene. *Environ. Microbiol.* 15, 1832–1841. doi: 10.1111/1462-2920.12076

El Amrani, A., Dumas, A.-S., Wick, L. Y., Yergeau, E., and Berthomé, R. (2015). Omics" Insights into PAH Degradation toward Improved Green Remediation Biotechnologies. *Environ. Sci. Technol.* 49, 11281–11291. doi: 10.1021/acs.est.5b01740

El Fantroussi, S., and Agathos, S. N. (2005). Is bioaugmentation a feasible strategy for pollutant removal and site remediation? *Curr. Opin. Microbiol.* 8, 268–275. doi: 10.1016/j.mib.2005.04.011

Fuchs, G., Boll, M., and Heider, J. (2011). Microbial degradation of aromatic compounds–from one strategy to four. *Nat. Rev. Microbiol.* 9, 803–816. doi: 10.1038/nrmicro2652

Gan, S., Lau, E., and Ng, H. (2009). Remediation of soils contaminated with polycyclic aromatic hydrocarbons (PAHs). *J. Hazard. Mater.* 172, 532–549. doi: 10.1016/j.jhazmat.2009.07.118

Gentili, A. R., Cubitto, M. A., Ferrero, M., and Rodriguéz, M. S. (2006). Bioremediation of crude oil polluted seawater by a hydrocarbon-degrading bacterial strain immobilized on chitin and chitosan flakes. *Int. Biodeterior. Biodegradation* 57, 222–228. doi: 10.1016/j.ibiod.2006.02.009

Ghazali, F. M., Rahman, R. N. Z. A., Salleh, A. B., and Basri, M. (2004). Biodegradation of hydrocarbons in soil by microbial consortium. *Int Biodeterior. Biodegradation* 54, 61–67. doi: 10.1016/j.ibiod.2004.02.002

Ghosal, D., Ghosh, S., Dutta, T. K., and Ahn, Y. (2016). Current state of knowledge in microbial degradation of polycyclic aromatic hydrocarbons (PAHs): a review. *Front. Microbiol.* 7:1837. doi: 10.3389/fmicb.2016.01837

Gieg, L. M., Davidova, I. A., Duncan, K. E., and Suflita, J. M. (2010). Methanogenesis, sulfate reduction and crude oil biodegradation in hot Alaskan oilfields. *Environ. Microbiol.* 12, 3074–3086. doi: 10.1111/j.1462-2920.2010.02282.x

Gieg, L. M., Fowler, S. J., and Berdugo-Clavijo, C. (2014). Syntrophic biodegradation of hydrocarbon contaminants. *Curr. Opin. Biotechnol.* 27, 21–29. doi: 10.1016/j.copbio.2013.09.002

Gilbert, J. A., and Dupont, C. L. (2011). Microbial metagenomics: beyond the genome. *Annu. Rev. Mar. Sci.* 3, 347–371. doi: 10.1146/annurev-marine-120709-142811

Grabowski, A., Nercessian, O., Fayolle, F., Blanchet, D., and Jeanthon, C. (2005). Microbial diversity in production waters of a low-temperature biodegraded oil reservoir. *FEMS Microbiol. Lett.* 54, 427–443. doi: 10.1016/j.femsec.2005.05.007

Greenwood, P. F., Wibrow, S., George, S. J., and Tibbett, M. (2009). Hydrocarbon biodegradation and soil microbial community response to repeated oil exposure. *Org. Geochem.* 40, 293–300. doi: 10.1016/j.orggeochem.2008.12.009

Gupta, S., Pathak, B., and Fulekar, M. H. (2015). Molecular approaches for biodegradation of polycyclic aromatic hydrocarbon compounds: a review. *Rev. Environ. Sci. Biotechnol.* 14, 241–269. doi: 10.1007/s11157-014-9353-3

Gutierrez, T., Singleton, D. R., Berry, D., Yang, T., Aitken, M. D., and Teske, A. (2013). Hydrocarbon-degrading bacteria enriched by the Deepwater Horizon oil spill identified by cultivation and DNA-SIP. *ISME J.* 7, 2091–2104. doi: 10.1038/ismej.2013.98

Habe, H., and Omori, T. (2003). Genetics of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. *Biosci. Biotechnol. Biochem.* 67, 225–243. doi: 10.1271/bbb.67.225

Haleyur, N., Shahsavari, E., Jain, S. S., Koshlaf, E., Ravindran, V. B., Morrison, P. D., et al. (2019). Influence of bioaugmentation and biostimulation on PAH degradation in aged contaminated soils: response and dynamics of the bacterial community. *J. Environ. Manage.* 238, 49–58. doi: 10.1016/j.jenvman.2019.02.115

Hallmann, C., Schwark, L., and Grice, K. (2008). Community dynamics of anaerobic bacteria in deep petroleum reservoirs. *Nat. Geosci.* 1, 588–591. doi: 10.1038/ngeo260

Han, Y., Zhang, Y., Xu, C., and Hsu, C. S. (2018). Molecular characterization of sulfur-containing compounds in petroleum. *Fuel* 221, 144–158. doi: 10.1016/j.fuel.2018.02.110

Harvey, R. G. (1998). "Environmental chemistry of PAHs," in *PAHs and Related Compounds*, ed. A. H. Neilson (Berlin: Springer), 1–54. doi: 10.1007/978-3-540-49697-7_1

Hassanshahian, M., and Cappello, S. (2013). "Crude oil biodegradation in the marine environments," in *Biodegradation-Engineering and Technology*, eds R. Chamy and F. Rosenkranz (Rijeka: InTech), 101–135.

Hazen, T. C., Dubinsky, E. A., Desantis, T. Z., Andersen, G. L., Piceno, Y. M., Singh, N., et al. (2010). Deep-sea oil plume enriches indigenous oil-degrading bacteria. *Science* 330, 204–208.

Hazen, T. C., Prince, R. C., and Mahmoudi, N. (2016). Marine oil biodegradation. *Environ. Sci. Technol.* 50, 2121–2129.

He, P., Li, L., Liu, J., Bai, Y., and Fang, X. (2016). Diversity and distribution of catechol 2, 3-dioxygenase genes in surface sediments of the Bohai Sea. *FEMS Microbiol. Lett.* 363:86.

Head, I., Larter, S., Gray, N., Sherry, A., Adams, J., Aitken, C., et al. (2010). "Hydrocarbon degradation in petroleum reservoirs," in *Handbook of Hydrocarbon and Lipid Microbiology*, ed. K. N. Timmis (Berlin: Springer).

Head, I. M. (2017). "Microorganisms in the Oil and Gas Industry," in *Microbiologically Influenced Corrosion in the Upstream Oil and Gas Industry*, eds T. L. Skovhus, D. Enning, and J. S. Lee (Boca Raton, FL: CRC Press), 59.

Head, I. M., Gray, N. D., and Larter, S. R. (2014). Life in the slow lane; biogeochemistry of biodegraded petroleum containing reservoirs and implications for energy recovery and carbon management. *Front. Microbiol.* 5:566. doi: 10.3389/fmicb.2014.00566

Head, I. M., Jones, D. M., and Röling, W. F. (2006). Marine microorganisms make a meal of oil. *Nat. Rev. Microbiol.* 4, 173–182.

Heider, J. (2007). Adding handles to unhandy substrates: anaerobic hydrocarbon activation mechanisms. *Curr. Opin. Chem. Biol.* 11, 188–194.

Hein, F. J., Leckie, D., Larter, S., and Suter, J. R. (2013). "Heavy oil and bitumen petroleum systems in Alberta and beyond: the future is nonconventional and the future is now," in *Heavyoil and Oil-Sand Petroleum Systems in Alberta and Beyond: AAPG Studies*, eds F. J. Hein, D. Leckie, S. Larter, and J. R. Suter (Tulsa, OK: American Association of Petroleum), 1–21.

Hentati, D., Chebbi, A., Loukil, S., Kchaou, S., Godon, J.-J., Sayadi, S., et al. (2016). Biodegradation of fluoranthene by a newly isolated strain of Bacillus stratosphericus from Mediterranean seawater of the Sfax fishing harbour, Tunisia. *Environ. Sci. Pollut. Res.* 23, 15088–15100.

Hidalgo, K. J., Teramoto, E. H., Soriano, A. U., Valoni, E., Baessa, M. P., Richnow, H. H., et al. (2019). Taxonomic and functional diversity of the microbiome in a jet fuel contaminated site as revealed by combined application of in situ microcosms with metagenomic analysis. *Sci. Total Environ.* 708:135152.

Hu, P., Tom, L., Singh, A., Thomas, B. C., Baker, B. J., Piceno, Y. M., et al. (2016). Genome-resolved metagenomic analysis reveals roles for candidate phyla and other microbial community members in biogeochemical transformations in oil reservoirs. *mBio* 7:e01669-15. doi: 10.1128/mBio.01669-15

Hubert, C. R., Oldenburg, T. B., Fustic, M., Gray, N. D., Larter, S. R., Penn, K., et al. (2012). Massive dominance of Epsilonproteobacteria in formation waters from a Canadian oil sands reservoir containing severely biodegraded oil. *Environ. Microbiol.* 14, 387–404.

Hugenholtz, P., Goebel, B. M., and Pace, N. R. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* 180, 4765–4774.

Hugenholtz, P., and Pace, N. R. (1996). Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. *Trends Biotechnol.* 14, 190–197.

ITOPF (2011). *Effects of Oil Pollution on Social and Economic Activities*. London: ITOPF.

Jin, H. M., Kim, J. M., Lee, H. J., Madsen, E. L., and Jeon, C. O. (2012). Alteromonas as a key agent of polycyclic aromatic hydrocarbon biodegradation in crude oil-contaminated coastal sediment. *Environ. Sci. Technol.* 46, 7731–7740.

Johansson, L., and Van Bavel, B. (2003). Levels and patterns of polycyclic aromatic hydrocarbons in incineration ashes. *Sci. Total Environ.* 311, 221–231.

Jones, D., Head, I., Gray, N., Adams, J., Rowan, A., Aitken, C., et al. (2008). Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. *Nature* 451, 176–180.

Juhasz, A. L., and Naidu, R. (2000). Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo [a] pyrene. *Int. Biodeterior. Biodegradation* 45, 57–88.

Juhasz, A. L., Waller, N., Lease, C., Bentham, R., and Stewart, R. (2005). Pilot scale bioremediation of creosote-contaminated soil—efficacy of enhanced natural attenuation and bioaugmentation strategies. *Bioremed. J.* 9, 139–154.

Keller, A. H., Kleinsteuber, S., and Vogt, C. (2018). Anaerobic benzene mineralization by nitrate-reducing and sulfate-reducing microbial consortia enriched from the same site: comparison of community composition and degradation characteristics. *Microb. Ecol.* 75, 941–953.

Kimes, N. E., Callaghan, A. V, Aktas, D. F., Smith, W. L., Sunner, J., Golding, B. Y., et al. (2013). Metagenomic analysis and metabolite profiling of deep–sea sediments from the Gulf of Mexico following the Deepwater Horizon oil spill. *Front. Microbiol.* 4:50. doi: 10.3389/fmicb.2013.00050

Kimes, N. E., Callaghan, A. V., Suflita, J. M., and Morris, P. J. (2014). Microbial transformation of the Deepwater Horizon oil spill—past, present, and future perspectives. *Front. Microbiol.* 5:603. doi: 10.3389/fmicb.2014.00603

King, G., Kostka, J., Hazen, T., and Sobecky, P. (2015). Microbial responses to the Deepwater Horizon oil spill: from coastal wetlands to the deep sea. *Annu. Rev. Mar. Sci.* 7, 377–401.

Kingston, P. F. (2002). Long-term environmental impact of oil spills. *Spill Sci. Technol. Bull.* 7, 53–61.

Kobayashi, H., Endo, K., Sakata, S., Mayumi, D., Kawaguchi, H., Ikarashi, M., et al. (2012). Phylogenetic diversity of microbial communities associated with the crude-oil, large-insoluble-particle and formation-water components of the reservoir fluid from a non-flooded high-temperature petroleum reservoir. *J. Biosci. Bioeng.* 113, 204–210.

Kryachko, Y., Dong, X., Sensen, C. W., and Voordouw, G. (2012). Compositions of microbial communities associated with oil and water in a mesothermic oil field. *Antonie Van Leeuwenhoek* 101, 493–506.

Kümmel, S., Herbst, F.-A., Bahr, A., Duarte, M., Pieper, D. H., Jehmlich, N., et al. (2015). Anaerobic naphthalene degradation by sulfate-reducing Desulfobacteraceae from various anoxic aquifers. *FEMS Microbiol. Lett.* 91:fiv006.

Kuppusamy, S., Thavamani, P., Megharaj, M., and Naidu, R. (2016). Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by novel bacterial consortia tolerant to diverse physical settings–assessments in liquid-and slurry-phase systems. *Int. Biodeterior. Biodegradation* 108, 149–157.

Lamendella, R., Strutt, S., Borglin, S., Chakraborty, R., Tas, N., Mason, O., et al. (2014). Assessment of the Deepwater Horizon oil spill impact on Gulf coast microbial communities. *Front. Microbiol.* 5:130. doi: 10.3389/fmicb.2014.00130

Lamichhane, S., Bal Krishna, K. C., and Sarukkalige, R. (2016). Polycyclic aromatic hydrocarbons (PAHs) removal by sorption: a review. *Chemosphere* 148, 336–353.

Lawal, A. T. (2017). Polycyclic aromatic hydrocarbons. A review. *Cogent Environ. Sci.* 3:1339841.

Leahy, J. G., and Colwell, R. R. (1990). Microbial degradation of hydrocarbons in the environment. *Microbiol. Rev.* 54, 305–315.

Li, D., Midgley, D. J., Ross, J. P., Oytam, Y., Abell, G. C., Volk, H., et al. (2012). Microbial biodiversity in a Malaysian oil field and a systematic comparison with oil reservoirs worldwide. *Arch. Microbiol.* 194, 513–523.

Li, G., Wu, S., Wang, L., and Akoh, C. C. (2016). Concentration, dietary exposure and health risk estimation of polycyclic aromatic hydrocarbons (PAHs) in youtiao, a Chinese traditional fried food. *Food Control* 59, 328–336.

Li, J.-J., Cai, W.-T., Zhao, Y.-S., Dong, J., and Lian, S. (2014). Effects of groundwater geochemical constituents on degradation of benzene, toluene, ethylbenzene, and xylene coupled to microbial dissimilatory Fe (III) reduction. *Environ. Eng. Sci.* 31, 202–208.

Li, X.-X., Mbadinga, S. M., Liu, J.-F., Zhou, L., Yang, S.-Z., Gu, J.-D., et al. (2017). Microbiota and their affiliation with physiochemical characteristics of different subsurface petroleum reservoirs. *Int. Biodeterior. Biodegradation* 120, 170–185.

Liao, X., Zhao, D., Yan, X., and Huling, S. G. (2014). Identification of persulfate oxidation products of polycyclic aromatic hydrocarbon during remediation of contaminated soil. *J. Hazard. Mater.* 276, 26–34.

Lin, B., Westerhoff, H. V., and Röling, W. F. M. (2009). How Geobacteraceae may dominate subsurface biodegradation: physiology of Geobacter metallireducens in slow-growth habitat-simulating retentostats. *Environ. Microbiol.* 11, 2425–2433.

Liu, K., Han, W., Pan, W.-P., and Riley, J. T. (2001). Polycyclic aromatic hydrocarbon (PAH) emissions from a coal-fired pilot FBC system. *J. Hazard. Mater.* 84, 175–188.

Loviso, C. L., Lozada, M., Guibert, L. M., Musumeci, M. A., Sarango Cardenas, S., Kuin, R. V., et al. (2015). Metagenomics reveals the high polycyclic aromatic hydrocarbon-degradation potential of abundant uncultured bacteria from chronically polluted subantarctic and temperate coastal marine environments. *J. Appl. Microbiol.* 119, 411–424.

Lovley, D. R. (2003). Cleaning up with genomics: applying molecular biology to bioremediation. *Nat. Rev. Microbiol.* 1, 35–44.

Lozada, M., Mercadal, J. P. R., Guerrero, L. D., Di Marzio, W. D., Ferrero, M. A., and Dionisi, H. M. (2008). Novel aromatic ring-hydroxylating dioxygenase genes from coastal marine sediments of Patagonia. *BMC Microbiol.* 8:50. doi: 10.1186/1471-2180-8-50

Lu, X.-Y., Zhang, T., and Fang, H. H.-P. (2011). Bacteria-mediated PAH degradation in soil and sediment. *Appl. Microbiol. Biotechnol.* 89, 1357–1371.

Ma, B., Lyu, X.-F., Zha, T., Gong, J., He, Y., and Xu, J.-M. (2015). Reconstructed metagenomes reveal changes of microbial functional profiling during PAHs degradation along a rice (Oryza sativa) rhizosphere gradient. *J. Appl. Microbiol.* 118, 890–900.

Ma, Q., Qu, Y., Shen, W., Zhang, Z., Wang, J., Liu, Z., et al. (2015). Bacterial community compositions of coking wastewater treatment plants in steel industry revealed by Illumina high-throughput sequencing. *Bioresour. Technol.* 179, 436–443.

Mackay, D., and Callcott, D. (1998). "Partitioning and physical chemical properties of PAHs," in *PAHs and Related Compounds*, ed. A. H. Neilson (Berlin: Springer), 325–345.

Madsen, E. L., Sinclair, J. L., and Ghiorse, W. C. (1991). In situ biodegradation: microbiological patterns in a contaminated aquifer. *Science* 252, 830–833.

Maithreepala, R., and Doong, R.-A. (2009). Transformation of carbon tetrachloride by biogenic iron species in the presence of Geobacter sulfurreducens and electron shuttles. *J. Hazard. Mater.* 164, 337–344.

Marcos, M. S., Lozada, M., and Dionisi, H. M. (2009). Aromatic hydrocarbon degradation genes from chronically polluted Subantarctic marine sediments. *Lett. Appl. Microbiol.* 49, 602–608.

Margesin, R., Labbé, D., Schinner, F., Geer, C. W., and Whyte, L. G. (2003). Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils. *Appl. Environ. Microbiol.* 69, 3085–3092.

Marston, C. P., Pereira, C., Ferguson, J., Fischer, K., Hedstrom, O., Dashwood, W.-M., et al. (2001). Effect of a complex environmental mixture from coal tar containing polycyclic aromatic hydrocarbons (PAH) on the tumor initiation, PAH–DNA binding and metabolic activation of carcinogenic PAH in mouse epidermis. *Carcinogenesis* 22, 1077–1086.

Mason, O. U., Hazen, T. C., Borglin, S., Chain, P. S., Dubinsky, E. A., Fortney, J. L., et al. (2012). Metagenome, metatranscriptome and single-cell sequencing reveal microbial response to Deepwater Horizon oil spill. *ISME J.* 6, 1715–1727.

Mason, O. U., Scott, N. M., Gonzalez, A., Robbins-Pianka, A., Bælum, J., Kimbrel, J., et al. (2014). Metagenomics reveals sediment microbial community response to Deepwater Horizon oil spill. *ISME J.* 8, 1464–1475.

Mbadinga, S. M., Wang, L.-Y., Zhou, L., Liu, J.-F., Gu, J.-D., and Mu, B.-Z. (2011). Microbial communities involved in anaerobic degradation of alkanes. *Int. Biodeterior. Biodegradation* 65, 1–13.

Mcfarlin, K. M., Prince, R. C., Perkins, R., and Leigh, M. B. (2014). Biodegradation of dispersed oil in arctic seawater at-1 C. *PLoS One* 9:e84297. doi: 10.1371/journal.pone.0084297

Mcgenity, T. J., Folwell, B. D., Mckew, B. A., and Sanni, G. O. (2012). Marine crude-oil biodegradation: a central role for interspecies interactions. *Aquat. Biosyst.* 8:10.

Meckenstock, R., Annweiler, E., Michaelis, W., Richnow, H., and Schink, B. (2000). Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. *Appl. Environ. Microbiol.* 66, 2743–2747.

Meckenstock, R. U., Boll, M., Mouttaki, H., Koelschbach, J. S., Cunha Tarouco, P., Weyrauch, P., et al. (2016). Anaerobic Degradation of Benzene and Polycyclic Aromatic Hydrocarbons. *J. Mol. Microbiol. Biotechnol.* 26, 92–118.

Meckenstock, R. U., Von Netzer, F., Stumpp, C., Lueders, T., Himmelberg, A. M., Hertkorn, N., et al. (2014). Water droplets in oil are microhabitats for microbial life. *Science* 345, 673–676.

Meslé, M., Dromart, G., and Oger, P. (2013). Microbial methanogenesis in subsurface oil and coal. *Res. Microbiol.* 164, 959–972.

Messina, E., Denaro, R., Crisafi, F., Smedile, F., Cappello, S., Genovese, M., et al. (2016). Genome sequence of obligate marine polycyclic aromatic hydrocarbons-degrading bacterium *Cycloclasticus* sp. 78-ME, isolated from petroleum deposits of the sunken tanker Amoco Milford Haven, Mediterranean Sea. *Mar. Genomics* 25, 11–13.

Muangchinda, C., Rungsihiranrut, A., Prombutara, P., Soonglerdsongpha, S., and Pinyakong, O. (2018). 16S metagenomic analysis reveals adaptability of a mixed-PAH-degrading consortium isolated from crude oil-contaminated seawater to changing environmental conditions. *J. Hazard. Mater.* 357, 119–127.

Müller, J. B., Ramos, D. T., Larose, C., Fernandes, M., Lazzarin, H. S. C., Vogel, T. M., et al. (2017). Combined iron and sulfate reduction biostimulation as a novel approach to enhance BTEX and PAH source-zone biodegradation in biodiesel blend-contaminated groundwater. *J. Hazard. Mater.* 326, 229–236.

Musat, F., Galushko, A., Jacob, J., Widdel, F., Kube, M., Reinhardt, R., et al. (2009). Anaerobic degradation of naphthalene and 2-methylnaphthalene by strains of marine sulfate-reducing bacteria. *Environ. Microbiol.* 11, 209–219.

Nagashima, H., Zulkharnain, A. B., Maeda, R., Fuse, H., Iwata, K., and Omori, T. (2010). Cloning and nucleotide sequences of carbazole degradation genes from marine bacterium *Neptunibacter* sp. strain CAR-SF. *Curr. Microbiol.* 61, 50–56. doi: 10.1007/s00284-009-9575-8

Naseri, M., Barabadi, A., and Barabady, J. (2014). Bioremediation treatment of hydrocarbon-contaminated Arctic soils: influencing parameters. *Environ. Sci. Pollut. Res.* 21, 11250–11265. doi: 10.1007/s11356-014-3122-2

Nduagu, E. I., and Gates, I. D. (2015). Unconventional heavy oil growth and global greenhouse gas emissions. *Environ. Sci. Technol.* 49, 8824–8832. doi: 10.1021/acs.est.5b01913

Neethu, C., Saravanakumar, C., Purvaja, R., Robin, R., and Ramesh, R. (2019). Oil-spill triggered shift in indigenous microbial structure and functional dynamics in different marine environmental matrices. *Sci. Rep.* 9:1354. doi: 10.1038/s41598-018-37903-x

Nelson, J. R., and Grubesic, T. H. (2018). Oil spill modeling: risk, spatial vulnerability, and impact assessment. *Prog. Phys. Geogr. Earth Environ.* 42, 112–127. doi: 10.1177/0309133317744737

Neufeld, J. D., Vohra, J., Dumont, M. G., Lueders, T., Manefield, M., Friedrich, M. W., et al. (2007). DNA stable-isotope probing. *Nat. Protoc.* 2, 860–866. doi: 10.1038/nprot.2007.109

Nikolaivits, E., Dimarogona, M., Fokialakis, N., and Topakas, E. (2017). Marine-derived biocatalysts: importance, accessing, and application in aromatic pollutant bioremediation. *Front. Microbiol.* 8:265. doi: 10.3389/fmicb.2017.00265

Nikolopoulou, M., Pasadakis, N., and Kalogerakis, N. (2013). Evaluation of autochthonous bioaugmentation and biostimulation during microcosm-simulated oil spills. *Mar. Pollut. Bull.* 72, 165–173. doi: 10.1016/j.marpolbul.2013.04.007

Nyyssönen, M., Kapanen, A., Piskonen, R., Lukkari, T., and Itävaara, M. (2009). Functional genes reveal the intrinsic PAH biodegradation potential in creosote-contaminated groundwater following in situ biostimulation. *Appl. Microbiol. Biotechnol.* 84, 169–182. doi: 10.1007/s00253-009-2022-8

Orphan, V. J., Taylor, L. T., Hafenbradl, D., and Delong, E. F. (2000). Culture-dependent and culture-independent characterization of microbial assemblages associated with high-temperature petroleum reservoirs. *Appl. Environ. Microbiol.* 66, 700–711. doi: 10.1128/AEM.66.2.700-711.2000

Pannekens, M., Kroll, L., Müller, H., Mbow, F. T., and Meckenstock, R. U. (2019). Oil reservoirs, an exceptional habitat for microorganisms. *New Biotechnol.* 49, 1–9. doi: 10.1016/j.nbt.2018.11.006

Peng, C., Tang, Y., Yang, H., He, Y., Liu, Y., Liu, D., et al. (2020). Time-and compound-dependent microbial community compositions and oil hydrocarbon degrading activities in seawater near the Chinese Zhoushan Archipelago. *Mar. Pollut. Bull.* 152:110907. doi: 10.1016/j.marpolbul.2020.110907

Pereira, E., Napp, A. P., Allebrandt, S., Barbosa, R., Reuwsaat, J., Lopes, W., et al. (2019). Biodegradation of aliphatic and polycyclic aromatic hydrocarbons in seawater by autochthonous microorganisms. *Int. Biodeterior. Biodegradation* 145:104789. doi: 10.1016/j.ibiod.2019.104789

Perelo, L. W. (2010). In situ and bioremediation of organic pollutants in aquatic sediments. *J. Hazard. Mater.* 177, 81–89. doi: 10.1016/j.jhazmat.2009.12.090

Phelps, C. D., Battistelli, J., and Young, L. (2002). Metabolic biomarkers for monitoring anaerobic naphthalene biodegradation in situ. *Environ. Microbiol.* 4, 532–537. doi: 10.1046/j.1462-2920.2002.00330.x

Pieper, D. H., and Reineke, W. (2000). Engineering bacteria for bioremediation. *Curr. Opin. Biotechnol.* 11, 262–270. doi: 10.1016/S0958-1669(00)00094-X

Poi, G., Shahsavari, E., Aburto-Medina, A., Mok, P. C., and Ball, A. S. (2018). Large scale treatment of total petroleum-hydrocarbon contaminated groundwater using bioaugmentation. *J. Environ. Manage.* 214, 157–163. doi: 10.1016/j.jenvman.2018.02.079

Rabus, R., Hansen, T. A., and Widdel, F. (2006). "Dissimilatory sulfate- and sulfur-reducing prokaryotes," in *The Prokaryotes*, eds M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (Berlin: Springer), 659–768. doi: 10.1007/0-387-30742-7_22

Ramos, D. T., Da Silva, M. L. B., Nossa, C. W., Alvarez, P. J. J., and Corseuil, H. X. (2014). Assessment of microbial communities associated with fermentative–methanogenic biodegradation of aromatic hydrocarbons in groundwater contaminated with a biodiesel blend (B20). *Biodegradation* 25, 681–691. doi: 10.1007/s10532-014-9691-4

Reddy, C. M., Arey, J. S., Seewald, J. S., Sylva, S. P., Lemkau, K. L., Nelson, R. K., et al. (2012). Composition and fate of gas and oil released to the water column during the Deepwater Horizon oil spill. *Proc. Natl. Acad. Sci. U.S.A.* 109, 20229–20234. doi: 10.1073/pnas.1101242108

Redmond, M. C., and Valentine, D. L. (2012). Natural gas and temperature structured a microbial community response to the Deepwater Horizon oil spill. *Proc. Natl. Acad. Sci. U.S.A.* 109, 20292–20297. doi: 10.1073/pnas.1108756108

Rockne, K. J., and Strand, S. E. (2001). Anaerobic biodegradation of naphthalene, phenanthrene, and biphenyl by a denitrifying enrichment culture. *Water Res.* 35, 291–299. doi: 10.1016/S0043-1354(00)00246-3

Rodríguez-Martínez, E. M., Pérez, E. X., Schadt, C. W., Zhou, J., and Massol-Deyá, A. A. (2006). Microbial diversity and bioremediation of a hydrocarbon-contaminated aquifer (Vega Baja, Puerto Rico). *Int. J. Environ. Res. Public Health* 3, 292–300. doi: 10.3390/ijerph2006030036

Röling, W. F., Head, I. M., and Larter, S. R. (2003). The microbiology of hydrocarbon degradation in subsurface petroleum reservoirs: perspectives and prospects. *Res. Microbiol.* 154, 321–328. doi: 10.1016/S0923-2508(03)00086-X

Ron, E. Z., and Rosenberg, E. (2014). Enhanced bioremediation of oil spills in the sea. *Curr. Opin. Biotechnol.* 27, 191–194. doi: 10.1016/j.copbio.2014.02.004

Safinowski, M., and Meckenstock, R. U. (2006). Methylation is the initial reaction in anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. *Environ. Microbiol.* 8, 347–352. doi: 10.1111/j.1462-2920.2005.00900.x

Saito, A., Iwabuchi, T., and Harayama, S. (1999). Characterization of genes for enzymes involved in the phenanthrene degradation in *Nocardioides* sp. KP7. *Chemosphere* 38, 1331–1337. doi: 10.1016/S0045-6535(98)00534-7

Schwarz, A., Adetutu, E. M., Juhasz, A. L., Aburto-Medina, A., Ball, A. S., and Shahsavari, E. (2019). Response of the fungal community to chronic petrogenic contamination in surface and subsurface soils. *Geoderma* 338, 206–215. doi: 10.1016/j.geoderma.2018.12.004

Seo, J.-S., Keum, Y.-S., and Li, Q. X. (2009). Bacterial degradation of aromatic compounds. *Int. J. Environ. Res. Public Health* 6, 278–309. doi: 10.3390/ijerph6010278

Sette, L. D., Simioni, K. C. M., Vasconcellos, S. P., Dussan, L. J., Neto, E. V. S., and Oliveira, V. M. (2007). Analysis of the composition of bacterial communities in oil reservoirs from a southern offshore Brazilian basin. *Antonie Van Leeuwenhoek* 91, 253–266. doi: 10.1007/s10482-006-9115-5

Shahsavari, E., Adetutu, E. M., and Ball, A. S. (2015). "Phytoremediation and necrophytoremediation of petrogenic hydrocarbon-contaminated soils," in *Phytoremediation*, eds S. S. Gill, R. Gill, G. R. Lanza, L. Newman, and A. A. Ansari (Cham: Springer), 321–334. doi: 10.1007/978-3-319-10969-5_26

Shahsavari, E., Schwarz, A., Aburto-Medina, A., and Ball, A. S. (2019). Biological degradation of polycyclic aromatic compounds (PAHs) in soil: a current perspective. *Curr. Pollut. Rep.* 5, 84–92. doi: 10.1007/s40726-019-00113-8

Sharma, T. (2014). In silico investigation of polycyclic aromatic hydrocarbons against bacterial 1-2 dioxygenase. *J. Chem. Pharm. Res.* 6, 873–877.

Shen, T., Pi, Y., Bao, M., Xu, N., Li, Y., and Lu, J. (2015). Biodegradation of different petroleum hydrocarbons by free and immobilized microbial consortia. *Environ. Sci. Process. Impacts* 17, 2022–2033. doi: 10.1039/C5EM00318K

Shi, K., Zhang, Q., Xue, J., Chen, X., Chen, Y., Qiao, Y., et al. (2020). Study on the degradation performance and bacterial community of bioaugmentation in petroleum-pollution seawater. *J. Environ. Chem. Eng.* 8:103900. doi: 10.1016/j.jece.2020.103900

Shin, B., Kim, M., Zengler, K., Chin, K.-J., Overholt, W. A., Gieg, L. M., et al. (2019). Anaerobic degradation of hexadecane and phenanthrene coupled to sulfate reduction by enriched consortia from northern Gulf of Mexico seafloor sediment. *Sci. Rep.* 9:1239. doi: 10.1038/s41598-018-36567-x

Siddique, T., Fedorak, P. M., and Foght, J. M. (2006). Biodegradation of short-chain n-alkanes in oil sands tailings under methanogenic conditions. *Environ. Sci. Technol.* 40, 5459–5464. doi: 10.1021/es060993m

Sierra-García, I. N., Alvarez, J. C., De Vasconcellos, S. P., De Souza, A. P., Dos Santos Neto, E. V., and De Oliveira, V. M. (2014). New hydrocarbon degradation pathways in the microbial metagenome from Brazilian petroleum reservoirs. *PLoS One* 9:e90087. doi: 10.1371/journal.pone.0090087

Sierra-García, I. N., Belgini, D. R., Torres-Ballesteros, A., Paez-Espino, D., Capilla, R., Neto, E. V. S., et al. (2020). In depth metagenomic analysis in

contrasting oil wells reveals syntrophic bacterial and archaeal associations for oil biodegradation in petroleum reservoirs. *Sci. Total Environ.* 715, 136646. doi: 10.1016/j.scitotenv.2020.136646

Sierra-Garcia, I. N., Dellagnezze, B. M., Santos, V. P., Chaves, B. M. R., Capilla, R., Santos Neto, E. V., et al. (2017). Microbial diversity in degraded and non-degraded petroleum samples and comparison across oil reservoirs at local and global scales. *Extremophiles* 21, 211–229. doi: 10.1007/s00792-016-0897-8

Silva, T. R., Verde, L. C. L., Santos Neto, E. V., and Oliveira, V. M. (2013). Diversity analyses of microbial communities in petroleum samples from Brazilian oil fields. *Int. Biodeterior. Biodegradation* 81, 14. doi: 10.1016/j.ibiod.2012.05.005

Simon, C., and Daniel, R. (2011). Metagenomic analyses: past and future trends. *Appl. Environ. Microbiol.* 77, 1153–1161. doi: 10.1128/AEM.02345-10

Smith, M. B., Rocha, A. M., Smillie, C. S., Olesen, S. W., Paradis, C., Wu, L., et al. (2015). Natural bacterial communities serve as quantitative geochemical biosensors. *mBio* 6:e00326-15. doi: 10.1128/mBio.00326-15

Souza, E. C., Vessoni-Penna, T. C., and De Souza Oliveira, R. P. (2014). Biosurfactant-enhanced hydrocarbon bioremediation: an overview. *Int. Biodeterior. Biodegradation* 89, 88–94. doi: 10.1016/j.ibiod.2014.01.007

Speight, J. (2015). "Chapter 1-occurrence and formation of crude oil and natural gas," in *Subsea and Deepwater Oil and Gas Science and Technology*, ed. J. G. Speight (Boston, MA: Gulf Professional Publishing), 1–43. doi: 10.1016/B978-1-85617-558-6.00001-5

Sun, M., Xiao, T., Ning, Z., Xiao, E., and Sun, W. (2015). Microbial community analysis in rice paddy soils irrigated by acid mine drainage contaminated water. *Appl. Microbiol. Biotechnol.* 99, 2911–2922. doi: 10.1007/s00253-014-6194-5

Taleghani, N. D., and Tyagi, M. (2017). Impacts of major offshore oil spill incidents on petroleum industry and regional economy. *J. Energy Resources Technol.* 139:022913-1. doi: 10.1115/1.4035426

Tan, B., Dong, X., Sensen, C. W., and Foght, J. (2013). Metagenomic analysis of an anaerobic alkane-degrading microbial culture: potential hydrocarbon-activating pathways and inferred roles of community members. *Genome* 56, 599–611. doi: 10.1139/gen-2013-0069

Tarhriz, V., Nouioui, I., Spröer, C., Verbarg, S., Ebrahimi, V., Cortés-Albayay, C., et al. (2019). *Pseudomonas khazarica* sp. nov., a polycyclic aromatic hydrocarbon-degrading bacterium isolated from Khazar Sea sediments. Antonie Van Leeuwenhoek 113, 521–532.

Techtmann, S. M., and Hazen, T. C. (2016). Metagenomic applications in environmental monitoring and bioremediation. *J. Ind. Microbiol. Biotechnol.* 43, 1345–1354.

Teramoto, M., Queck, S. Y., and Ohnishi, K. (2013). Specialized hydrocarbonoclastic bacteria prevailing in seawater around a port in the Strait of Malacca. *PLoS One* 8:e66594. doi: 10.1371/journal.pone.0066594

Thomas, T., Gilbert, J., and Meyer, F. (2012). Metagenomics-a guide from sampling to data analysis. *Microb. Informatics Exp.* 2:3.

Tiralerdpanich, P., Sonthiphand, P., Luepromchai, E., Pinyakong, O., and Pokethitiyook, P. (2018). Potential microbial consortium involved in the biodegradation of diesel, hexadecane and phenanthrene in mangrove sediment explored by metagenomics analysis. *Mar. Pollut. Bull.* 133, 595–605.

U.S. Department of Health and Human Services (1999). *Agency for Toxic Substances and Disease Registry-ATSDR*. Washington, DC: U.S. Department of Health and Human Services.

Uad, I., Silva-Castro, G. A., Pozo, C., González-López, J., and Calvo, C. (2010). Biodegradative potential and characterization of bioemulsifiers of marine bacteria isolated from samples of seawater, sediment and fuel extracted at 4000 m of depth (Prestige wreck). *Int. Biodeterior. Biodegradation* 64, 511–518. doi: 10.1016/j.ibiod.2010.06.005

Uhlik, O., Leewis, M.-C., Strejcek, M., Musilova, L., Mackova, M., Leigh, M. B., et al. (2013). Stable isotope probing in the metagenomics era: a bridge towards improved bioremediation. *Biotechnol. Adv.* 31, 154–165. doi: 10.1016/j.biotechadv.2012.09.003

Urgun-Demirtas, M., Stark, B., and Pagilla, K. (2006). Use of genetically engineered microorganisms (GEMs) for the bioremediation of contaminants. *Crit. Rev. Biotechnol.* 26, 145–164. doi: 10.1080/07388550600842794

Van De Kamp, J., Hook, S. E., Williams, A., Tanner, J. E., and Bodrossy, L. (2019). Baseline characterization of aerobic hydrocarbon degrading microbial communities in deep-sea sediments of the Great Australian Bight, Australia. *Environ. Microbiol.* 21, 1782–1797. doi: 10.1111/1462-2920.14559

Van Hamme, J. D., Singh, A., and Ward, O. P. (2003). Recent advances in petroleum microbiology. *Microbiol. Mol. Biol. Rev.* 67, 503–549. doi: 10.1128/MMBR.67.4.503-549.2003

Varjani, S. (2014). *Hydrocarbon Degrading and Biosurfactants (bio-emulsifiers) Producing Bacteria from Petroleum Oil Wells*. Ph.D. thesis, Kadi Sarva Vishwavidyalaya, Gandhinagar.

Varjani, S., and Upasani, V. (2012). Characterization of hydrocarbon utilizing *Pseudomonas* strains from crude oil contaminated samples. *Int. J. Sci. Comput.* 6, 120–127.

Varjani, S. J. (2017). Microbial degradation of petroleum hydrocarbons. *Bioresour. Technol.* 223, 277–286. doi: 10.1016/j.biortech.2016.10.037

Varjani, S. J., Rana, D. P., Jain, A. K., Bateja, S., and Upasani, V. N. (2015). Synergistic ex-situ biodegradation of crude oil by halotolerant bacterial consortium of indigenous strains isolated from on shore sites of Gujarat, India. *Int. Biodeterior. Biodegradation* 103, 116–124. doi: 10.1016/j.ibiod.2015.03.030

Varjani, S. J., and Upasani, V. N. (2013). Comparative studies on bacterial consortia for hydrocarbon degradation. *Screening* 2, 5377–5383.

Varjani, S. J., and Upasani, V. N. (2016). Biodegradation of petroleum hydrocarbons by oleophilic strain of *Pseudomonas aeruginosa* NCIM 5514. *Bioresour. Technol.* 222, 195–201. doi: 10.1016/j.biortech.2016.10.006

Varjani, S. J., and Upasani, V. N. (2017). A new look on factors affecting microbial degradation of petroleum hydrocarbon pollutants. *Int. Biodeterior. Biodegradation* 120, 71–83. doi: 10.1016/j.ibiod.2017.02.006

Vasconcellos, S. P. D., Angolini, C. F. F., García, I. N. S., Martins Dellagnezze, B., Silva, C. C. D., Marsaioli, A. J., et al. (2010). Screening for hydrocarbon biodegraders in a metagenomic clone library derived from Brazilian petroleum reservoirs. *Org. Geochem.* 41, 675–681. doi: 10.1016/j.orggeochem.2010.03.014

Vedler, E., Heinaru, E., Jutkina, J., Viggor, S., Koressaar, T., Remm, M., et al. (2013). *Limnobacter* spp. as newly detected phenol-degraders among Baltic Sea surface water bacteria characterised by comparative analysis of catabolic genes. *Syst. Appl. Microbiol.* 36, 525–532. doi: 10.1016/j.syapm.2013.07.004

Von Netzer, F., Kuntze, K., Vogt, C., Richnow, H. H., Boll, M., and Lueders, T. (2016). Functional Gene Markers for Fumarate-Adding and Dearomatizing Key Enzymes in Anaerobic Aromatic Hydrocarbon Degradation in Terrestrial Environments. *J. Mol. Microbiol. Biotechnol.* 26, 180–194. doi: 10.1159/000441946

Voordouw, G., Armstrong, S. M., Reimer, M. F., Fouts, B., Telang, A. J., Shen, Y., et al. (1996). Characterization of 16S rRNA genes from oil field microbial communities indicates the presence of a variety of sulfate-reducing, fermentative, and sulfide-oxidizing bacteria. *Appl. Environ. Microbiol.* 62, 1623–1629. doi: 10.1128/AEM.62.5.1623-1629.1996

Waigi, M. G., Kang, F., Goikavi, C., Ling, W., and Gao, Y. (2015). Phenanthrene biodegradation by sphingomonads and its application in the contaminated soils and sediments: a review. *Int. Biodeterior. Biodegradation* 104, 333–349. doi: 10.1016/j.ibiod.2015.06.008

Wang, F., Li, C., Wang, H., Chen, W., and Huang, Q. (2016). Characterization of a phenanthrene-degrading microbial consortium enriched from petrochemical contaminated environment. *Int. Biodeterior. Biodegradation* 115, 286–292. doi: 10.1016/j.ibiod.2016.08.028

Wang, L.-Y., Gao, C.-X., Mbadinga, S. M., Zhou, L., Liu, J.-F., Gu, J.-D., et al. (2011). Characterization of an alkane-degrading methanogenic enrichment culture from production water of an oil reservoir after 274 days of incubation. *Int. Biodeterior. Biodegradation* 65, 444–450. doi: 10.1016/j.ibiod.2010.12.010

Wang, L.-Y., Ke, W.-J., Sun, X.-B., Liu, J.-F., Gu, J.-D., and Mu, B.-Z. (2014). Comparison of bacterial community in aqueous and oil phases of water-flooded petroleum reservoirs using pyrosequencing and clone library approaches. *Appl. Microbiol. Biotechnol.* 98, 4209–4221. doi: 10.1007/s00253-013-5472-y

Wang, W., Zhong, R., Shan, D., and Shao, Z. (2014). Indigenous oil-degrading bacteria in crude oil-contaminated seawater of the Yellow sea, China. *Appl. Microbiol. Biotechnol.* 98, 7253–7269. doi: 10.1007/s00253-014-5817-1

Ward, D. M., Atlas, R. M., Boehm, P. D., and Calder, J. A. (1980). Microbial biodegradation and chemical evolution of oil from the Amoco spill. *Ambio* 277–283.

Watanabe, K., Kodama, Y., and Kaku, N. (2002). Diversity and abundance of bacteria in an underground oil-storage cavity. *BMC Microbiol.* 2:23. doi: 10.1186/1471-2180-2-23

Wentzel, A., Lewin, A., Cervantes, F. J., Valla, S., and Kotlar, H. K. (2013). *Deep Subsurface Oil Reservoirs as Poly-Extreme Habitats for Microbial Life. A Current Review. Polyextremophiles*, (Dordrecht: Springer), 439–466. doi: 10.1007/978-94-007-6488-0_19

Wiedemeier, T. H., Rifai, H. S., Newell, C. J., and Wilson, J. T. (1999). *Natural Attenuation of Fuels and Chlorinated Solvents in the Subsurface*. New York, NY: John Wiley & Sons. doi: 10.1002/9780470172964

Wilhelm, R. C., Hanson, B. T., Chandra, S., and Madsen, E. (2018). Community dynamics and functional characteristics of naphthalene-degrading populations in contaminated surface sediments and hypoxic/anoxic groundwater. *Environ. Microbiol.* 20, 3543–3559. doi: 10.1111/1462-2920.14309

Wilkes, H., Buckel, W., Golding, B. T., and Rabus, R. (2016). Metabolism of hydrocarbons in n-alkane-utilizing anaerobic bacteria. *J. Mol. Microbiol. Biotechnol.* 26, 138–151. doi: 10.1159/000442160

Wolf, D., Cryder, Z., and Gan, J. (2019). Soil bacterial community dynamics following surfactant addition and bioaugmentation in pyrene-contaminated soils. *Chemosphere* 231, 93–102. doi: 10.1016/j.chemosphere.2019.05.145

Wu, G., He, L., and Chen, D. (2013). Sorption and distribution of asphaltene, resin, aromatic and saturate fractions of heavy crude oil on quartz surface: molecular dynamic simulation. *Chemosphere* 92, 1465–1471. doi: 10.1016/j.chemosphere.2013.03.057

Xia, W., Shen, W., Yu, L., Zheng, C., Yu, W., and Tang, Y. (2016). Conversion of petroleum to methane by the indigenous methanogenic consortia for oil recovery in heavy oil reservoir. *Appl. Energy* 171, 646–655. doi: 10.1016/j.apenergy.2016.03.059

Xu, W., Zhang, Y., Cao, H., Sheng, Y., Li, H., Li, Y., et al. (2018). Metagenomic insights into the microbiota profiles and bioaugmentation mechanism of organics removal in coal gasification wastewater in an anaerobic/anoxic/oxic system by methanol. *Bioresour. Technol.* 264, 106–115. doi: 10.1016/j.biortech.2018.05.064

Xu, Z., Hansen, M. A., Hansen, L. H., Jacquiod, S., and Sørensen, S. J. (2014). Bioinformatic approaches reveal metagenomic characterization of soil microbial community. *PLoS One* 9:e93445. doi: 10.1371/journal.pone.0093445

Xue, J., Yu, Y., Bai, Y., Wang, L., and Wu, Y. (2015). Marine oil-degrading microorganisms and biodegradation process of petroleum hydrocarbon in marine environments: a review. *Curr. Microbiol.* 71, 220–228. doi: 10.1007/s00284-015-0825-7

Yagi, J. M., Neuhauser, E. F., Ripp, J. A., Mauro, D. M., and Madsen, E. L. (2010). Subsurface ecosystem resilience: long-term attenuation of subsurface contaminants supports a dynamic microbial community. *ISME J.* 4, 131–143. doi: 10.1038/ismej.2009.101

Yakimov, M. M., Timmis, K. N., and Golyshin, P. N. (2007). Obligate oil-degrading marine bacteria. *Curr. Opin. Biotechnol.* 18, 257–266. doi: 10.1016/j.copbio.2007.04.006

Yang, S., Yu, M., and Chen, J. (2017). Draft genome analysis of Dietzia sp. 111N12-1, isolated from the South China Sea with bioremediation activity. *Braz. J. Microbiol.* 48, 393–394. doi: 10.1016/j.bjm.2016.10.029

Yergeau, E., Bokhorst, S., Kang, S., Zhou, J., Greer, C. W., Aerts, R., et al. (2012a). Shifts in soil microorganisms in response to warming are consistent across a range of Antarctic environments. *ISME J.* 6, 692–702. doi: 10.1038/ismej.2011.124

Yergeau, E., Sanschagrin, S., Beaumier, D., and Greer, C. W. (2012b). Metagenomic analysis of the bioremediation of diesel-contaminated Canadian high arctic soils. *PLoS One* 7:e30058. doi: 10.1371/journal.pone.0030058

Youssef, N., Elshahed, M. S., and McInerney, M. J. (2009). "Microbial processes in oil fields: culprits, problems, and opportunities. *Adv. Appl. Microbiol.* 66, 141–251. doi: 10.1016/S0065-2164(08)00806-X

Yu, S. H., Ke, L., Wong, Y. S., and Tam, N. F. S. (2005). Degradation of polycyclic aromatic hydrocarbons (PAHS) by a bacterial consortium enriched from mangrove sediments. *Environ. Int.* 31, 149–154. doi: 10.1016/j.envint.2004.09.008

Zafra, G., Taylor, T. D., Absalón, A. E., and Cortés-Espinosa, D. V. (2016). Comparative metagenomic analysis of PAH degradation in soil by a mixed microbial consortium. *J. Hazard. Mater.* 318, 702–710. doi: 10.1016/j.jhazmat.2016.07.060

Zander, M. (1983). *Physical and Chemical Properties of Polycyclic Aromatic Hydrocarbons*. New York, NY: Marcel Dekker Inc.

Zengler, K., Richnow, H. H., Rosselló-Mora, R., Michaelis, W., and Widdel, F. (1999). Methane formation from long-chain alkanes by anaerobic microorganisms. *Nature* 401, 266–269. doi: 10.1038/45777

Zhang, T., Bain, T. S., Nevin, K. P., Barlett, M. A., and Lovley, D. R. (2012). Anaerobic benzene oxidation by Geobacter species. *Appl. Environ. Microbiol.* 78, 8304–8310. doi: 10.1128/AEM.02469-12

Zhang, X., Sullivan, E. R., and Young, L. Y. (2000). Evidence for aromatic ring reduction in the biodegradation pathwayof carboxylated naphthalene by a sulfate reducing consortium. *Biodegradation* 11, 117–124. doi: 10.1023/A:1011128109670

Zhang, X., and Young, L. Y. (1997). Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. *Appl. Environ. Microbiol.* 63, 4759–4764. doi: 10.1128/AEM.63.12.4759-4764.1997

Zhang, Z., Hou, Z., Yang, C., Ma, C., Tao, F., and Xu, P. (2011). Degradation of n-alkanes and polycyclic aromatic hydrocarbons in petroleum by a newly isolated *Pseudomonas aeruginosa* DQ8. *Bioresour. Technol.* 102, 4111–4116. doi: 10.1016/j.biortech.2010.12.064

Zhao, D., Liu, C., Liu, L., Zhang, Y., Liu, Q., and Wu, W.-M. (2011). Selection of functional consortium for crude oil-contaminated soil remediation. *Int. Biodeterior. Biodegradation* 65, 1244–1248. doi: 10.1016/j.ibiod.2011.07.008

Zhou, H. W., Guo, C. L., Wong, Y. S., and Tam, N. F. Y. (2006). Genetic diversity of dioxygenase genes in polycyclic aromatic hydrocarbon-degrading bacteria isolated from mangrove sediments. *FEMS Microbiol. Lett.* 262, 148–157. doi: 10.1111/j.1574-6968.2006.00379.x

Zhou, L., Li, K.-P., Mbadinga, S. M., Yang, S.-Z., Gu, J.-D., and Mu, B.-Z. (2012). Analyses of n-alkanes degrading community dynamics of a high-temperature methanogenic consortium enriched from production water of a petroleum reservoir by a combination of molecular techniques. *Ecotoxicology* 21, 1680–1691. doi: 10.1007/s10646-012-0949-5

Zhuang, W.-Q., Tay, J.-H., Maszenan, A., and Tay, S. (2002). *Bacillus naphthovorans* sp. nov. from oil-contaminated tropical marine sediments and its role in naphthalene biodegradation. *Appl. Microbiol. Biotechnol.* 58, 547–554. doi: 10.1007/s00253-001-0909-0

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Manuscript 2 – Review 2: Recent advances in bioremediation of biofuel blends

K.J. Hidalgo[a,b1], A.J. Giachini[c] , M.R. Schneider[c], A.U. Soriano[d], M.P. Baessa[d], L.F. Martins[d], and V.M. Oliveira[a]

[a] Microbial Resources Division, Research Center for Chemistry, Biology and Agriculture (CPQBA), University of Campinas – UNICAMP, Av. Alexandre Cazellato, 999, ZIP 13148-218, Paulínia, SP, Brazil,

[b] Graduate Program in Genetics and Molecular Biology, Institute of Biology, University of Campinas (UNICAMP), Rua Monteiro Lobato 255, Cidade Universitária ZIP 13083-862, Campinas, SP, Brazil.

[c] Núcleo Ressacada de Pesquisas Em Meio Ambiente (REMA) - Department of Microbiology, Federal University of Santa Catarina (UFSC), Campus Universitário Sul da Ilha - Rua José Olímpio da Silva, 1326 - Bairro Tapera, 88049-500 Florianópolis, SC, Brazil

[d] PETROBRAS/ R&D Center (CENPES), Av. Horácio Macedo, 950. ZIP 21941-915, Cidade Universitária, Ilha do Fundão, Rio de Janeiro, Brazil.

[1] Corresponding author: K.J. Hidalgo
E-mail: khidalgo@javeriana.edu.co
Telephone: +55 19 98172 1510

Abstract

Modern society is highly dependent on petroleum as the main source for the global energy demands. The non-renewable nature of oil and the huge environmental impact caused by spills of oil and/or its derivatives drove the society to searching for alternative fuels aiming to reduce pollution and promoting sustainability. Biofuels such as biodiesel and ethanol are some of these alternatives. In some countries, as Brazil, ethanol and biodiesel have been increasingly integrated into the energy matrix, as blends with gasoline and diesel, respectively. With the growing use of such blends, cases of pollution in soil, surface and groundwater are expected. Due to the different biodegradation dynamics when ethanol and/or biodiesel are present, it is necessary to understand the behavior and fate of the petroleum hydrocarbons under different bioremediation approaches through geochemical and microbial analyses. The biological, physical, and chemical characteristics of each biofuel blend may alter the structure, composition, and metabolic potential of the microbiome present in affected areas. This review presents the current understanding about how the presence of biofuels and the applied bioremediation approach can affect the biodegradation of petroleum hydrocarbons. Furthermore, the insights discussed in this review brings to light the knowledge gap on how the microbiome is impacted by the biofuel blend composition and how this information might pave the way to improving the bioremediation strategy efficiency.

Keywords: Biodiesel, gasohol, ethanol, microbiome, microbial degradation, biofuels blends.

INTRODUCTION

Spills of petroleum or its derivatives usually occur as a result of leakage from underground storage tanks, rupture of pipelines and transport accidents (Islam et al., 2013), delivering compounds that can cause major impacts to the environment and human health for their toxicity, mutagenicity and carcinogenicity (Tahhan and Abu-Ateih, 2009). Moreover, climate and security energy issues have encouraged the replacement of fossil fuels by cost-benefit renewable and clean energy sources such as biofuels. In Brazil, government initiatives since the 1970s have led to a considerable presence of biofuels in the energy matrix. Currently, biodiesel is blended with diesel (12% biodiesel – B12), and ethanol is blended with gasoline (27% ethanol – E27) or sold as hydrated ethanol fuel (E100) (Canabarro et al., 2023). Nonetheless, increasing the share of biofuels is part of Brazil's strategy to achieve its greenhouse gas emissions reduction targets over the following years (Grangeia et al., 2022).

The increasing use of biodiesel/diesel and gasohol blends (OECD/FAO, 2023) implies that eventually most of the environmental pollution caused by accidental fuel spills or leaks will have at least partially a biofuel component. Given the different dynamics in the biodegradation of petroleum hydrocarbons when ethanol or biodiesel are present (Chen et al., 2008a; Corseuil et al., 2011; Costa et al., 2009; da Silva and Corseuil, 2012; Rama et al., 2019; Ramos et al., 2014; Steiner et al., 2018), there is an urgent need to develop specific alternatives for the remediation of environments impacted by these mixtures.

Currently, several technologies can be applied for the remediation of environments impacted with hydrocarbons. Bioremediation based-approaches have been well studied and shown to be the most cost-effective to treat hydrocarbon-polluted sites (Baniasadi and Mousavi, 2018; Ng et al., 2015; Zhao et al., 2011). In the case of areas impacted by biofuel/petrofuel blends, there has been growing interest in studies of the biodegradation processes of such mixtures (Alvarez and Hunt, 2002; Chen et al., 2008a; Corseuil et al., 1998; Cyplik et al., 2011; Da Silva and Alvarez, 2004; Da Silva et al., 2005; Heermann and Powers, 1998; Ng et al., 2015; Rama et al., 2019; Ramos, 2012; Satapanajaru et al., 2017).

Considerable work has already been undertaken to assess the effect of biofuels on petroleum hydrocarbon degradation dynamics, rate, and efficiency (Alvarez and Hunt, 2002; Cyplik et al., 2011; Rama et al., 2019; Ramos, 2012). The behavior of some relevant microbial degraders in areas affected with biofuel/petrofuels blends has been studied in a few cases (Colla et al., 2014; Luisa et al., 2015; Müller et al., 2017; Ramos, 2013); however, in these studies, there is no information about the metabolic potential of these microbial communities. In conclusion, there is still a lack of knowledge on the functional profiles of the microbiome and how that can impact the bioremediation efficiency. Thus, the main aim of this review is to present the updates and current knowledge on the bioremediation of oil hydrocarbons in the presence of biofuels.

1. Biofuels

In the recent years, several problems of modern society, such as the increase in world energy demand, the depletion of petroleum reserves, and the notorious climate changes due to the production of greenhouse gases, have increased the interest in the use of renewable energy sources (Belincanta et al., 2016). One of the solutions for such challenges is the progressive replacement of petroleum-based fuels with feedstock-based fuels (biofuels). Currently, the main biofuels available are biodiesel (produced from vegetable oils), ethanol (produced from sugarcane and corn), and biogas (produced from biomass).

1.1. Ethanol blends

Ethanol, as pure hydrous ethanol (E100) or in blends of anhydrous ethanol plus gasoline (gasohol), is an advanced biofuel that can expressively reduce greenhouse gas emissions compared with pure gasoline (EPA, 2009). For each megajoule of generated energy, the combustion of E100 emits approximately 75% less CO_2 equivalent than the combustion of pure gasoline. Therefore, the higher the proportion of ethanol in gasohol, the lower the CO_2 emission (Grassi and Pereira, 2019). In Brazil, commercialized gasoline has 27% ethanol (E27), while in the United States has 10% (E10) (Steiner et al., 2018).

1.1.1. Gasoline

Gasoline is a by-product of the oil refining, composed by a complex mixture of hydrocarbons (usually C4 – C12) (Speight, 2020), additives and blending agents. The composition of the gasoline is highly variable, depending on the crude petroleum origin and type used, the refining process and the use of additives. Typically, gasoline can contain among 150 to 1,000 compounds (Stauffer et al., 2008). The hydrocarbon composition in gasoline is reported in Table 1 (Stauffer et al., 2008).

Table 1. Typical composition of gasoline hydrocarbons.

Compounds	Proportion (% volume)
Alkanes	4 – 8
Alkenes	2 – 5
Isoalkanes	25 – 40
Cycloalkanes	3 – 7
Total aromatics	20 – 50 (0.5 – 2.5% Benzene)

Gasoline leaks can become a health and environmental issue mainly due to the high proportion of highly toxic BTEX (Benzene, Toluene, Ethylbenzene, and Xylenes) (Bolden et al., 2015). Despite the high volatility of such molecules, the monoaromatic structure (Figure 1) confers relatively high-water solubility (compared to other hydrocarbons) (ITRC), thus facilitating their migration through soil and groundwater. Due to the physicochemical characteristics of BTEX compounds, the deeper the leak, the lower the volatilization, and the more challenging to decontaminate (Costa, 2008). Benzene is recognized as a human carcinogen (Weelink et al., 2010).

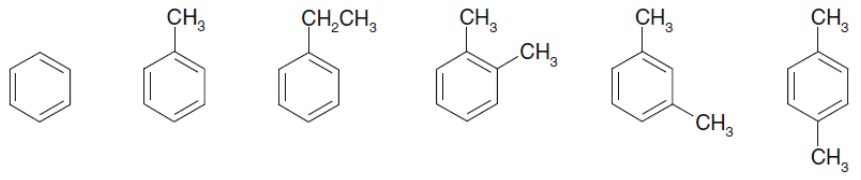


Figure 1. From left to right, molecular structure of the monoaromatics benzene, toluene, ethylbenzene, ortho-xylene, meta-xylene, and para-xylene.

1.1.2. Ethanol

Ethanol is a renewable fuel made from starch- or sugar-based feedstocks, such as corn grain and sugar cane, or from cellulosic biomass, such as wood chips or crop

residues (EERE). Ethanol is characterized as a short chain organic compound with high solubility. The process of first-generation ethanol production from sugar cane begins with milling the plant for juice extraction (which results in bagasse as a residue destined for electricity generation or second-generation ethanol production). The extracted juice is filtered (retained solids are used as fertilizer) and mixed with molasse (a liquid by-product of sugar crystallization). This blend is fermented by yeasts, which use the saccharose and other carbohydrates as carbon source to produce ethanol. In the last step, the fermented broth is distilled to recover the ethanol (vinasse is produced and used as fertilizer) (Sydney et al., 2021).

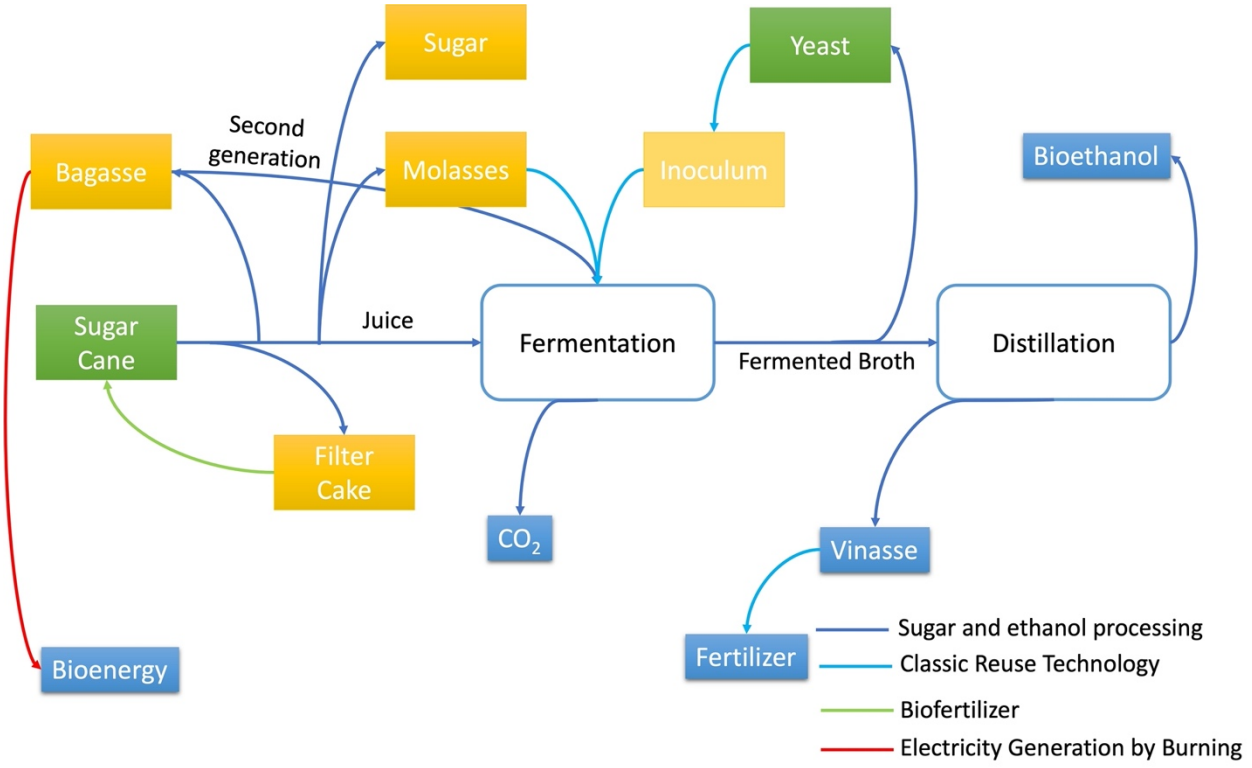


Figure 2. Sugarcane processing to generate ethanol and other energy sources.

1.2. Biodiesel blends

Biodiesel is a renewable fuel with combustion properties similar to those of petroleum-derived diesel (DeMello et al., 2007; Hollebone et al., 2008; Huang et al., 2012; Müller, 2011), which allows biodiesel in diesel engines without any modification (Knothe et al., 2006). Because of that, biodiesel was viewed as an alternative to target security

energy and greenhouse gas emissions, boosting biodiesel industry in several countries (Debnath and Whistance, 2023). Biodiesel has been used as a blend with diesel oil, and the blending mandates have changed over time and among countries influenced by domestic production incentives, trading policies, resources availability and environmental regulations (Canabarro et al., 2023; Debnath and Whistance, 2023). For example, Brazil started with B2 (2% biodiesel, 98% diesel) in 2008 and currently commercializes B12 (Canabarro et al., 2023), while in the US, the biodiesel blends have varied from B2 to B20 (Administration, 2022).

1.2.1. Diesel

Diesel is the second most used fuel, especially for transportation and electric power generation. The compression ignition engines, known as diesel engines, were named after Dr. Rudolf Diesel, who invented such engines (Huang et al., 2012). Diesel is a member of the class of crude oil products known as middle distillates, in other words, diesel is higher boiling than gasoline but lower boiling than gas oil. Diesel is a hydrocarbon mixture (C10 to C19) composed by saturated hydrocarbons (approximately 75% v/v, primarily paraffins), monoaromatics (including alkyl benzenes) and polyaromatic hydrocarbons (PAHs) (mainly naphthalenes) (Speight, 2020).

PAHs are molecules composed by more than one benzene ring (Figure 3). They are the most abundant organic pollutants in the environment. Because of PAH hydrophobicity, recalcitrancy, toxicity, mutagenicity, and carcinogenicity (Bolden et al., 2015), sixteen of them are considered of high priority contaminants (Figure 3) (Head et al., 2006; Lee et al., 2018). PAHs account for 25 to 35% of crude oil constituents. Due to their characteristics, PAHs can persist in the environment for several years. The solubility of such compounds decreases as the number of carbons increases, making them more difficult to be degraded by microorganisms (Head et al., 2006; Lee et al., 2018).

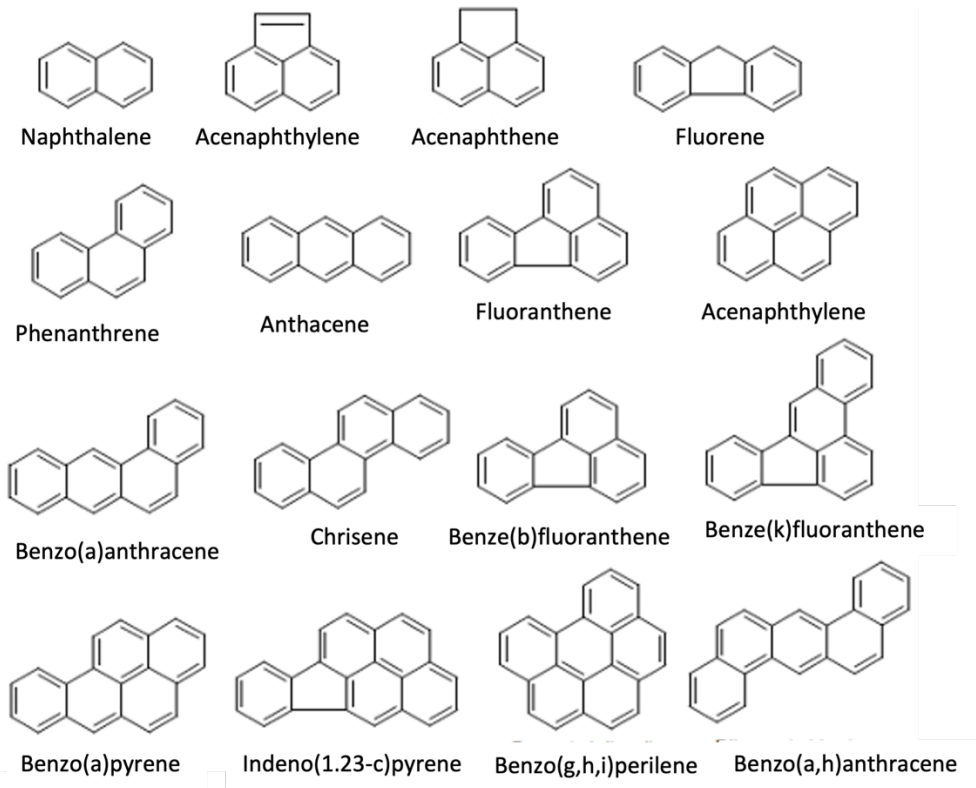


Figure 3. Molecular structures of the sixteen priority PAHs according to the United States EPA (Hussar et al., 2012)

1.2.2. Biodiesel

Biodiesel is produced from vegetable or animal oils, mainly via transesterification of triglycerides with short-chain alcohols in the presence of acid or alkali catalysts, yielding monoesters and glycerin (Figure 4) (Knothe and Razon, 2017; Mittelbach and Remschmidt, 2004; Moser, 2011). Methanol, ethanol, propanol, and butanol are usually short-chain alcohols used for this process. However, methanol is the most commonly used because of its low price (Ramadhas et al., 2005). In Brazil, the ethanol obtained from the sugar cane can be used as the alcohol in the transesterification reaction (ANP, 2020).

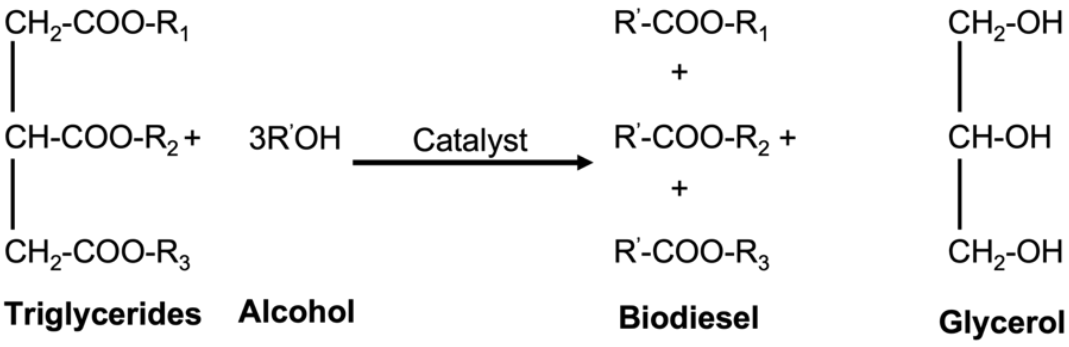


Figure 4. Triglycerides transesterification process to obtain Biodiesel

The primary oil sources used to produce biodiesel worldwide are palm, soybean and rapeseed (Akram et al., 2022; Ramos, 2013). In Brazil, soybean is the principal feedstock for biodiesel production, followed by waste animal fatty materials (Lima et al., 2020). Biodiesel is composed of several fatty acid methyl esters (FAMEs), the proportion of them depending on the feedstock source (Figure 5). In soybean biodiesel, methyl linoleate (C18:2) comprises about half of the content, and methyl oleate (C18:1) is the second most abundant compound. On the other hand, methyl oleate is thrice as high as methyl linoleate in rapeseed biodiesel (Martínez et al., 2014). Methyl palmitate (C16:0) is a low proportion of biodiesel from both soybean and rapeseed (Martínez et al., 2014) but can be more than 40% of the palm oil biodiesel content (Nagi et al., 2008). Such differences have economic and environmental implications. A higher proportion of unsaturated methyl esters improves fuel combustion but increases NOx emission (Jiaqiang et al., 2016). In addition, a higher degree of unsaturation is expected to support a higher biodegradation rate (Raczyk et al., 2017).

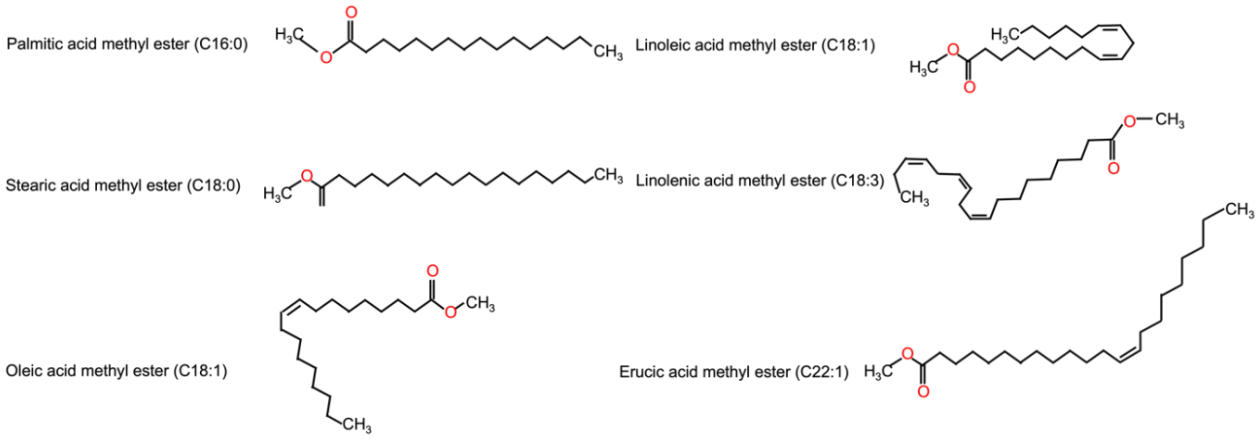


Figure 5. Molecular structures of FAMEs present in biodiesel.

1.3. Bioremediation approaches

Bioremediation is the process of detoxification of polluted environments mediated by microorganisms or their products. As a result, contaminants are transformed into less toxic or inert compounds, such as carbon dioxide and water, harmless to humans and the environment.

Depending on the level of intervention there are three main categories into the bioremediation approaches. Natural attenuation or intrinsic bioremediation is the least invasive and comprises the use of native organisms to transform toxic organic compounds into others of lower toxicity or even inert. Biostimulation also uses native organisms, but their growth and metabolic rates are increased through the addition of nutrients that are otherwise limited in the matrix, resulting in higher pollutant degradation rates. Finally, bioaugmentation uses the addition of enzymes or exogenous organisms to the system to improve the biodegradation rates. (Neuhauser et al., 2009; Techtmann and Hazen, 2016; Varjani and Upasani, 2012; Zhao et al., 2011).

Natural attenuation is mainly used to clean environments with BTEX and some chlorinated hydrocarbons. This remediation approach is notable for its low cost and little intervention of the natural ecosystem, but the effectiveness of natural attenuation depends on intrinsic matrix characteristics, such as subsurface geology, hydrology and microbiology (Mulligan and Yong, 2004).

However, the bioremediation also presents some disadvantages that are important to be noted: i) the bioremediation only can be applied to compounds that are biodegradable; ii) the processes of biodegradation are highly affected by environmental factors (i.e., pH, temperature, oxygen, bioavailability, etc); iii) microbial degradation of some compounds may lead to the production of more toxic and mobile molecules than the initial pollutant; iv) bioremediation is often a slower than other physical remediation processes, such as excavation and incineration (Alves et al,. 2019; Daccò, 2020; Jabbar et al., 2022)

Actually, microbial composition and activity in the affected sites can have a major impact on the fate of the contaminant in the environment (Lovley, 2003). The use of molecular biology and metagenomics has allowed expanding the knowledge of the biological systems in polluted environments, thus providing subsidies for the optimization of strategies to mitigate environmental pollution (Techtmann and Hazen, 2016).

1.4. Aerobic degradation of hydrocarbons

Alkanes with less than 14 carbons are rapidly volatilized (Chikere et al., 2011). Alkanes and alkenes with more carbons are easily biodegraded by oxygenases, which add oxygen to the hydrocarbon to form alcohol. This later is metabolized to water and carbon dioxide through fatty acid biosynthesis to obtain acetyl-CoA (Abbasian et al., 2015).

On the other hand, aromatic hydrocarbons are more difficult to be degraded than short chain aliphatic hydrocarbons. The recalcitrance of aromatic hydrocarbons is directly related with the increase of molecular size and ring numbers, mainly because of the decrease in solubility (Chikere et al., 2011). The first stage in aerobic biodegradation of aromatic hydrocarbons is the activation via the addition of oxygen by mono- and/or dioxygenases (Figure 6) (Baboshin and Golovleva, 2012). After this reaction, intermediate metabolites are produced, such as phenol, benzyl alcohol and catechol (Fuchs et al., 2011). These aromatic intermediates are cleaved by oxygenase enzymes, producing carboxylic acids (Vaillancourt et al., 2006). The degradation continues until acetyl-CoA and succinyl-CoA, which can enter in the Krebs Cycle (Fuchs et al., 2011).

1.5. Anaerobic degradation of aromatic hydrocarbons

Aromatic hydrocarbon degradation is very slow in the absence of oxygen (Meckenstock et al., 2016). Studies with oilfield microbial consortia showed that many hydrocarbons can be metabolized under anaerobic conditions, coupled to iron reduction, denitrification or sulfate reduction pathways, as a syntrophic process[2] between anoxygenic bacteria with methanogenic archaea (Keller et al., 2018; Van Hamme et al., 2003). These microorganisms use ferric ion, sulfate or nitrate as electron acceptor for anaerobic respiration and co-exist with other syntrophs (Keller et al., 2018; Widdel and Rabus, 2001).

In recent years, the ecology of complex communities involved in anaerobic hydrocarbon degradation has been further explored in their own habitats using molecular

[2] The syntrophic process is characterized by cooperative interaction between at least two metabolically different species of microorganisms that depend on each other to metabolize a single compound (Marietou, 2021; Vincent et al., 2021)

biology tools. Thus, it has been possible to identify several marker genes, allowing to reveal the diversity of autochthonous potentially degrading microorganisms for remediation of hydrocarbon-impacted areas. Marker genes currently used may code for: i) enzymes of peripheral pathways, ii) central enzymes, or iii) enzymes for dearomatization.

Anaerobic degradation begins with the activation of the hydrocarbon molecule (Figure 6). This can occur in several ways, which converge into central metabolites that are metabolized by conserved pathways to acetyl-CoA for assimilation (β-oxidation) producing CO_2 that can be used for methanogenic microorganisms to carry out the conversion to methane (Fuchs et al., 2011; von Netzer et al., 2016). Four enzymatic reactions have been proposed for this first step: i) addition of fumarate to monosubstituted aromatics (i.e. toluene, xylene, ethylbenzene, methyl-naphthalene)(Widdel and Rabus, 2001), catalyzed by fumarate-adding enzymes (FAEs) such as benzyl succinate synthase (BSS) and naphthyl-2-methylsuccinate synthase (NMS) (Musat et al., 2009); ii) methylation in unsubstituted aromatics as benzene, producing toluene via methyltransferases (Safinowski and Meckenstock, 2006); iii) oxygen-independent hydroxylation of benzene via dehydrogenase, producing phenol (Rabus and Widdel, 1995); and iv) direct carboxylation of compounds as benzene, phenanthrene and naphthalene by a carboxylase (Zhang and Young, 1997). The second stage begins with the dearomatization of the central intermediate (i.e. Benzoyl-CoA). This reaction can be catalyzed by two types of enzyme: i) benzoyl-CoA reductase ATP-dependent (*bcr* genes) in facultative anaerobes; and ii) benzoyl-CoA reductase ATP-independent (*bam* genes) in strictly anaerobes (von Netzer et al., 2016). Finally, the last step is the β-oxidation to produce hydrogen and acetyl-CoA.

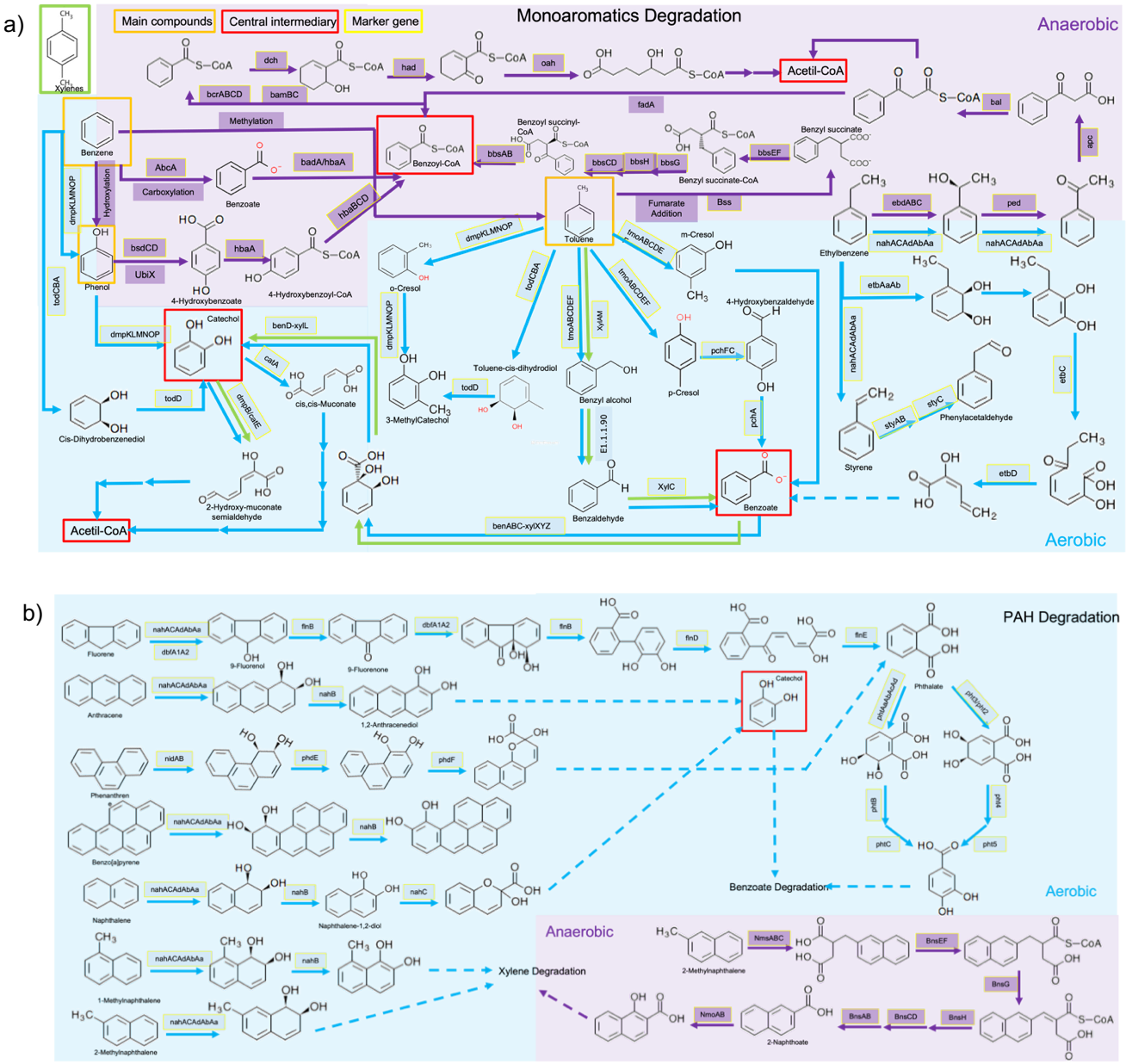


Figure 6. Aromatic hydrocarbon degradation. a) monoaromatics, b) polyaromatics (PAHs). Aerobic pathways are in blue and anaerobic in purple.

Syntrophy is very common in anaerobic hydrocarbon degradation. Once acetate and hydrogen are produced, nitrate-, iron- and/or sulfate-reducing bacteria use them as electron donors to completely mineralize hydrocarbon compounds. These microorganisms compete with methanogenic archaeas for substrate uptake. When electron acceptors (i.e. NO_3^-, Fe^{3+}, SO_4) are in low concentrations or not available, will decrease the nitrate, iron and/or sulfate reduction rates and will enhance the

methanogenesis. Thus, methanogenic microorganisms utilize hydrogen and consume acetate to produce methane (Figure 7) (Gieg et al., 2014).

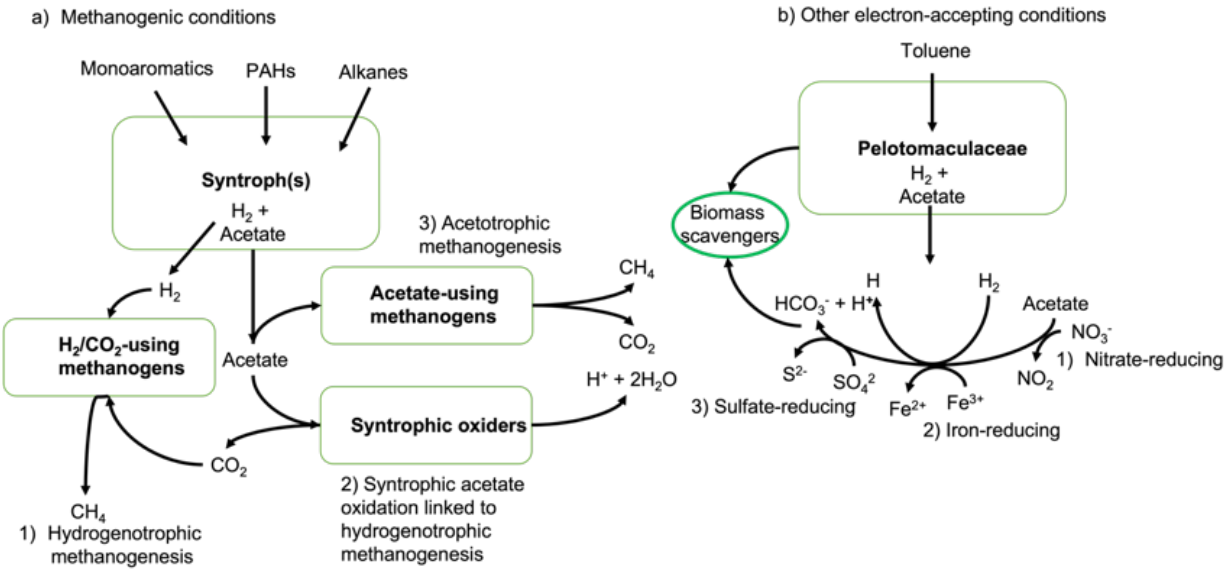


Figure 7. Conceptual models for the syntrophic biodegradation of hydrocarbons under (a) methanogenic conditions (absence of electron acceptors) and (b) in presence of electron acceptors (Nitrate, Fe(III), or sulfate).

1.6. Ethanol degradation

Ethanol or ethylic alcohol is a short-chain organic compound, highly hydrophilic. Ethanol is a common substrate for anaerobic microorganisms, even to its high redox potential making it difficult to reduce NAD+ (Bertsch et al., 2016). Several aerobic bacteria can mineralize ethanol to CO_2 and H_2O in the Kreb's cycle. Ethanol degradation begins with the oxidation to acetaldehyde by an alcohol dehydrogenase (adh) (Hektor et al., 2000; Österreicher-Cunha et al., 2009). Other oxidation reaction produces acetyl-CoA, either directly from acetaldehyde by an acetylating acetaldehyde dehydrogenase or via acetate by an acetaldehyde dehydrogenase and an acetate-CoA ligase (Alvarez and Hunt, 2002). The acetyl-CoA is converted to CO_2 inside the Kreb's cycle (Powers et al., 2001).

Oxygen is the preferred electron acceptor for ethanol degradation, favoring aerobic degradation over anaerobic metabolic pathways (Alvarez and shunt 1999). For that reason, aerobic degradation is faster than anaerobic. However, different microorganisms can metabolize ethanol, using different electron acceptors under limitation of oxygen (Bertsch et al., 2016). Ethanol is an intermediate metabolite in the organic matter anaerobic degradation. Under anaerobic conditions, ethanol can be fermented to acetate

by acetic acid bacteria or sulfate reducers (Powers et al., 2001). Acetate can be further converted to carbon dioxide and methane by methanogenic archaea (Karakashev et al., 2006). Similar to the hydrocarbon degradation, in absence of oxygen, other electron acceptors can be used. Some acetogenic bacteria can use nitrate (Karakashev et al., 2006) or CO_2 (Beaty and Ljungdahl, 1991) as electron acceptors. Viulu et al. (2013) were able to cultivate *Geobacter* species in ethanol, using Fe(II) as final electron acceptor (Viulu et al., 2013).

1.7. Biodiesel degradation

Pure biodiesel is composed of fatty acid methyl esters (FAMEs), which are similar in structure to their parent fatty acid esters (Thomas et al., 2017). The latter are natural products and ubiquitous components of cellular membranes, easily metabolized by several microorganisms in soil and water. Contrarily, FAMEs are not so common in nature, because many fatty acids exist in microorganisms as glycerol esters (Thomas et al., 2017).

Under aerobic and anaerobic conditions, FAME degradation begins with de-esterification to produce free fatty acids and methanol (Aktas et al., 2010; Sousa et al., 2007; Stolz et al., 1995). Then, by β-oxidation, two carbons are sequentially removed in the fatty acids (Figure 8) (Aktas et al., 2010; Stolz et al., 1995).

On the other hand, FAME degradation may follow the well-known metabolism to degrade glycerides (mono-, di-, or triglycerides). Degradation of glyceride esters starts with de-esterification to produce free fatty acids and glycerol by lipases, or also known as esterases (Ghaly et al., 2010). Lipases can perform both esterification and de-esterification reactions involving fatty acid methyl esters (Thomas et al., 2017). Aktas et al. (2010) studied the degradation of soybean biodiesel by different inoculum under anaerobic conditions. They observed the production of short-chain molecules as would be expected from the β-oxidation metabolism (Aktas et al., 2010). Another hypothesis about FAME degradation is focused on alkane metabolism because they have long hydrophobic alkyl like FAME. The addition of hydroxyl group to the terminal carbon from alkanes to form aldehyde are the first stage in the degradation of aliphatic hydrocarbons.

This hydroxylation can occur also at subterminal carbon. In anaerobic conditions, fumarate addition has been postulated (Rojo, 2009; Rojo, 2010). Thus, due to the similarity between the structure of FAME and alkanes, these alkane degradation pathways have been proposed as likely pathways also for biodiesel degradation (Thomas et al., 2017).

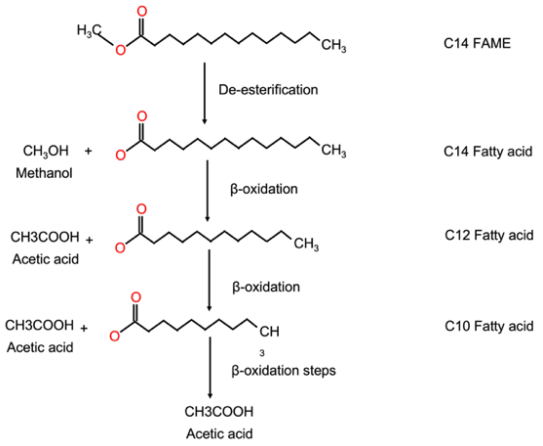


Figure 8. Metabolic pathway of FAMEs degradation.

Similar to anaerobic degradation of petroleum hydrocarbons, other final electron acceptors can be coupled to the bioconversion process under anaerobic conditions. Typically, reduction of nitrate, sulfate and ferric iron or manganese, as well as methanogenesis, can take place (Thomas et al., 2017).

1.8. Studies of biofuel/petrofuels blends bioremediation

Many laboratory and field studies on petrofuels bioremediation have been performed, where different alternatives were explored aiming at the contaminant removal and the characterization of microbial metabolic processes (Baniasadi and Mousavi, 2018; Ng et al., 2015; Zhao et al., 2011). As ethanol or biodiesel may change petroleum hydrocarbon fate, degradation rates and dynamics, the effect of biofuels in the remediation of petroleum spills have received great attention (Alvarez and Hunt, 2002; Cyplik et al., 2011; Rama et al., 2019; Ramos, 2012). However, only few works have been reported in literature on microbial community composition and metabolic potential assessment in cases of biofuel/petrofuels blends contamination (Table 2).

Table 2. Microbial community and functional potential assessment in biodegradation of biofuel/petrofuel blends in laboratory and field scale studies.

Biofuel/ petrofuel blend	Scale	Matrix	Microorganisms	Bioremediation approach	Genes	Molecular Technique	References
B0; B20; B100	Lab	soil microcosms	**Bacillus megaterium* **Bacillus pumilus* **Pseudomonas aeruginosa* **Stenotrophomonas maltophilia*	Bioaugmentation		DGGE	Meyer, Satastevan et al, 2012
B10	Lab	soil microcosms	**Achromobacter xylosoxidans* **Ochrobacterium intermedium* **Pseudomonas aeruginosa*	Bioaugmentation / Biostimulation			Colla, Andreazeza, 2014
B10	Lab	soil microcosms	Autochthonous consortium: **Klebsiella pneumoniae* **Ochrobacterium anthropic* Allochthonous consortium: **A. xylosoxidans* **O. intermedium*	Bioaugmentation / Biostimulation		16S rRNA gene	Luisa, Leticia et al 2015
B20	Field	Groundwater	**Geobacteriaceae* (iron reducer) *Sulfate reducing bacteria *Total Archaea	Natural Attenuation / Biostimulation	*bss*A	qPCR	Ramos, 2013
B20	Field	Groundwater	**Geobacteriaceae* (iron reducer) **Sulfate reducing bacteria*	Natural Attenuation / Biostimulation	*bss*A	qPCR	Muller, Ramos et al 2017
Gasohol	Field	Groundwater	**Geobacteriaceae* (iron reducer) *Sulfate reducing bacteria *Nitrate reducing bacteria *Methanogenic archaea	Biostimulation	*tod nah rmo phe bph*	qPCR	da Silva and Corseuil, 2012
Gasohol	Field	Groundwater	*Sulfate reducing bacteria *Methanogenic archaea	Natural Attenuation		qPCR	Feris, Mackay, 2008

1.8.1. Biodiesel/diesel blends bioremediation

Biodiesel can be more easily transformed than diesel because it is a natural product composed by fatty acids, which are very biologically active, while diesel contains recalcitrant petroleum hydrocarbons, that demand adapted microorganisms able to produce specialized enzymes (Zhang et al., 1998).

The impact of biodiesel on the bioremediation of conventional diesel has been largely investigated. Natural attenuation and bioaugmentation/biostimulation approaches were evaluated as strategies to remove diesel (B0), biodiesel (B100) and 20% biodiesel-diesel blend (B20) from soil in a laboratory-scale experiment (Meyer et al., 2014). A consortium containing four bacteria (*Bacillus megaterium, Bacillus pumilus, Pseudomonas aeruginosa and Stenotrophomonas maltophilia*) was used as bioaugmentation strategy and biostimulation was performed adjusting the C:N:P ratio (Meyer et al., 2012). As indicator of degradation, CO_2 production, dehydrogenase activity and quantification of total petroleum hydrocarbons (TPH) were evaluated and DGGE was used to monitor the microbial community. Bioaugmentation/biostimulation assays showed higher production of CO_2 when compared with natural attenuation experiments, suggesting that the bacterial consortium and the nutrients favored biodegradation. B100 microcosm showed higher respiratory rates than B20 microcosm, suggesting that the presence of biodiesel increases the biodegradation of conventional diesel, and the greater the quantity of the biofuel the more biodegradable is the diesel. This finding was also observed in other similar studies (Junior et al., 2009; Mariano et al., 2008; Pasqualino et al., 2006; Zhang et al., 1998). Pasqualino et al. (2006) evaluated the biodegradability by natural attenuation of biodiesel (B100) and mixtures with diesel (B0, B5, B12.5, B20, B25, B37.5, B50, B62.5, B75 and B87.5) and gasoline (B0, B17, B33, B50, B67, B83) using as inoculum samples of activated sludge from a water treatment plant. The authors observed that adding biodiesel to the fossil fuels increased the biodegradability in all assays and hypothesized that biodiesel could promote and increase the speed of the biodegradation of diesel by co-metabolisms. This process is characterized by microorganisms that use a second substrate (easily degradable) as carbon and energy source to degrade the first

substrate, that under other conditions would not be degraded by the microorganism as a sole carbon source (Pasqualino et al., 2006).

An interesting work carried out by Elazhari-Ali and co-workers in 2013 aimed to test the hypothesis that the biodegradation of volatile petroleum hydrocarbons (VPHs) in soil is affected by the presence of ethanol (E10) and biodiesel B20 (Elazhari-Ali et al., 2013). For this, soil microcosms amended with gasoline and kerosene (referred in the study as pure petroleum hydrocarbons - PP) and E10 or B20 were subjected to natural attenuation and biostimulation (adding of NH4Cl and KH2PO4). Bacterial community analysis was performed by using DGGE and GC-MS was used to quantify the components in the fuels and blends. Toluene was the only hydrocarbon not detected after 24 days. The removal rates were similar in the PP and PP+B20 assays and lower in the PP+E10 assay, suggesting that the inhibitory effect of biofuels on VPH biodegradation are minor with biodiesel than with ethanol compounds. This finding is consistent with the study of Lovanh et al. (2002), which showed that the metabolic flux of ethanol hinders that of toluene and other BTEX compounds (Lovanh et al., 2002). The nutrients added allowed faster biodegradation, suggesting that VPHs biodegradation was limited by inorganic compound availability. Pearson correlation of the DGGE analysis profile showed that lack of inorganic nutrients was the predominant factor driving the differentiation of bacterial communities in all the assays (Elazhari-Ali et al., 2013).

Other studies focused on evaluating the influence of different factors (i.e. biodiesel blend, contaminated matrix, area size, etc) on the efficiency of bioremediation techniques. Colla and collaborators (2014) evaluated the efficiency of natural attenuation, biostimulation, conventional bioaugmentation and successive bioaugmentation in the degradation of TPH in soil microcosms contaminated with B10 (Colla et al., 2014). NH_4NO_3 and KH_2PO_4 were added to adjust the C:N:P ratio as a biostimulation approach. Bioaugmentation experiments were conducted inoculating a bacterial consortium, that included *Achromobacter xylosoxidans, Pseudomonas aeruginosa and Ochrobactrum intermedium*, at the initial setup and at the 11^{th} day of incubation for the successive bioaugmentation and only at the beginning for

conventional bioaugmentation. CO_2 production, TPH levels and microbial growth were measured. TPH removal was higher with biostimulation and successive bioaugmentation approaches than with natural attenuation and conventional bioaugmentation (Colla et al., 2014). Soil microcosms amended with B10 were used to compare degradation efficiency under natural attenuation, bioaugmentation with autochthonous or allochthonous bacteria and biostimulation approaches (Luisa et al., 2015). Autochthonous consortium included *Klebsiella pneumoniae, Burkholdeira and Ochrobactrum anthropic*, whereas allochthonous consortium comprised *A. xylosoxidans, P. aeruginosa* and *O. intermedium*. The biostimulation assay consisted in the addition of NH_4NO_3 and KH_2PO_4 to adjust the C:N:P ratio. Microbial growth, CO_2 and TPH quantification were used as indicators of microbial activity and degradation. To assess the microbial diversity, 16S rRNA gene was sequenced by Illumina high-throughput sequencing. Bioaugmentation with allochthonous bacteria and biostimulation presented similar results with natural attenuation experiment, suggesting that the native microbiota even though not previously exposed to contamination showed ability to degrade the hydrocarbons present. Results of the microbial diversity analysis showed different dynamics according to the bioremediation approach used, showing that the type of treatment may drive the microbial community structure (Luisa et al., 2015).

As biodiesel can be produced from different vegetable sources, Corseuil and colleagues (2011) studied natural attenuation of monoaromatic hydrocarbons in groundwater contaminated with soybean and castor oil biodiesel (Corseuil et al., 2011). Two sets of anaerobic microcosms composed by sediment and groundwater from an uncontaminated area and spiked with pure soybean or castor oil biodiesel (B100) were implemented. The first set was used to assess the natural attenuation of each type of biodiesel by quantification of the removal of fatty acid methyl esters and hydrocarbons compared with a sterile control. Soybean biodiesel was 80% removed in 41 days compared with 40% of the castor biodiesel in 90 days. Those differences were attributed to the higher viscosity and lower bioavailability of the castor biodiesel. In the second set of microcosms, benzene and toluene were spiked with and without soybean biodiesel, to evaluate the impact of the soybean biodiesel in the

monoaromatic hydrocarbon removal. Results showed that the biodiesel had an inhibitory effect in the degradation of benzene and toluene, since the time of removal was increased compared with the microcosms without biodiesel.

Although there are relatively a great number of studies on biodiesel remediation in laboratory scale with important results, it is not possible to extrapolate all findings to field scale due to the multiple factors that are involved in environmental degradation processes. However, some *in situ* studies have been developed. Ramos et al. (2013) performed one of the pioneering field studies on biodiesel remediation to investigate the potential of anaerobic biostimulation to enhance BTEX biodegradation under methanogenic conditions (Ramos, 2013). For that, two soil areas were contaminated with 100 L of biodiesel B20 (20% v/v biodiesel and 80% v/v diesel) at 1.6 m below the water table. One of them was biostimulated with ammonium acetate. BTEX concentration was quantified by gas chromatography and qPCR technique was used to quantify total bacteria, iron- (Geobacteraceae) and sulfate-reducing bacteria and total archaea. BTEX removal began 8 months after contamination in the biostimulated area, while in the natural attenuation area the concentration of BTEX still increased two years after the release. Also, it was observed that abundance of archaea, Geobacteraceae and sulfate-reducing bacteria was higher in the biostimulated area. The authors concluded that the methanogenic biostimulation could effectively improve the source zone bioremediation of groundwater polluted with biodiesel blends. In the same field study, the effect of combined biostimulation of iron and sulfate reducing bacteria to improve BTEX and PAH biodegradation in a B20-contaminated groundwater was investigated. Another area impacted with B20 under monitored natural attenuation conditions was used as control. Benzene and naphthalene hydrocarbons were quantified by gas chromatography, and dissolved oxygen, acetate, bromide, sulfate, ferrous iron, and sulfide were measured. qPCR was used to quantify total bacteria, iron and sulfate reducers and the gene *bss*A that encodes to benzylsuccinate synthase α-subunit. This gene is used as biomarker for the presence of anaerobic aromatic hydrocarbon degraders (Winderl et al., 2007). Additionally, 16S rRNA gene sequencing was performed to assess microbial community structure and composition. Results showed that the combined

biostimulation of iron and sulfate reducing bacteria accelerated BTEX and PAHs biodegradation. Shifts in microbial community were observed, with the increase of Geobacter spp. and GOUTA 19 spp., which are key microorganisms in the anaerobic biodegradation of hydrocarbons under iron and sulfate reduction, showing that the injection of electron acceptors may modulate the community structure (Müller et al., 2017).

The same authors performed another long-term (6.2 years) field study using B20 impacted area (without ammonium acetate injections) and another area contaminated with 100 L of E24 (24% v/v of ethanol and 76% v/v of gasoline). The objective was to compare BTEX and PAHs degradation under natural attenuation approach. The results showed that each biofuel had different behavior depending on their characteristics and mobility. Contrarily to the results obtained on laboratory scale (Chen et al., 2008a; Da Silva et al., 2005), ethanol was degraded faster than biodiesel blends due to higher mobility and dissolution. Biodiesel at the source zone was more persistent, and it showed preferential degradation, causing a long-term negative effect on BTEX and PAH removal (Ramos et al., 2016).

1.8.2. Gasohol blends bioremediation

Laboratory studies aiming to evaluate different bioremediation approaches and to analyze the effect of ethanol in the degradation of petroleum compounds have been performed in the last two decades (Chen et al., 2008a; Da Silva et al., 2005). Ethanol is rapidly metabolized by oxygen consumption, and the main intermediate, acetate, is normally degraded to methane. After oxygen is depleted, other electron acceptors can be used to remove ethanol by anaerobic degradation. Depending on the conditions, ethanol metabolism makes petroleum hydrocarbon degradation slower, due to electron acceptors depletion (Ma et al., 2013).

Da Silva and colleagues used flow-through synthetic aquifer columns to evaluate the effect of the addition of electron acceptors (sulfate, Fe(III) and nitrate) on the degradation of BTEX and gasohol blends (Da Silva et al., 2005). Results showed that in the presence of ethanol, BTEX biodegradation efficiencies decreased, due to a

rapid oxygen depletion in the conversion of ethanol, stimulating the methanogenic metabolism. On the other hand, when anaerobic electron acceptors were added BTEX biodegradation rates increased, because methanogenesis was suppressed and ethanol mineralization was accelerated (Da Silva et al., 2005). However, Chen et al. (2008) obtained contrasting results. The authors evaluated BTEX degradation in ethanol and electron acceptor-amended microcosms and observed that electron donors (acetate and/or propionate/butyrate) were produced from the mineralization process and these products competed for the electron acceptors added affecting BTEX degradation rates (Chen et al., 2008b). Depending on the ethanol concentration added, BTEX degradation dynamics was affected. High concentration (5000 mg/L) completely inhibited BTEX degradation even under biostimulation with electron acceptors. Whereas under low ethanol concentration (500 mg/L), after ethanol and its intermediate acetate were removed, toluene was slightly degraded under nitrate reducing conditions (Chen et al., 2008b).

In soils, there are additional factors that can influence the degradation of hydrocarbons in the presence of ethanol. Österreicher-Cunha et al. (2009) investigated the impact of ethanol on the degradation and distribution processes of BTEX in tropical soil under unsaturated conditions. Chemical analyses comprised quantification of gasoline compounds and BTEX contents by gas chromatography. Microbial degradation was evaluated measuring fluorescein diacetate (FDA) hydrolysis. Culturable heterotrophic bacteria were quantified by colony forming units (CFU) count. All parameters measured suggested higher retention and delay of BTEX degradation when ethanol was present (Österreicher-Cunha et al., 2009).

In another study, a polyurethan-immobilized methanogenic consortium was used in a pilot-scale horizontal-flow bioreactor for the anaerobic treatment of gasoline and ethanol-contaminated groundwater (Souza et al., 2009). First, the bioreactor was metabolically activated in laboratory, until a specific methane production rate. Groundwater contaminated with the gasohol blend was sampled in a gas station and pumped into a storage tank. The separated liquid phase was treated in the bioreactor. BTEX concentration was measured in influent and effluent samples. The microbial

community diversity was assessed by sampling the immobilized biofilm at the metabolic activation stage and at the end of the trial (70 days) by DGGE technique. Results showed that BTEX was significantly removed (59% to 80%). Additionally, the bioreactor could effectively maintain an anaerobic consortium, which probably mineralized hydrocarbons through syntrophic interactions (Souza et al., 2009).

Results at lab-scale showed the importance of investigating and testing as many factors as possible, since the dynamics of degradation vary greatly depending on the parameters. Field studies allow to obtain more accurate results of the influence of the conditions prevailing in the impacted area.

Several field-scale works also have focused on the effect of ethanol on the degradation of petroleum hydrocarbons, as BTEX and PAHs. In 2011, Corseuil and collaborators performed a study releasing 100 L the E24 into a sandy aquifer to evaluate natural attenuation of BTEX in the presence of ethanol. Groundwater samples were analyzed by quantification of BTEX, ethanol and acetate concentrations. They observed that BTEX degradation was affected by the presence of ethanol, mainly under methanogenic conditions. This effect is mainly attributed to the accelerated depletion of dissolved oxygen and catabolite repression. The results demonstrated that the inhibitory effect of the ethanol was relatively short. However, acetate accumulation was longer, and this fact may contribute to decrease the thermodynamic feasibility of BTEX degradation. After inhibition (2.7 years) by the presence of ethanol, BTEX natural attenuation proceeded at similar rates compared with unblended gasoline polluted areas. The authors concluded that monitored natural attenuation can be an option to remove gasohol blend spills (Corseuil et al., 2011). In the same study area (E24 impacted), BTEX and ethanol degradation rates were compared, under monitored natural attenuation (MNA) or biostimulation by nitrate injection. The required time for total ethanol removal was significantly different between the two areas. In the MNA area, ethanol disappeared after 3 years, while in the biostimulated area ethanol removal took place after 1.4 years. After these periods, BTEX began to be degraded. The addition of nitrate increased electron acceptor availability, accelerating ethanol degradation and rising BTEX biodegradation rates

(Corseuil et al., 2011). Similar results were obtained by Costa et al. (2009), where nitrate addition in ethanol and BTEX degradation in groundwater contaminated with E25 was monitored for 32 months. Results showed that 90% of the ethanol concentration reduction was coupled to nitrate reduction pathway. The nitrate supplied the high electron acceptor demand for the ethanol metabolism and avoided the formation of high redox potential areas that negatively impact the degradation of BTEX. These findings indicated that biostimulation with nitrate injections is a plausible alternative for the recovery of areas impacted with gasohol blends (Costa et al., 2009).

BTEX degradation in areas impacted with different gasohol blends (E10 and E25) and treated with different bioremediation approaches (MNA and nitrate biostimulation) was studied by Steiner and colleagues (2018). Groundwater samples taken from different depths of the plume were analyzed by chemical (pH, redox potential, dissolved oxygen, acetate, nitrate, sulfate, methane, ethanol, BTEX and ferrous iron) and microbiological techniques (total bacteria, nitrate-, sulfate- and iron reducers and archaea by qPCR and 16S rRNA next generation sequencing). The authors observed that the geochemical and microbial conditions in the area with E10 blend subjected to MNA supported anaerobic ethanol and BTEX biodegradation. While in E25 blend area, even with nitrate injection, BTEX removal rates were lower than the one in the MNA area and ethanol was degraded at a similar speed (Steiner et al., 2018). However, Rama and collaborators compared BTEX migration and biodegradation in groundwater impacted by gasohol E24 and E85 and observed higher BTEX degradation rates in E85 impacted area than in E24 area. Results obtained in these two works showed that distinct gasohol blend proportions have impact, as well as the bioremediation strategy on hydrocarbon degradation, and there are important aspects that should be considered when choosing the most cost-effective and best remediation technology for different gasohol spills (Steiner et al., 2018).

In addition to the impact of nitrate injection on biodegradation rates, it is also important to understand its effects on microbial communities involved in BTEX degradation. Groundwater microbiome analysis in gasohol-contaminated site

subjected to biostimulation with nitrate was performed by (da Silva and Corseuil, 2012). Total bacteria, nitrate-, iron-, sulfate-reducing bacteria and methanogenic archaea were quantified by qPCR technique. Additionally, in order to assess the anaerobic BTEX degradation potential, *bss*A gene was quantified and, for the aerobic degradation potential, toluene dioxygenase (*tod*), naphthalene dioxygenase (*nah*), ring hydroxylating monooxygenase (*rmo*), phenol hydroxylase (*phe*), and biphenyl dioxygenase (*bph*) genes were assessed. Nitrate reducing bacteria was not stimulated by nitrate injection. The authors hypothesized that nitrate alleviate the high consumption of oxygen during ethanol and BTEX degradation, generating microaerophilic niches supporting growth of BTEX degrading bacteria due to the high amount of BTEX aerobic degradation genes (da Silva and Corseuil, 2012). Also, the ethanol presence may modify the microbial community structure and diversity. A previous study investigated the effect of ethanol on the microbial community structure and function in BTX and BTX + ethanol plumes in an aquifer (Feris et al., 2008). Total bacteria and archaea and sulfate reducing bacteria were quantified using qPCR based on 16S rRNA and *aps* gene, respectively. Chemical analyses included BTX, MTBE, ethanol, methane, sulfate, pH, ferrous iron, and sulfide quantifications. In the BTX + ethanol plume, was observed an increase in the archaea abundance was observed, BTX plume size, as well as depletion of sulfate and methanogenic conditions compared with the BTX experiment. Altogether, results demonstrated that due to the ethanol degradation, oxygen and other electron acceptors were depleted and methanogenic conditions were favored, reducing natural attenuation rates of BTX (Feris et al., 2008).

Conclusions

The presence of biodiesel associated to diesel and ethanol to gasoline, change the degradation rates. Depending on the conditions the biofuels can increase or decrease the velocity of petroleum hydrocarbons degradation. At the same time, overall, variation in the structure, composition and metabolic potential of the microbial communities occurs according to the biofuel type and proportion present in the blend, since these compounds show different physical and chemical properties. All these

knowledges are important to the selection of bioremediation strategies, and it may help improving the degradation of these compounds. This knowledge may pave the way for the design of further public policies for the sustainable use of biofuel blends and the efficient recovery of affected areas.

Acknowledgments

The authors are grateful to Petrobras for their financial and technical support and authorization to publish this work. K.J.H. thanks the Colombian Department of Science - COLCIENCIAS for providing a doctoral scholarship (860/2020).

Disclosure statement

All authors have approved the manuscript. The authors have declared that no conflict interests exist.

Funding

This work was funded by Petróleo Brasileiro S.A. (PETROBRAS), regulated by the Brazilian National Agency of Petroleum, Natural Gas and Biofuels (ANP, resolution 05/2015).

ORCID

K.J. Hidalgo https://orcid.org/0000-0003-4607-3750

A.J. Giachini https://orcid.org/0000-0001-8347-003X

M.R.S Schneider https://orcid.org/0000-0002-8377-5236

A.U. Soriano https://orcid.org/0000-0002-7629-4573

M.P. Baessa https://orcid.org/0000-0001-7857-060X

L.F. Martins https://orcid.org/0000-0002-0277-9834

V.M. Oliveira https://orcid.org/0000-0001-8817-4758

References

Abbasian F, Lockington R, Mallavarapu M, Naidu R. A Comprehensive Review of Aliphatic Hydrocarbon Biodegradation by Bacteria. Applied Biochemistry and Biotechnology 2015; 176: 670-699.

Administration UEI. Biofuels explained. Biodiesel, renewable diesel, and other biofuels, 2022.

Akram F, ul Haq I, Raja SI, Mir AS, Qureshi SS, Aqeel A, et al. Current trends in biodiesel production technologies and future progressions: A possible displacement of the petro-diesel. Journal of Cleaner Production 2022: 133479.

Aktas DF, Lee JS, Little BJ, Ray RI, Davidova IA, Lyles CN, et al. Anaerobic metabolism of biodiesel and its impact on metal corrosion. Energy & Fuels 2010; 24: 2924-2928.

Alvarez PJ, Hunt CS. The effect of fuel alcohol on monoaromatic hydrocarbon biodegradation and natural attenuation. Revista Latinoamericana de Microbiologia 2002; 44: 83.

Alves, D.; Villar, I.; Mato, S., Thermophilic composting of hydrocarbon residue with sewage sludge and fish sludge as cosubstrates: Microbial changes and TPH reduction. Journal of environmental management 2019**;** 239, 30-37.ANP. Agência Nacional do Petróleo, Gás Natural e Biocombustíveis. Biocombustíveis, 2020.

Baboshin M, Golovleva L. Aerobic bacterial degradation of polycyclic aromatic hydrocarbons (PAHs) and its kinetic aspects. Microbiology 2012; 81: 639-650.

Baniasadi M, Mousavi SM. A Comprehensive Review on the Bioremediation of Oil Spills. In: Kumar V, Kumar M, Prasad R, editors. Microbial Action on Hydrocarbons. Springer Singapore, Singapore, 2018, pp. 223-254.

Beaty P, Ljungdahl L. Growth of Clostridium thermoaceticum on methanol, ethanol or dimethylsulfoxide, abstr. K-131. Abstr. 91st Annu. Meet. Am. Soc. Microbiol, 1991, pp. 236.

Belincanta J, Alchorne J, Teixeira da Silva M. The Brazilian experience with ethanol fuel: aspects of production, use, quality and distribution logistics. Brazilian Journal of Chemical Engineering 2016; 33: 1091-1102.

Bertsch J, Siemund AL, Kremp F, Müller V. A novel route for ethanol oxidation in the acetogenic bacterium Acetobacterium woodii: the acetaldehyde/ethanol dehydrogenase pathway. Environmental Microbiology 2016; 18: 2913-2922.

Bolden AL, Kwiatkowski CF, Colborn T. New Look at BTEX: Are Ambient Levels a Problem? Environmental Science & Technology 2015; 49: 5261-5276.

Canabarro NI, Silva-Ortiz P, Nogueira L, Cantarella H, Maciel-Filho R, Souza GM. Sustainability assessment of ethanol and biodiesel production in Argentina, Brazil, Colombia, and Guatemala. Renewable and Sustainable Energy Reviews 2023; 171: 113019.

Chen YD, Barker JF, Gui L. A strategy for aromatic hydrocarbon bioremediation under anaerobic conditions and the impacts of ethanol: A microcosm study. Journal of Contaminant Hydrology 2008a; 96: 17-31.

Chen YD, Barker JF, Gui L. A strategy for aromatic hydrocarbon bioremediation under anaerobic conditions and the impacts of ethanol: A microcosm study. Journal of Contaminant Hydrology 2008b; 96: 17-31.

Chikere C, Okpokwasili G, Chikere B. Monitoring of microbial hydrocarbon remediation in the soil. 3 Biotech 2011; 1: 117-138.

Colla TS, Andreazza R, Bücker F, de Souza MM, Tramontini L, Prado GR, et al. Bioremediation assessment of diesel–biodiesel-contaminated soil using an alternative bioaugmentation strategy. Environmental Science and Pollution Research 2014; 21: 2592-2602.

Corseuil HX, Hunt CS, Dos Santos RCF, Alvarez PJ. The influence of the gasoline oxygenate ethanol on aerobic and anaerobic BTX biodegradation. Water Research 1998; 32: 2065-2072.

Corseuil HX, Monier AL, Gomes AP, Chiaranda HS, do Rosario M, Alvarez PJ. Biodegradation of soybean and castor oil biodiesel: implications on the natural attenuation of monoaromatic hydrocarbons in groundwater. Groundwater Monitoring & Remediation 2011; 31: 111-118.

Costa AHR. Bioestimulação com injeção de nitrato em águas subterrâneas impactadas por derramamento de gasolina com etanol. 2008.

Costa AHR, Nunes CC, Corseuil HX. Biorremediação de águas subterrâneas impactadas por gasolina e etanol com o uso de nitrato. Engenharia Sanitária e Ambiental 2009; 14: 265-274.

Cyplik P, Schmidt M, Szulc A, Marecik R, Lisiecki P, Heipieper HJ, et al. Relative quantitative PCR to assess bacterial community dynamics during biodegradation of diesel and biodiesel fuels under various aeration conditions. Bioresource Technology 2011; 102: 4347-4352.

Da Silva ML, Alvarez PJ. Enhanced anaerobic biodegradation of benzene-toluene-ethylbenzene-xylene-ethanol mixtures in bioaugmented aquifer columns. Applied and Environmental Microbiology 2004; 70: 4720-4726.

Da Silva ML, Ruiz-Aguilar GM, Alvarez PJ. Enhanced anaerobic biodegradation of BTEX-ethanol mixtures in aquifer columns amended with sulfate, chelated ferric iron or nitrate. Biodegradation 2005; 16: 105-114.

da Silva MLB, Corseuil HX. Groundwater microbial analysis to assess enhanced BTEX biodegradation by nitrate injection at a gasohol-contaminated site. International Biodeterioration & Biodegradation 2012; 67: 21-27.

Daccò, C.; Girometta, C.; Asemoloye, M.; Carpani, G.; Picco, A.; Tosi, S., Key fungal degradation patterns, enzymes and their applications for the removal of aliphatic hydrocarbons in polluted soils: A review. International Biodeterioration & Biodegradation 2020; 147, 104866.

Debnath D, Whistance J. The Biofuel Industry and Global Trade Nexus. Biofuels in Circular Economy. Springer, 2023, pp. 303-319.

DeMello JA, Carmichael CA, Peacock EE, Nelson RK, Arey JS, Reddy CM. Biodegradation and environmental behavior of biodiesel mixtures in the sea: an initial study. Marine pollution bulletin 2007; 54: 894-904.

EERE EERE. Ethanol Fuel Basics. Alternative Fuels Data Center. 2022. US Department of Energy.

Elazhari-Ali A, Singh AK, Davenport RJ, Head IM, Werner D. Biofuel components change the ecology of bacterial volatile petroleum hydrocarbon degradation in aerobic sandy soil. Environmental Pollution 2013; 173: 125-132.

EPA U. EPA Lifecycle Analysis of Greenhouse Gas Emissions from Renewable Fuels. In: Quality OoTaA, editor. United States Environmental Protection Agency, 2009.

Feris K, Mackay D, Sieyes Nd, Chakraborty I, Einarson M, Hristova K, et al. Effect of Ethanol on Microbial Community Structure and Function During Natural Attenuation of Benzene, Toluene, and o-Xylene in a Sulfate-reducing Aquifer. Environmental Science & Technology 2008; 42: 2289-2294.

Fuchs G, Boll M, Heider J. Microbial degradation of aromatic compounds--from one strategy to four. Nature reviews. Microbiology 2011; 9: 803.

Ghaly A, Dave D, Brooks M, Budge S. Production of biodiesel by enzymatic transesterification. Am J Biochem Biotechnol 2010; 6: 54-76.

Gieg LM, Fowler SJ, Berdugo-Clavijo C. Syntrophic biodegradation of hydrocarbon contaminants. Current Opinion in Biotechnology 2014; 27: 21-29.

Grangeia C, Santos L, Lazaro LLB. The Brazilian biofuel policy (RenovaBio) and its uncertainties: An assessment of technical, socioeconomic and institutional aspects. Energy Conversion and Management: X 2022; 13: 100156.

Grassi M, Pereira G. Energy-cane and RenovaBio: Brazilian vectors to boost the development of Biofuels. Industrial crops and products 2019; 129: 201-205.

Head IM, Jones DM, Röling WF. Marine microorganisms make a meal of oil. Nature Reviews Microbiology 2006; 4: 173.

Heermann SE, Powers SE. Modeling the partitioning of BTEX in water-reformulated gasoline systems containing ethanol. Journal of Contaminant Hydrology 1998; 34: 315-341.

Hektor HJ, Kloosterman H, Dijkhuizen L. Nicotinoprotein methanol dehydrogenase enzymes in Gram-positive methylotrophic bacteria. Journal of Molecular Catalysis B: Enzymatic 2000; 8: 103-109.

Hollebone B, Fieldhouse B, Landriault M, Doe K, Jackman P. Aqueous solubility, dispersibility and toxicity of biodiesels. International Oil Spill Conference. 2008. American Petroleum Institute, 2008, pp. 929-936.

Huang D, Zhou H, Lin L. Biodiesel: an Alternative to Conventional Fuel. Energy Procedia 2012; 16: 1874-1885.

Hussar E, Richards S, Lin Z-Q, Dixon RP, Johnson KA. Human health risk assessment of 16 priority polycyclic aromatic hydrocarbons in soils of Chattanooga, Tennessee, USA. Water, Air, & Soil Pollution 2012; 223: 5535-5548.

Islam TH, Ghosh B, Magnet MMH, Fatema K, Akter S, Khan MAR, et al. Isolation and identification of petroleum degrading bacteria from oil contaminated soil & water and assessment of their potentiality in bioremediation. Journal of Environmental Science, toxocology and Food Technology 2013; 5: 55 - 58.

ITRC. TPH Risk Evaluation at Petroleum-Contaminated Sites.

Jabbar, N. M.; Alardhi, S. M.; Mohammed, A. K.; Salih, I. K.; Albayati, T. M., Challenges in the implementation of bioremediation processes in petroleum-contaminated soils: A review. Environmental Nanotechnology, Monitoring & Management 2022**;** 18, 100694.

Jiaqiang E, Liu T, Yang W, Li J, Gong J, Deng Y. Effects of fatty acid methyl esters proportion on combustion and emission characteristics of a biodiesel fueled diesel engine. Energy Conversion and Management 2016; 117: 410-419.

Junior JS, Mariano AP, de Angelis D. Biodegradation of biodiesel/diesel blends by Candida viswanathii. African Journal of Biotechnology 2009; 8.

Karakashev D, Batstone DJ, Trably E, Angelidaki I. Acetate Oxidation Is the Dominant Methanogenic Pathway from Acetate in the Absence of Methanosaetaceae. Applied and Environmental Microbiology 2006; 72: 5138-5141.

Keller AH, Kleinsteuber S, Vogt C. Anaerobic Benzene Mineralization by Nitrate-Reducing and Sulfate-Reducing Microbial Consortia Enriched From the Same Site: Comparison of Community Composition and Degradation Characteristics. Microbial Ecology 2018; 75: 941-953.

Knothe G, Razon LF. Biodiesel fuels. Progress in Energy and Combustion Science 2017; 58: 36-59.

Knothe G, van-Gerpen J, Pereira L, Krahl J. Manual de biodiesel, 2006.

Lee DW, Lee H, Lee AH, Kwon B-O, Khim JS, Yim UH, et al. Microbial community composition and PAHs removal potential of indigenous bacteria in oil contaminated sediment of Taean coast, Korea. Environmental Pollution 2018; 234: 503-512.

Lima M, Mendes L, Mothé G, Linhares F, de Castro M, Da Silva M, et al. Renewable energy in reducing greenhouse gas emissions: Reaching the goals of the Paris agreement in Brazil. Environmental Development 2020; 33: 100504.

Lovanh N, Hunt CS, Alvarez PJ. Effect of ethanol on BTEX biodegradation kinetics: aerobic continuous culture experiments. Water Research 2002; 36: 3739-3746.

Luisa W, Letícia T, Francielle B, Raquel D, Patricia DdQ, Kateryna Z, et al. Culture-independent analysis of bacterial diversity during bioremediation of soil contaminated with a diesel-biodiesel blend (B10) S. Journal of Bioremediation and Biodegradation 2015; 6.

Ma J, Rixey WG, Alvarez PJJ. Microbial processes influencing the transport, fate and groundwater impacts of fuel ethanol releases. Current Opinion in Biotechnology 2013; 24: 457-466.

Mariano AP, Tomasella RC, De Oliveira LM, Contiero J, De Angelis DdF. Biodegradability of diesel and biodiesel blends. African Journal of Biotechnology 2008; 7.

Marietou A. Chapter Two - Sulfate reducing microorganisms in high temperature oil reservoirs. In: Gadd GM, Sariaslani S, editors. Advances in Applied Microbiology. 116. Academic Press, 2021; 99-131.

Martínez G, Sánchez N, Encinar JM, González JF. Fuel properties of biodiesel from vegetable oils and oil mixtures. Influence of methyl esters distribution. Biomass and Bioenergy 2014; 63: 22-32.

Meckenstock RU, Boll M, Mouttaki H, Koelschbach JS, Cunha Tarouco P, Weyrauch P, et al. Anaerobic Degradation of Benzene and Polycyclic Aromatic Hydrocarbons. Journal of Molecular Microbiology and Biotechnology 2016; 26: 92-118.

Meyer DD, Beker SA, Bücker F, Peralba MdCR, Guedes Frazzon AP, Osti JF, et al. Bioremediation strategies for diesel and biodiesel in oxisol from southern Brazil. International Biodeterioration & Biodegradation 2014; 95: 356-363.

Meyer DD, Santestevan NA, Bücker F, Salamoni SP, Andreazza R, De Oliveira Camargo FA, et al. Capability of a selected bacterial consortium for degrading diesel/biodiesel blends (B20): enzyme and biosurfactant production. Journal of Environmental Science and Health, Part A 2012; 47: 1776-1784.

Mittelbach M, Remschmidt C. Biodiesel—The Comprehensive Handbook, published by M. Mittelbach, Karl-Franzens-Universität Graz, Graz, Austria 2004.

Moser BR. Biodiesel production, properties, and feedstocks. Biofuels. Springer, 2011, pp. 285-347.
Müller JB. Avaliação da toxicidade da fração solúvel em água do biodiesel, diesel e da mistura binária diesel/biodiesel de 5%(B5). 2011.
Müller JB, Ramos DT, Larose C, Fernandes M, Lazzarin HSC, Vogel TM, et al. Combined iron and sulfate reduction biostimulation as a novel approach to enhance BTEX and PAH source-zone biodegradation in biodiesel blend-contaminated groundwater. Journal of hazardous materials 2017; 326: 229-236.
Mulligan CN, Yong RN. Natural attenuation of contaminated soils. Environment International 2004; 30: 587-601.
Musat F, Galushko A, Jacob J, Widdel F, Kube M, Reinhardt R, et al. Anaerobic degradation of naphthalene and 2-methylnaphthalene by strains of marine sulfate-reducing bacteria. Environmental Microbiology 2009; 11: 209-219.
Nagi J, Ahmed SK, Nagi F. Palm Biodiesel an Alternative Green Renewable Energy for the Energy Demands of the Future. International Conference on Constuction and Building Technology ICCBT, 2008.
Neuhauser EF, Ripp JA, Azzolina NA, Madsen EL, Mauro DM, Taylor T. Monitored natural attenuation of manufactured gas plant tar mono-and polycyclic aromatic hydrocarbons in ground water: a 14-year field study. Groundwater Monitoring & Remediation 2009; 29: 66-76.
Ng YF, Ge L, Chan WK, Tan SN, Yong JWH, Tan TTY. An environmentally friendly approach to treat oil spill: Investigating the biodegradation of petrodiesel in the presence of different biodiesels. Fuel 2015; 139: 523-528.
OECD/FAO. Agricultural Outlook. 2023.
Österreicher-Cunha P, do Amaral Vargas Jr E, Guimarães JRD, Lago GP, dos Santos Antunes F, da Silva MIP. Effect of ethanol on the biodegradation of gasoline in an unsaturated tropical soil. International Biodeterioration & Biodegradation 2009; 63: 208-216.
Pasqualino JC, Montané D, Salvadó J. Synergic effects of biodiesel in the biodegradability of fossil-derived fuels. Biomass and Bioenergy 2006; 30: 874-879.
Powers SE, Hunt CS, Heermann SE, Corseuil HX, Rice D, Alvarez PJ. The transport and fate of ethanol and BTEX in groundwater contaminated by gasohol. Critical Reviews in Environmental Science and Technology 2001; 31: 79-123.
Rabus R, Widdel F. Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Archives of Microbiology 1995; 163: 96-103.
Raczyk M, Kmiecik D, Przybylski R, Rudzińska M. Effect of Fatty Acid Unsaturation on Phytosteryl Ester Degradation. Journal of the American Oil Chemists' Society 2017; 94: 701-711.
Rama F, Ramos DT, Müller JB, Corseuil HX, Miotliński K. Flow field dynamics and high ethanol content in gasohol blends enhance BTEX migration and biodegradation in groundwater. Journal of contaminant hydrology 2019; 222: 17-30.
Ramadhas AS, Jayaraj S, Muraleedharan C. Biodiesel production from high FFA rubber seed oil. Fuel 2005; 84: 335-340.
Ramos DT. Bioestimulação de processos metanogênicos com acetato de amônio para degradação acelerada de hidrocarbonetos de petróleo em águas subterrâneas contaminadas com diesel B20. Engenharia Ambiental. Doutor. Universidade Federal de Santa Catarina, Florianóplis, 2013.
Ramos DT, da Silva MLB, Nossa CW, Alvarez PJJ, Corseuil HX. Assessment of microbial communities associated with fermentative–methanogenic biodegradation of aromatic hydrocarbons in groundwater contaminated with a biodiesel blend (B20). Biodegradation 2014; 25: 681-691.
Ramos DT, Lazzarin HSC, Alvarez PJJ, Vogel TM, Fernandes M, do Rosário M, et al. Biodiesel presence in the source zone hinders aromatic hydrocarbons attenuation in a B20-contaminated groundwater. Journal of Contaminant Hydrology 2016; 193: 48-53.
Ramos SM. Análise comparativa da influência do etanol em diferentes processos de remediação de águas subterrâneas impactadas por gasolina. 2012.
Rojo F. Degradation of alkanes by bacteria. Environmental Microbiology 2009; 11: 2477-2490.
Rojo F. Enzymes for aerobic degradation of alkanes. Handbook of hydrocarbon and lipid microbiology 2010; 2: 781-797.

Safinowski M, Meckenstock RU. Methylation is the initial reaction in anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. Environmental Microbiology 2006; 8: 347-352.

Satapanajaru T, Chokejaroenrat C, Sakulthaew C, Yoo-Iam M. Remediation and restoration of petroleum hydrocarbon containing alcohol-contaminated soil by persulfate oxidation activated with soil minerals. Water, Air, & Soil Pollution 2017; 228: 1-15.

Sousa DZ, Pereira MA, Stams AJ, Alves MM, Smidt H. Microbial communities involved in anaerobic degradation of unsaturated or saturated long-chain fatty acids. Applied and environmental microbiology 2007; 73: 1054-1064.

Souza DA, Chinalia FA, Foresti E, Zaiat M. Bioremediation of gasoline-contaminated groundwater in a pilot-scale packed-bed anaerobic reactor. International Biodeterioration & Biodegradation 2009; 63: 747-751.

Speight JG. Chapter 3 - Hydrocarbons from crude oil. In: Speight JG, editor. Handbook of Industrial Hydrocarbon Processes (Second Edition). Gulf Professional Publishing, Boston, 2020, pp. 95-142.

Stauffer E, Dolan JA, Newman R. CHAPTER 7 - Flammable and Combustible Liquids. In: Stauffer E, Dolan JA, Newman R, editors. Fire Debris Analysis. Academic Press, Burlington, 2008, pp. 199-233.

Steiner LV, Ramos DT, Liedke AMR, Serbent MP, Corseuil HX. Ethanol content in different gasohol blend spills influences the decision-making on remediation technologies. Journal of environmental management 2018; 212: 8-16.

Stolz J, Follis P, Floro G, Donofrio R, Buzzelli J, Griffin W. Aerobic and anaerobic biodegradation of the methyl esterified fatty acids of soy diesel in freshwater and soil environments. Duquesne University, Pittsburg 1995.

Sydney EB, Carvalho JCd, Letti LAJ, Magalhães AI, Karp SG, Martinez-Burgos WJ, et al. Current developments and challenges of green technologies for the valorization of liquid, solid, and gaseous wastes from sugarcane ethanol production. Journal of Hazardous Materials 2021; 404: 124059.

Tahhan RA, Abu-Ateih RY. Biodegradation of petroleum industry oily-sludge using Jordanian oil refinery contaminated soil. International Biodeterioration & Biodegradation 2009; 63: 1054-1060.

Techtmann SM, Hazen TC. Metagenomic applications in environmental monitoring and bioremediation. Journal of Industrial Microbiology & Biotechnology 2016; 43: 1345-1354.

Thomas AO, Leahy MC, Smith JW, Spence MJ. Natural attenuation of fatty acid methyl esters (FAME) in soil and groundwater. Quarterly Journal of Engineering Geology and Hydrogeology 2017; 50: 301-317.

Vaillancourt FH, Bolin JT, Eltis LD. The ins and outs of ring-cleaving dioxygenases. Critical Reviews in Biochemistry and Molecular Biology 2006; 41: 241-267.

Van Hamme JD, Singh A, Ward OP. Recent Advances in Petroleum Microbiology. Microbiology and Molecular Biology Reviews 2003; 67: 503-549.

Varjani S, Upasani V. Characterization of hydrocarbon utilizing Pseudomonas strains from crude oil contaminated samples. Int. J. of Scientific Computing 2012; 6: 120-127.

Vincent SGT, Jennerjahn T, Ramasamy K. Chapter 3 - Environmental variables and factors regulating microbial structure and functions. In: Vincent SGT, Jennerjahn T, Ramasamy K, editors. Microbial Communities in Coastal Sediments. Elsevier; 2021, 79-117.

Viulu S, Nakamura K, Kojima A, Yoshiyasu Y, Saitou S, Takamizawa K. Geobacter sulfurreducens subsp. ethanolicus, subsp. nov., an ethanol-utilizing dissimilatory Fe (III)-reducing bacterium from a lotus field. The Journal of General and Applied Microbiology 2013; 59: 325-334.

von Netzer F, Kuntze K, Vogt C, Richnow HH, Boll M, Lueders T. Functional Gene Markers for Fumarate-Adding and Dearomatizing Key Enzymes in Anaerobic Aromatic Hydrocarbon Degradation in Terrestrial Environments. Journal of Molecular Microbiology and Biotechnology 2016; 26: 180-194.

Weelink SAB, van Eekert MHA, Stams AJM. Degradation of BTEX by anaerobic bacteria: physiology and application. Reviews in Environmental Science and Bio/Technology 2010; 9: 359-385.

Widdel F, Rabus R. Anaerobic biodegradation of saturated and aromatic hydrocarbons. Current Opinion in Biotechnology 2001; 12: 259-276.

Winderl C, Schaefer S, Lueders T. Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (bssA) genes as a functional marker. Environmental microbiology 2007; 9: 1035-1046.

Zhang X, Peterson C, Reece D, Haws R, Möller G. Biodegradability of biodiesel in the aquatic environment. Transactions of the ASAE 1998; 41: 1423.

Zhang X, Young LY. Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. Applied and Environmental Microbiology 1997; 63: 4759-64.

Zhao D, Liu C, Liu L, Zhang Y, Liu Q, Wu W-M. Selection of functional consortium for crude oil-contaminated soil remediation. International Biodeterioration & Biodegradation 2011; 65: 1244-1248.

Chapter II - Manuscript 3: Shifts in structure and dynamics of the soil microbiome in fuel/biofuel blends-affected areas triggered by different bioremediation treatments

Environmental Pollution

Shifts in structure and dynamics of the soil microbiome in fuel/biofuel blends-affected areas triggered by different bioremediation treatments

--Manuscript Draft--

Manuscript Number:	ENVPOL-D-23-04406
Article Type:	Research Paper
Section/Category:	Soil, water, sediment, plant, biochar
Keywords:	Biodiesel ethanol gasohol biostimulation bioaugmentation microbial degradation
Corresponding Author:	Kelly Hidalgo Campinas, São Paulo BRAZIL
First Author:	Kelly Hidalgo
Order of Authors:	Kelly Hidalgo
	Admir Gianchi
	Marcio Schneider
	Adriana Soriano
	Marcus Baessa
	Luiz Martins
	Valéria Maia de Oliveira
Abstract:	The use of biofuels has grown in the last decades, due to climate and security energy issues and environmental impacts of fossil fuels use. Currently in Brazil, biodiesel is blended with diesel (12% Biodiesel named B12) and ethanol is blended with gasoline (27% ethanol, named E27). With increased use of those blends, cases of biofuel pollution in soil, surface and groundwater are expected. Elucidating structure, diversity, species interactions and assembly mechanisms of microbiomes is crucial for understanding the influence of environmental disturbances. However, little is known about how contamination with biofuel/petrofuel blends alters the soil microbiome. This study aimed to characterize the soil microbiome of four long term field experimental areas that received controlled releases of E10, E25 or B20 and were submitted to different interventions in contaminants source zone, using 16S rRNA gene amplicon high throughput sequencing. Results indicated that the soil microbiome of biodiesel affected areas is more diverse, resilient, and complex, likely due to the presence of syntrophic microorganisms, such as Clostridium and methanogenic archaea. It was also observed that in soils with low diversity and richness, the impact of bioremediation treatments on the microbial communities was higher. The network analysis showed that after applying the bioremediation treatment, hub species[1] appeared and the proportion of generalist taxa (more linked species) increased, suggesting that the treatment contributed to a more connected and dynamic assembly. All abundant keystone taxa are well-known degraders, suggesting that the abundant species are core targets for biostimulation in soil remediation. Overall, these findings extend our knowledge of the soil microbiome response triggered by pollution stress and bioremediation treatments, paving the way for future rationalized and efficient pollutant mitigation strategies. [1] Nodes that have the highest centrality degree in the network are therefore associated with a high number of other species (Rottjers and Faust, 2018)

Shifts in structure and dynamics of the soil microbiome in fuel/biofuel blends-affected areas triggered by different bioremediation treatments

K.J. Hidalgo[a,b3], A.J. Giachini[c] , M.R. Schneider[c], A.U. Soriano[d], M.P. Baessa[d], L.F. Martins[d], and V.M. Oliveira[a]

[a] Microbial Resources Division, Research Center for Chemistry, Biology and Agriculture (CPQBA), University of Campinas – UNICAMP, Av. Alexandre Cazellato, 999, ZIP 13148-218, Paulínia, SP, Brazil,

[b] Graduate Program in Genetics and Molecular Biology, Institute of Biology, University of Campinas (UNICAMP), Rua Monteiro Lobato 255, Cidade Universitária ZIP 13083-862, Campinas, SP, Brazil.

[c] Núcleo Ressacada de Pesquisas Em Meio Ambiente (REMA) - Department of Microbiology, Federal University of Santa Catarina (UFSC), Campus Universitário Sul da Ilha - Rua José Olímpio da Silva, 1326 - Bairro Tapera, 88049-500 Florianópolis, SC, Brazil.

[d] PETROBRAS/ R&D Center (CENPES), Av. Horácio Macedo, 950. ZIP 21941-915 Cidade Universitária, Ilha do Fundão, Rio de Janeiro, Brazil.

[3] Corresponding author: K.J. Hidalgo
E-mail: khidalgo@javeriana.edu.co
Telephone: +55 19 98172 1510

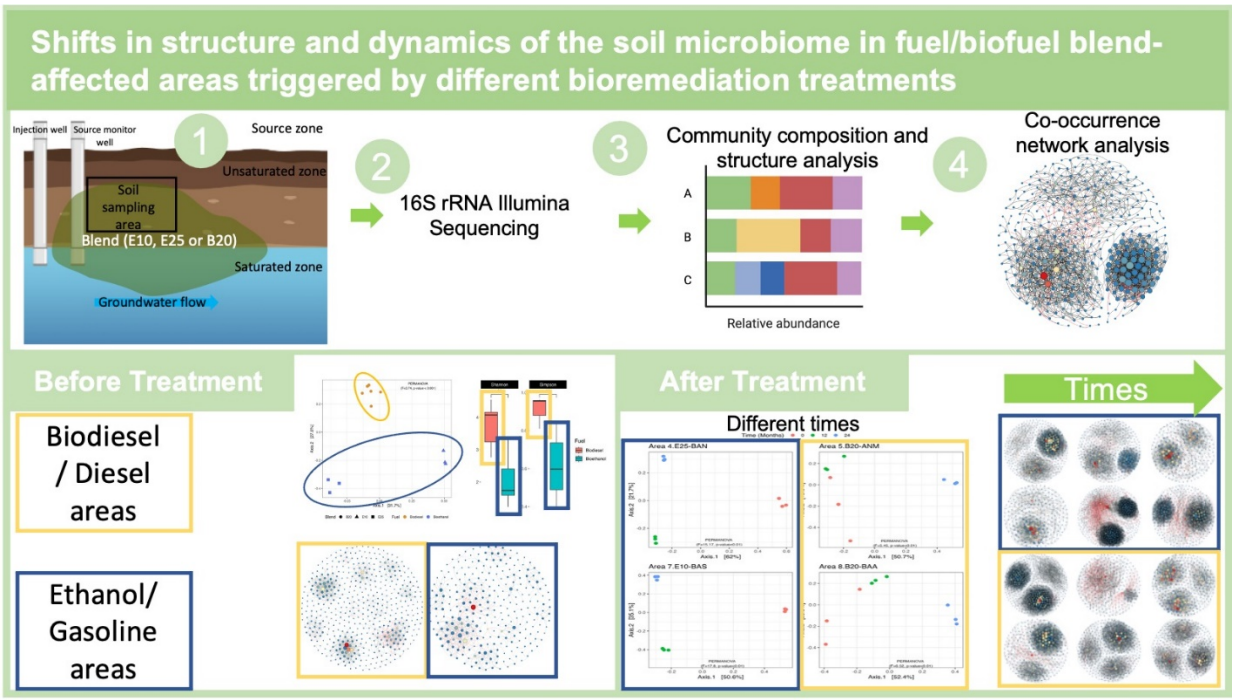
Abstract

The use of biofuels has grown in the last decades, due to climate and security energy issues and environmental impacts of fossil fuels use. Currently in Brazil, biodiesel is blended with diesel (12% Biodiesel named B12) and ethanol is blended with gasoline also named gasohol (27% ethanol, named E27). With increased use of those blends, cases of biofuel pollution in soil, surface and groundwater are expected. Elucidating structure, diversity, species interactions and assembly mechanisms of microbiomes is crucial for understanding the influence of environmental disturbances. However, little is known about how contamination with biofuel/petrofuel blends alters the soil microbiome. This study aimed to characterize the soil microbiome of four long term field experimental areas that received controlled releases of E10, E25 or B20 and were submitted to different interventions in contaminants source zone, using 16S rRNA gene amplicon high throughput sequencing. Results indicated that the soil microbiome of biodiesel affected areas is more diverse, resilient, and complex, likely due to the presence of syntrophic microorganisms, such as *Clostridium* and methanogenic archaea. It was also observed that in soils with low diversity and richness, the impact of bioremediation treatments on the microbial communities was higher. The network analysis showed that after applying the bioremediation treatment, hub species[4] appeared and the proportion of generalist taxa (more linked species) increased, suggesting that the treatment contributed to a more connected and dynamic assembly. All abundant keystone taxa are well-known degraders, suggesting that the abundant species are core targets for biostimulation in the remediation of these affected areas. Overall, these findings extend our knowledge of the soil microbiome response triggered by pollution stress and bioremediation treatments, paving the way for future rationalized and efficient pollutant mitigation strategies.

Keywords: Biodiesel, ethanol, gasohol, biostimulation, bioaugmentation, microbial degradation

[4] Nodes that have the highest centrality degree in the network and are therefore associated with a high number of other species (Rottjers and Faust, 2018)

Graphical abstract



1. INTRODUCTION

In the last decades, multiple accidental spills of fossil occurred due to leakages from underground storage tanks, rupture of pipelines and transport accidents (Baniasadi and Mousavi, 2018; Islam et al., 2013). As a result, the compounds released into the environment caused huge impacts to the ecosystems and human health for their toxicity, mutagenicity, and carcinogenicity (Baniasadi and Mousavi, 2018; Tahhan and Abu-Ateih, 2009). Climate and security energy crises have encouraged the replacement of fossil fuels by cost-benefit renewable and clean energy sources, such as biodiesel and ethanol. In some countries, such as Brazil, biofuels are used as blends. Currently, biodiesel is being used in blends with diesel (also called B12 - 12% biodiesel / 88% Diesel), and ethanol is blended with gasoline (also called E27 - 27% hydrated ethanol / 73% gasoline) (Canabarro et al., 2023).

The growing use of biofuel/fossil fuel blends (OECD/FAO, 2023) encourages investigating the implications of biofuels on the remediation of hydrocarbon-impacted sites. Several studies have demonstrated that the biodegradation of petroleum

hydrocarbons is affected by the presence of ethanol or biodiesel (Chen et al., 2008; Corseuil et al., 2011b; Costa et al., 2009; da Silva and Corseuil, 2012; Rama et al., 2019; Ramos et al., 2014; Steiner et al., 2018). Thus, it is important to develop specific protocols for the detoxification of ecosystems polluted with such blends. Since bioremediation treatments are well known as a cost-effective solution to treat hydrocarbon-polluted areas (Baniasadi and Mousavi, 2018; Ng et al., 2015; Zhao et al., 2011), studies addressing biodegradation of biofuel/petrofuel pollution have grown (Alvarez and Hunt, 2002; Chen et al., 2008; Corseuil et al., 1998; Cyplik et al., 2011; Da Silva et al., 2005; Da Silva and Alvarez, 2004; Heermann and Powers, 1998; Ng et al., 2015; Rama et al., 2019; Ramos, 2012; Satapanajaru et al., 2017). However, a considerable number of studies have focused only on the assessment of the effect of biofuels on petroleum hydrocarbon degradation dynamics, rate, and efficiency (Alvarez and Hunt, 2002; Cyplik et al., 2011; Rama et al., 2019; Ramos, 2012) and the behavior of the degrading microbial community has been studied in few cases (Luisa et al., 2015; Müller et al., 2017). Additionally, in these studies, the community dynamics and how it impacts the bioremediation efficiency are not covered.

Numerous field and laboratorial works have demonstrated the efficiency of the bioremediation treatments in the recovery of impacted soils (Chen et al., 2015; Norris, 2017; Stepanova et al., 2022; Wu et al., 2017). These treatments include addition of nutrients or electron acceptors (i.e. biostimulation), adding microbial degraders (i.e. bioaugmentation) or air injection (i.e. biosparging), among others (Koshlaf and Ball, 2017; Okoh et al., 2020), thus modulating the microbial community to increase the degradation rates. Soil microorganisms have important roles in biogeochemical processes such as the carbon, nitrogen, sulfur and phosphorus cycles (Falkowski et al., 2008), forming complex co-occurrence networks through indirect and direct interactions (Hallam and McCutcheon, 2015). However, there are no reports in literature on the assessment of the ecological processes (i.e., deterministic or stochastic) driving microbial community assembly, complexity, diversity and dynamics in areas affected by biofuel blends and bioremediation treatments.

In order to go beyond the classic compositional and diversity microbial community analyses, new analytical tools such as co-occurrence networks have recently offered strong methods for deciphering the intricate relationships among microorganisms, biogeographical patterns, shared ecological niches and keystone taxa (Barberán et al., 2012; Berry and Widder, 2014; Faust et al., 2012). The topological characteristics of nodes are employed in a co-occurrence network analysis to assess the potential significance of microorganisms, such as keystone species (Eiler et al., 2012; Steele et al., 2011), which are highly connected microorganisms that, besides their abundance, have a strong influence on the composition and function of microbial communities (Banerjee et al., 2018). For instance, node betweenness centrality shows the influence of one node on the co-occurrence of other nodes in the network (Greenblum et al., 2012). The core and central location of a node is determined by a high betweenness centrality, while low value indicates a peripheral location (Ma et al., 2016). The number of direct co-occurrences for a specific node is represented by the node degree (Greenblum et al., 2012). Additionally, the community assembly, i.e. the process that shapes the traits and abundance of taxa in communities, can be of great relevance in evaluating the impact of pollutants on the soil microbial community, that can affect the transfer and biodegradation (Guittar et al., 2019; Stegen et al., 2013). Thus, deep understanding of the polluted soil associated microbiome is crucial since microbial dynamics can have a great influence on the fate of the contaminants in the environment (Fowler et al., 2016; Hidalgo et al., 2019; Jin et al., 2010; Lovley, 2003).

This study aimed to assess the microbial community assembly, taxonomy and ecology in soils of four experimental areas that received controlled releases of different blends of biodiesel/diesel and gasohol to evaluate several bioremediation treatments (i.e. biostimulation with electron acceptors and nutrients, biosparging, and bioaugmentation). The hypotheses that guided our study were: (i) The microbiome composition and dynamics vary according to the different fuel/biofuel blends; (ii) The bioremediation treatments influence the microbial metabolism, resulting in shifts in microbial community structure and composition; (iii) The microbiome co-occurrence patterns and keystone species vary according to the different blends and

bioremediation treatments, reflecting the level of complexity and strength of the interactions among microbial members depending on the substrate, nutrients and electron acceptor availability. It should be mentioned that our hypotheses did not consider environmental factors (e.g. soil type, chemistry, humidity, precipitation, temperature, water table, etc), because all four areas were under the same pedological and climatological conditions.

2. MATERIAL AND METHODS

2.1. Description of experimental areas and bioremediation treatments

The experimental site is located at the Ressacada Experimental Farm in Florianópolis, owned by the Federal University from Santa Catarina (UFSC), in the southeast of Santa Catarina Island, Brazil (Figure S1A). The climate is mesothermic humid with an annual average precipitation of 1600 mm. The average groundwater temperature is 26°C in the summer and 22°C in the winter, and the water table varies from 0.7 to 2.0 m throughout the year. The subsurface soil consists of hydromorphic quartz sands with less than 5% clay. Soil organic carbon ranges between 0.16 and 0.68% (Ramos, 2013; Ramos et al., 2013; Ramos et al., 2010).

Controlled releases of blends of biofuels/petrofuels were conducted in four experimental areas throughout time (2004 – 2010) at Ressacada Experimental Farm to evaluate environmental behavior of contaminants in subsurface and several bioremediation strategies (Phase 1). The contamination source zones were established by releasing 100 L of a particular blend into a one- square-meter pit deep enough to reach the water table (1.0 – 1.6 m deep) (Figure S1C and Figure S2). Each experimental area covered 330 - 549 m^2 and encompassed several monitoring wells (MW) (Figure S1B and Figure S1C). Each MW had polyethylene tubes to sampling different depths of groundwater (2, 3, 4, 5 and 6 m below the ground surface) (Figure S1C). The results of that first phase of studies have been published elsewhere (see references in sections 2.1.1 – 2.1.4).

Recently (2020 – 2022), a second phase of remediation studies was performed (Phase 2), focusing on the source zones to achieve hydrocarbon concentration lower than Brazilian legal standards (BRASIL, 2009) (results not published yet). The present paper comprised the investigation of the soil microbiome dynamics in the source zone of those four experimental areas: two that received gasohol (i.e. areas 4.E25-BAN and 7.E10-BAS), and two that received biodiesel plus diesel (8.B20-BAA and 5.B20-ANM) (Figure S1and Figure S2).

2.1.1. Area 4.E25-BAN

This area was contaminated in 2004 with the blend E25 (Ethanol 25% and gasoline 75%). The first bioremediation treatment applied to the contaminant dissolved phase was biostimulation with nitrate (Costa et al., 2009; da Silva and Corseuil, 2012). In 2020, the second phase of bioremediation treatment consisted of adding nitrate (as an anaerobic electron acceptor) as well as niacin or nicotinic acid ($C_6H_5NO_2$) and phosphate. This bioremediation approach was repeated in 2022 (21 months later) (Figure S2) because xylenes concentrations above environmental legal standards had been observed at the source zone.

2.1.2. Area 7.E10-BAS

This area received the blend E10 in 2009 to fulfill experiments of biostimulation with sulfate for contaminants degradation in the dissolved phase (Ramos et al., 2010). In 2020, the source zone bioremediation was based on anaerobic biostimulation by adding ammonium acetate and iron oxide from acid mine drainage. In 2022 (21 months later), due to the odors increase, a new addition of biostimulants was needed (Figure S2).

2.1.3. Area 5.B20-ANM

The blend B20 (soybean biodiesel 20% and diesel 80%) was released in 2008 to evaluate the influence of biodiesel in diesel natural attenuation (Ramos, 2013; Ramos et al., 2013). This area was maintained with no activate treatment until 2020. In May/2020, aiming at accelerating the degradation of some petroleum hydrocarbons

found in the source zone in concentrations above legal standards, a bioremediation treatment based on bioaugmentation and biosparging was initiated. Using an internal loop airlift reactor (IALR), indigenous microorganisms were grown and reinjected into the source zone. In addition, periodic air injections were done into the source zone. In section 2.4, the operation of the IALR is detailed. In 2022, the culture medium was renewed in the source zone and in the reactor (21 months later) (Figure S2).

2.1.4. Area 8.B20-BAA

In this area, a controlled release of the B20 blend (soybean biodiesel 20% and diesel 80%) was done in 2010. The first bioremediation treatment employed at the dissolved phase was anaerobic biostimulation by adding ammonium acetate (Ramos et al., 2013; Ramos et al., 2014). As done in area 5.B20-ANM, the air injection (directly into the source zone) and the operation of an airlift reactor started in May/2020 to enrich microbial activity within the source zone. However, in this case, an external loop airlift reactor (EALR) was employed instead of an IALR (Figure S2), in order to favor, even more, the oxygen transference to the recirculating groundwater, since high concentrations of some petroleum hydrocarbons were still present. Moreover, in 2022 (21 months later), the culture medium was renewed within the reactor and in the source zone.

2.2. Groundwater analyses

Physicochemical and chemical analyses were performed in the Laboratory of Groundwater Remediation at the Ressacada Experimental Nucleus (REMA/UFSC) in Florianópolis, SC, Brazil. Samples were collected from the source zone at all depths (Figure S1B) using a peristaltic pump and Teflon tubing. However, for comparisons with microbiological data, only measures from 2m-depth were considered because most of the contamination was situated at such depth. Temperature, pH, and dissolved oxygen parameters were measured on site using a Micropurge Flow Cell (MP20-1380). BTEX were quantified in soils and water was performed by using a combination of the EPA/5021A and EPA/8015D methods ((USEPA). 2000). Principal Coordinates Analysis (PCA) was performed based on the chemical (BTEX

concentration) and physicochemical measurements (DO, temperature, pH) of the groundwater in all areas.

2.3. Soil Samples

Soil samples were collected in triplicate from the source zone of each area in three campaigns, time 0 (immediately before the first intervention in the source zone - 2020), time 12 (12 months after the first intervention in the source zone) and time 24 (3 months after the second intervention in the source zone) (Figure S2).

Samples were taken using a soil bucket auger of 6 cm (Figure S3) at the same depth (1.0 – 1.6 m below the ground) of the blend release. Samples were stored in sterile bags and kept at -80°C until further processing for molecular analyses. A total of 36 samples were collected. Additionally, parameters such as BTEX were measured in the soil samples.

2.4. Reactor Samples

In 2020, airlift reactor (ALR) was installed in areas 5.B20-ANM (IALR) and 8.B20-BAA (EALR) and operated continuously until the end of the study (Figure S2). Thirty days before the operation, injection of air and culture medium was done through the source zone-injection wells in both areas. The culture medium (20 L) was composed of molasse solution (2% w/v), niacin (0.1% w/v), soy oil (2% v/v), ammonium phosphate (0.25% w/v), isolated soy protein (0.5% w/v) and commercial biodegradable detergent (0.4% v/v), with pH adjusted to 6.5. Just before the operation start, reactors were added with 20 L of culture medium containing molasses solution (2% v/v), isolated soy protein (0.5% w/v), yeast extract (0.1% w/v), ammonium phosphate (0.5% w/v) and the pH adjusted to 6.5. After the preparation stage, the enriched community (inoculum) in the source zone was pumped into the reactor and the recirculation flow rate was 15 L/day. The reactors were employed to stimulate and modulate the microbial community, mainly for biosurfactants prion and aerobic hydrocarbon degradation. In the last campaign (2022), one sample of each reactor

was taken and stored in sterile conic tubes (Falcon®) and kept at -80°C until further processing for molecular analyses.

2.5. DNA extraction, 16S rRNA gene amplicon sequencing and bioinformatic analyses

DNA from soil and the reactor samples was extracted using a combination of two commercial kits, FastDNA™ Spin kit for soil and DNeasy PowerSoil® Pro kit. Basically, the manufacturer's protocol of the DNeasy PowerSoil® Pro kit was followed, with some modifications. First, instead of PowerBead Pro tube, the Lysing Matrix E tube from the FastDNA™ Spin kit was used, since it provided more efficient lysis, possibly due to the mixture of ceramic and silica particles of different sizes. Second, elution was performed using 30 μL of C6 Solution, instead of 100 μL, due to the low microbial abundance in the samples. A better performance was observed when using a combination of both kits, one being more efficient in the lysis step and the other more efficient in cleaning and removing contaminants. DNA quality was checked by electrophoresis in 1.0% agarose gel. DNA purity and concentration were verified using NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and Qubit (Thermo Fisher Scientific, Waltham, MA, USA), respectively. The V3 – V4 variable region of 16S rRNA genes was sequenced using the Illumina HiSeq 2500 2 x 250 bp platform with primers 341F (CCTACGGGNGGCWGCAG) and 785R (GACTACHVGGGTATCTAATCC; fragment length of 444 bp) (Klindworth et al., 2012) at the NGS – Soluções Genômicas in Piracicaba – São Paulo, Brazil.

The sequences were processed following the DADA2 V1.26.0 pipeline (Callahan et al., 2016) in R language platform (R Development Core Team, 2021). This pipeline includes all steps, *i.e.* primers remotion, quality control, trimming (Phred Score > 30), denoising, sequence merging, chimera removal and taxonomic annotation. Taxonomic affiliation was assigned by using the SILVA ribosomal RNA gene database (Release 138.1 August 2020) (Quast et al., 2012) and the generated matrix was further used for statistical analyses. All the steps of this pipeline presented here are found at https://github.com/khidalgo85/metataxonomics. Sequences yield in

this study were deposited at the National Center for Biotechnology Information (NCBI) database under the accession numbers SAMN35439697-SAMN35439734.

2.6. Statistical Analyses

Compositional and diversity analyses were performed using Phyloseq R package (McMurdie and Holmes, 2013). Principal Correspondence Analysis was performed to compare the microbial community composition of biodiesel vs. gasohol polluted areas before the bioremediation treatment. Then, permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2014) was applied to test the significance of the clusters in PCoA. For this analysis, adonis function from the vegan (Oksanen et al., 2013) R package was used, including the pollutant blend as an independent variable with default parameters (Bray-Curtis' dissimilarity matrix (Beals, 1984) and 999 permutations). Shannon's diversity and Simpson's dominance indexes were calculated on the rarefied ASV matrix and used to show the biofuel blend effect on the microbial α-diversity. Normal distribution was tested using Shapiro-Wilk Normality Test and t-test was used to test the significance of the mean differences ($p < 0.05$) between the biodiesel and gasohol areas. The relationship between the community dissimilarity and the time of sampling was analyzed using Principal Coordinates Analysis (PCoA) in vegan package. A PERMANOVA using Bray-Curtis distances was applied to analyze the differences in the microbial communities between the sampling times (before and after the bioremediation treatment applications). The normalized stochasticity ratio (NST) was calculated to discriminate the different soil microbial community assembly processes (Ning et al., 2019).

2.7. Network analyses

Co-occurrence network analysis was performed to unravel the microbial interactions in the different areas polluted with biodiesel or gasohol and across time. For this, non-random co-occurrence patterns were carried out using the Python module SparCC (Friedman and Alm, 2012) and the ASVs frequency table. For each network, SparCC correlations were calculated and only SparCC >0.8 or <-0.8 and highly significant (p <0.01) correlations were selected. The Fruchterman-Reingold

layout algorithm with 1000 permutations in Gephi software was used to visualize the network (Bastian et al., 2009). The topological characteristics, such as number of nodes, edges (positive and negative) and communities, betweenness centrality (number of shortest paths through nodes), degree (number of adjacent edges), closeness centrality, modularity, average path length, diameter, average degree, among others, were calculated using Gephi. Some of these topological properties were used to test the significant differences across time in each area. Keystone taxa in the network could act as a hub for community structure and function. Keystone taxa have a greater degree and smaller betweenness centrality. Node network connectivity was assessed by calculation of the within-module connectivity (*Zi-score*) and among-module connectivity (*Pi-score*) (Guimera and Nunes Amaral, 2005). The nodes of each network were classified into four categories according the *Zi-Pi* scores (Poudel et al., 2016): peripherals ($Zi < 2.5$, $Pi < 0.62$), networks hubs ($Zi \geq 2.5$, $Pi \geq 0.62$), module hubs ($Zi \geq 2.5$, $Pi < 0.62$), and connectors ($Zi < 2.5$, $Pi \geq 0.62$) (Olesen et al., 2007). According to their roles in network topology, connectors, module hubs and network hubs are considered keystone species or generalists in the community (Banerjee et al., 2018).

The abundant and rare species were analyzed at local (i.e., in one sample) and regional level (i.e. across all samples), according with other studies (Liu et al., 2015; Logares et al., 2014). ASVs at local level with relative abundances < 0.01% were considered as rare, those with relative abundance > 0.5% were considered as abundant. At regional level, ASVs with average relative abundance < 0.001% were considered as rare, and > 0.05% as abundant (Jiao et al., 2017).

3. RESULTS AND DISCUSSION

3.1. Bioremediation treatments effectively reduced BTEX concentrations

In soil, BTEX concentrations were at least ten times higher than in water (Figure 1), because the compounds are absorbed and accumulated in the soil matrix (Ossai et al., 2020). However, hydrocarbon concentrations dropped significantly in soils in almost all areas at time 12 (12 months after the first source zone intervention made

in 2020) (Figure 1A). Regarding the groundwater, BTEX concentrations were more fluctuating, probably due to residual contaminants desorption from soil coupled to groundwater level fluctuation, especially in 7.E10-BAS area. However, at time 24 (24 months after the first source zone intervention and 3 months after the second zone intervention made in 2022) all compounds in the four areas were below the maximum contaminant levels (MCL) (Resolution CONAMA 420/2009, Brazil) (Figure 1B).

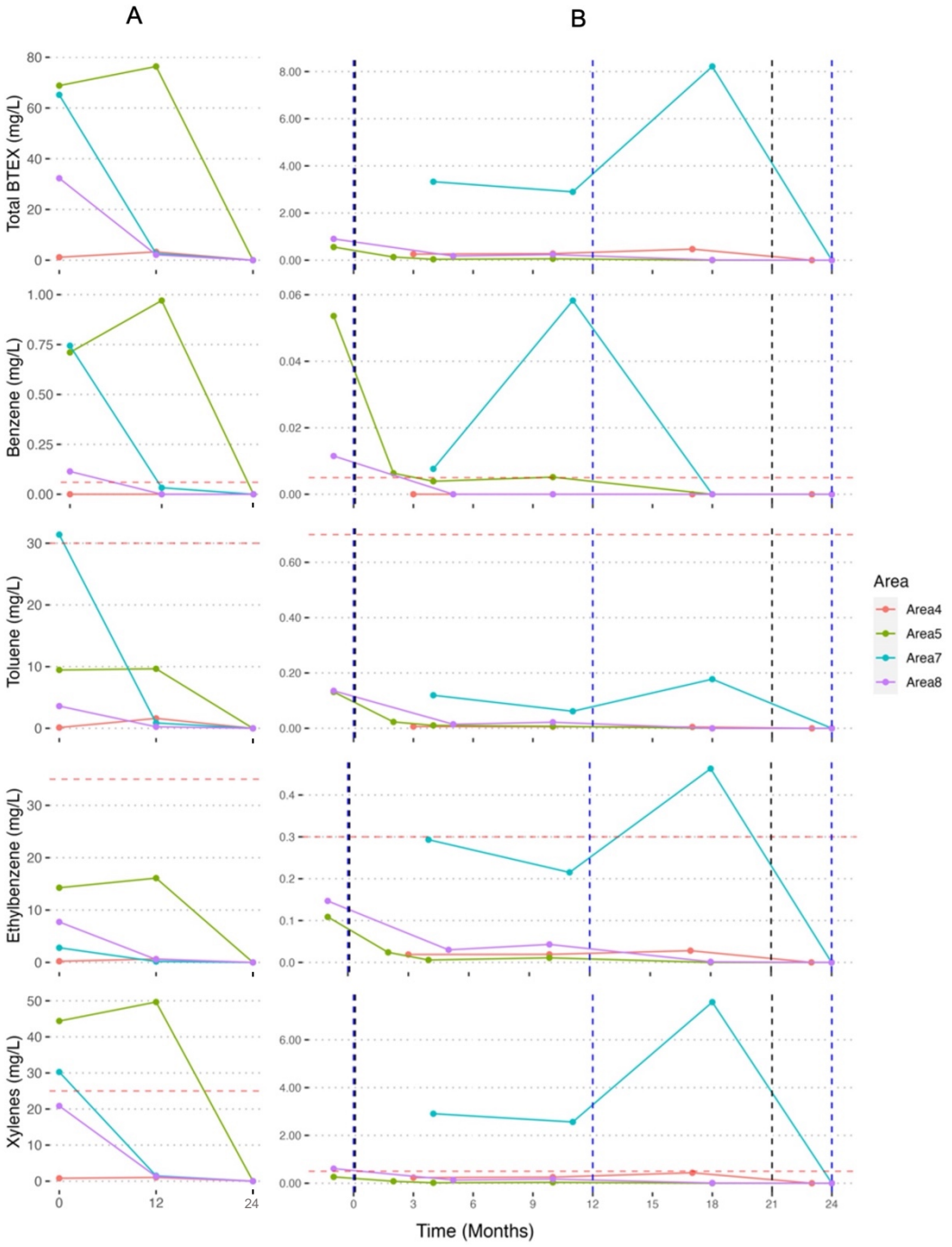


Figure 1. BTEX concentrations (mgL^{-1}) in soil (A) and groundwater (B) as a function of time (months after the source zone intervention). Red dashed line represents the MCLs for benzene, toluene, ethylbenzene, and xylenes. Blue dashed lines represent soil microbiome sampling. Black dashed line represents the time of the bioremediation treatment.

Differently from the other areas, in 5.B20-ANM area, hydrocarbon concentrations decreased in soil only after time 12 (Figure 1A). Due to this area was

not intervened for 12 years (Figure S2), it is probably that this contributed to the slow hydrocarbon degradation rates. On the other hand, as it was showed in a microcosms study, the nutrient depletion also is a main limiting factor in the oil removal through bioaugmentation technique (Sun et al., 2021). Nevertheless, the use of loop airlift reactor to perform the bioaugmentation could help to improve the nutrients and oxygen supply for the microorganisms. Additionally, the efficacy of the biosparging (injection of air) can vary greatly, since predicting the direction of the airflow is impossible. The airflow behavior depends on soil permeability, among other factors (Ossai et al., 2020; Philp and Atlas, 2005). Thus, we hypothesized that all these reasons could be contribute to the slow hydrocarbon degradation rates.

In 4.E25-BAN area the initial hydrocarbon concentrations were very low; this fact makes the removal of the residual concentrations difficult, due to the low availability of the hydrocarbons to the support the growth and activity of microorganisms (Al-Hawash et al., 2018; Ren et al., 2018). On the other hand, in 7.E10-BAS area, the highest concentrations of almost all BTEX compounds were observed in soil at time 0 (immediately before the first source zone intervention). However, at time 12, hydrocarbon concentrations were close to zero (removal > 93%). Nevertheless, in groundwater the trend was different. There was an increase in all hydrocarbon concentrations in the same period. We hypothesized that this can be related with the hydrocarbon release from the absorbed and adsorbed-phase in soil to the dissolved-phase in water and in the same way part of the hydrocarbon removal in soil can be attributed to migration to the water (Leharne, 2021). Twenty-one months after the first intervention in the source zone, new addition of biostimulants was performed. After that, hydrocarbon concentrations decreased until below the MCLs in the groundwater (Figure 1B). As it was expected, area 7.E10-BAS had low dissolved oxygen concentrations (Figure S1A). This potentially directly impacted the hydrocarbon degradation rate in the groundwater in this area, since anaerobic degradation is slower than aerobic metabolism (Haritash and Kaushik, 2009).

A Principal Coordinates Analysis (PCA) was performed using BTEX concentrations of the soil source zones in the four studying areas (Figure 2),

considering data from times 0 (2020), 12 months (2021) and 24 months (2022) after source zone intervention (Phase 2). Area 7.E10-BAS at time 0, and area 5.B20-ANM at times 0 and 12 formed separate clusters from the other areas. The biplot graph showed that toluene was strongly related to area7_0 (area 7.E10-BAS, time 0). As shown before, in this area the concentration of the BTEX compounds were higher before at the source zone anaerobic bioremediation, especially toluene. Meanwhile, xylene and ethylbenzene concentrations were highly related with area5_0 and area5_12 (area 5.B20-ANM, times 0 and 12) and moderately related with area8_0 (area 8.B20-BAA, time 0). The rest of the samples clustered together and were negatively related with the pollutant concentrations. Benzene was the compound with the lowest contribution in explaining the observed variation in soil, probably due to its higher volatility, water solubility and mobility among volatile hydrocarbons (Corseuil et al., 2011a) (see vars Contributions scale, Figure 2).

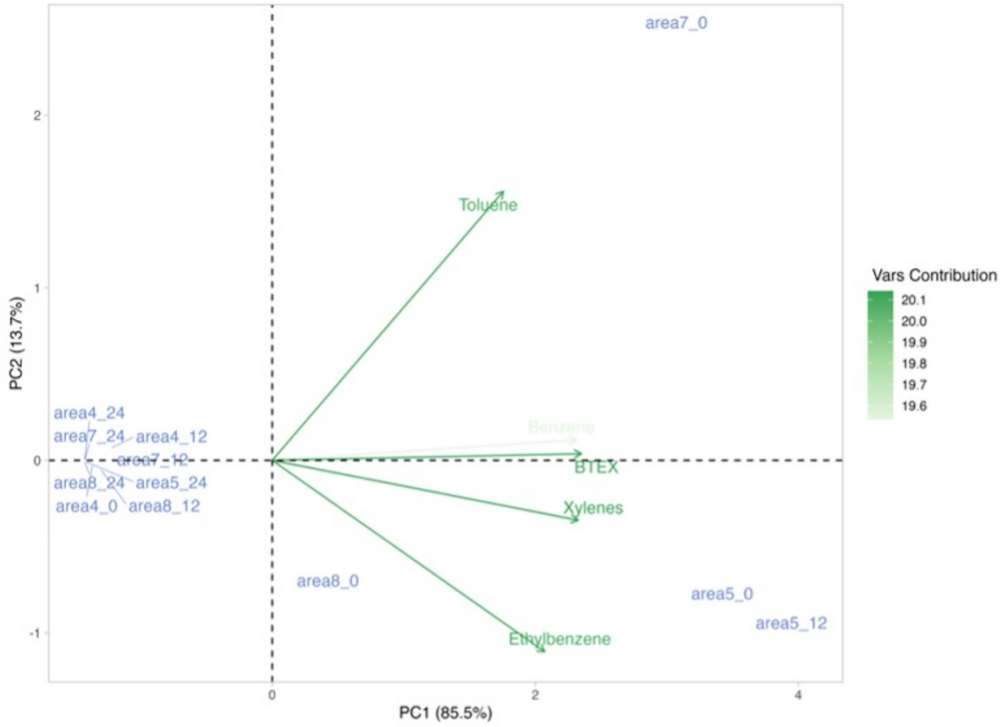


Figure 2. Principal coordinates analysis (PCA) based on BTEX concentrations in the soil source zones of the four areas under study at at times 0, 12 and 24. The color scale represents the variable contribution on the sample's distribution.

3.2. Microbial community profiles in polluted areas varied according to fuel/biofuel blends

The composition and structure of the microbial communities associated to soils contaminated with gasohol and biodiesel/diesel blends before and after the bioremediation treatment of source zones were evaluated via massive sequencing of the 16S rRNA gene V3-V4 variable region, yielding 1.5 million read pairs. After bioinformatic processing, 5664 ASVs were obtained in total, which were affiliated to 34 bacterial and 7 archaeal phyla (Figure 3A).

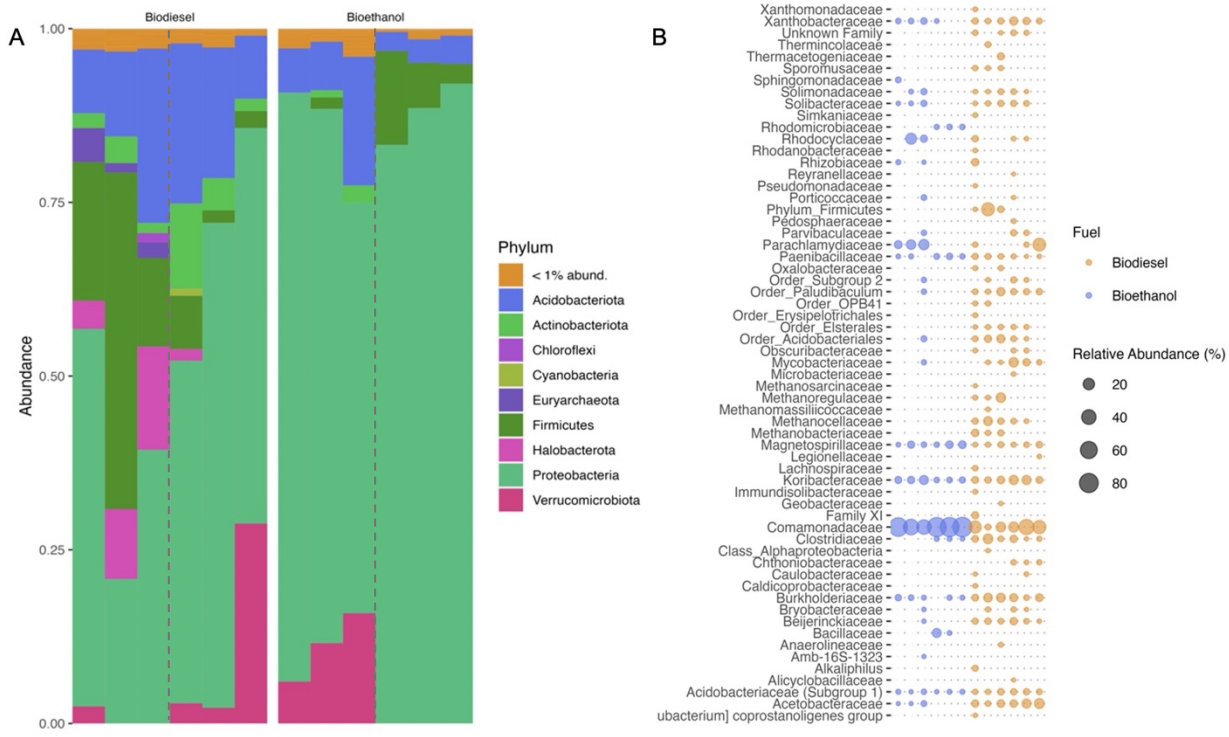


Figure 3. Relative abundances of microbial community taxa based on 16S rRNA gene sequencing in time 0, at phylum (A) and family (B) levels. Only families with > 0.5% of relative abundance are represented.

Proteobacteria was the most abundant phylum in both biofuel-type impacted areas (Figure 3A). However, this phylum was much more abundant in areas contaminated with gasohol, accounting for more than 80% of the total phylum abundance. Other differences in phyla composition were observed between the fuel/biofuel blends impacted areas. Archaeal phyla, such as Euryarchaeota, Halobacterota and Nanoarchaeota, were more abundant in areas contaminated with

biodiesel (B20) compared with the gasohol-associated areas. At family level, Comamonadaceae was the most prevalent family in gasohol polluted areas, with at least 70% of total community members (Figure 3B), mainly represented by *Extensimonas* genus. This genus has been previously isolated from activated sludge from a pesticide-manufacturing wastewater (Peng et al., 2020). Wang et al. (2021) showed that *Extensimonas* was one of the most abundant genera in the microbiome of ecopiles used to remediate petroleum contaminated soil (Wang et al., 2021). Other studies also reported the participation of members of this genus in hydrocarbon degradation (Gauchotte-Lindsay et al., 2019; Zhang et al., 2013). On the other hand, families such as *Xanthobacteraceae, Burkholderiaceae* and Acetobacteraceae were more abundant in biodiesel impacted areas. Xanthobacteraceae has been reported in oil polluted-soil microcosms as one of the most abundant families (Dörr de Quadros et al., 2016) and Burkholderiaceae was found to be associated with anaerobic benzene degradation in a chemostat injected with benzene and nitrate as electron acceptor (Zaan et al., 2012). Methanogenic archaea were found exclusively in biodiesel impacted areas (i.e., Methanocellaceae and Methanobacteriaceae). The absence of methanogenic microorganisms in gasohol impacted areas could be related to the presence of anaerobic electron acceptors that were added in Phase I (nitrate in area 4.E25-BAN and sulfate in area 7.E10-BAS) (Figure S2), thus suppressing methanogenic metabolism (Da Silva et al., 2005).

Statistical comparison of the composition of the microbial communities associated to gasohol and biodiesel/diesel polluted areas, which considered the relative abundance of the prokaryotic species, revealed that the microbiomes of these areas were significantly different (Table S1, Figure 4A). These results are supported by previous studies that compared microbial communities in soils polluted with blends of petroleum hydrocarbons and E10 or B20 (Elazhari-Ali et al., 2013).

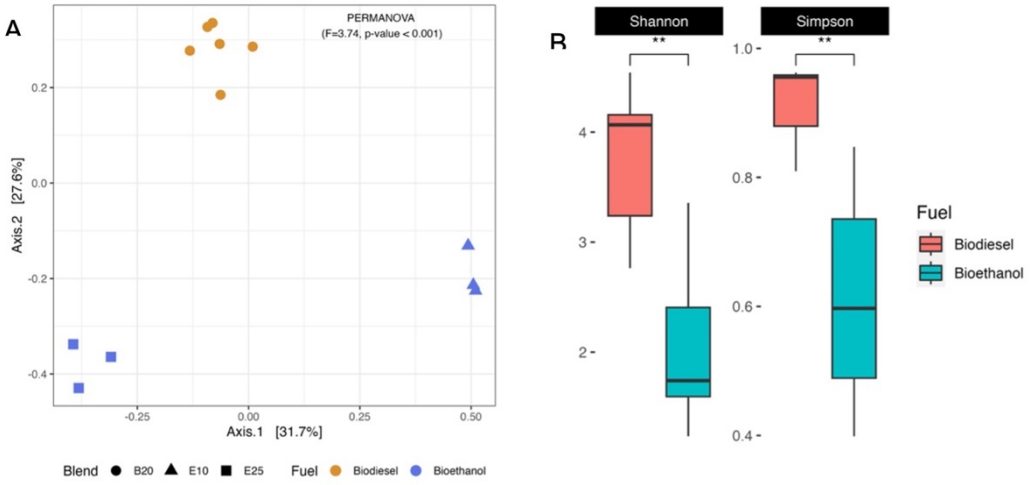


Figure 4. PCoA using Bray-Curtis distance of microbial communities in soil samples according to the fuel contaminant before the bioremediation treatment (A); and alpha diversity indexes (B).

Additionally, microbial communities between areas contaminated with different proportions of ethanol (area 4.E25-BAN and area 7.E10-BAS) were also different (Figure 4A). These results corroborated findings of previous studies where BTEX degradation and microbial community profiles in E10, E24 and E85 polluted sites were compared (Rama et al., 2019; Steiner et al., 2018). All these findings support the hypothesis that the type of contaminant and blend ratios (biofuel/fossil fuel) may drive microbial community composition.

Alpha diversity (Shannon and Simpson) indexes of microbial communities were significantly different between the gasohol and biodiesel/diesel impacted areas (T-test p-value < 0.001, Table S2-S4, Figure 4B), suggesting that the type of biofuel had an impact in the community structure. In the presence of biodiesel, the diversity indexes were higher when compared to the gasohol-impacted area. We hypothesized that due that the composition of the biodiesel is more complex than the ethanol, with the presence of different chemical functional groups (i.e. ester, fatty acids), being necessary different microorganisms to decomposed these compounds, resulting in the increase of species diversity (Bücker, et al., 2011).

3.3. Biostimulation with electron acceptors and nutrients triggered significant shifts in the microbial communities

Soil samples from the biofuel/fuel blend impacted areas were taken before and one year after source zone intervention, applied in 2020 (Figure S2). As mentioned above, this intervention treatment was repeated in all areas 21 months later (2022), due to the appearance of odors and increase in some hydrocarbon concentrations. A third sampling campaign (month 24) was performed three months after this second intervention in the source zone (Figure S2), in order to unravel the dynamics of the microbial community over time.

The PERMANOVA and ordination analyses revealed that microbial communities from areas polluted with gasohol and treated through injection of anaerobic electron acceptors showed a shift in the microbial composition, following a long or short period of time (one year – time 12 or 3 months- time 24) (Figure 5A and Table S5). On the other hand, in areas polluted with B20 and submitted to biosparging and bioaugmentation, samples from time 0 and time 12 clustered together, whereas samples from time 24 formed a separate group (Figure 5A and Table S5). Regarding the community structure, two-way ANOVAs were performed to compare Shannon indexes between sampling times in each area, showing statistical significance in all areas (Table S6 and Table S7, p-value < 0.05). Tukey HSD was used to determine which comparisons were significant (Table S7). The microbial community structure and diversity changed following one year (time 12) after the first intervention in the source zone and three months (time 24) after the second intervention in the source zone in area 7.E10-BAS, and only one year after (time 12) in area 4.E25-BAN. On the other hand, in area 5.B20-ANM, microbial community significantly changed in time 24, after the third bioremediation (Table S7). As the alpha diversity index in the areas polluted with biodiesel were high before the bioremediation treatment (Table S2), we hypothesized that the microbiomes in these soils are highly resilient, i.e. an altered microbial community that shows only transient shifts and bounces back to its original composition. For that reason, changes in the community at month 12 could not be observed (Griffiths et al., 2000; Shade et al., 2012; Van Elsas et al., 2012). As mentioned previously, this area was only monitored along the time (to evaluate natural attenuation) after the biofuel blend (B20) was released (Phase 1). Thus, the microbial community likely adapted to the pollutants and reached an equilibrium state. The

assumption that a higher microbial diversity implies a more resistant and/or resilient community is broadly accepted, because there is a higher probability of positive taxa interactions (Beyter et al., 2016; Briones and Raskin, 2003) and functional redundancy (Louca et al., 2018). Contrarily, the gasohol impacted areas showed lower diversity indexes values at time 0, being communities more susceptible to the disturbances such as a bioremediation treatment.

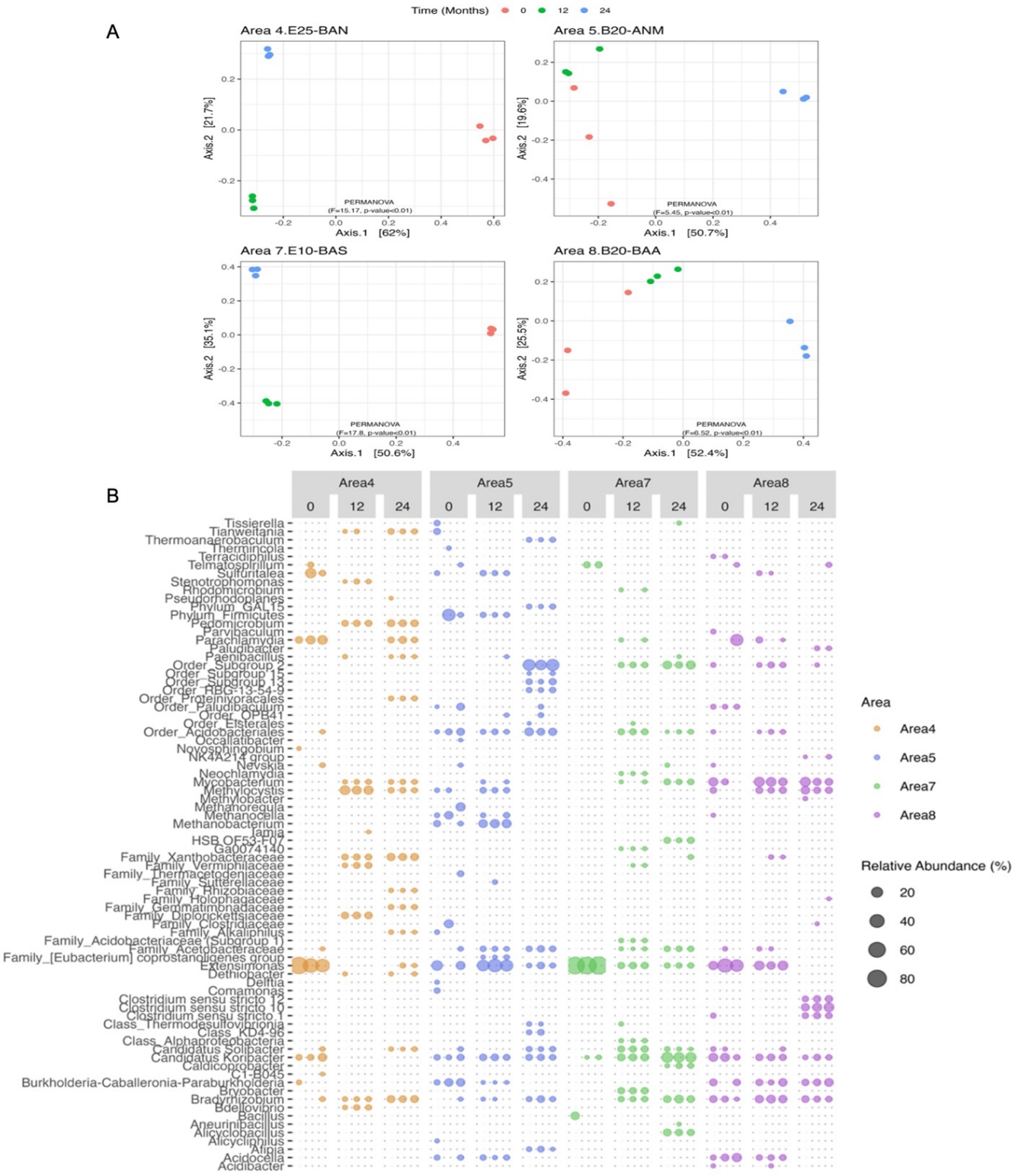


Figure 5. PCoA using Bray-Curtis distance by area and sampling year (A) and community composition of the polluted areas before and after the bioremediation treatments at genus level (B).

Comamonadaceae, represented mainly by members of the genus *Extensimonas*, was the most abundant and important family in both areas 4 and 7 and almost disappeared (Figure 5B) after the biostimulant injection (See sections 2.1.1 and 2.1.2). Probably the biostimulation promoted the microbial metabolisms leading to a depletion of oxygen summed by that the injection of electron acceptors promotes the anaerobic metabolism and *Extensimonas* is strictly aerobic (Zhang et al., 2013). After the bioremediation treatment, the diversity increased, and the abundance of several families related to hydrocarbon degradation processes were enriched. In area 4.E25-BAN, amended with niacin, nitrate and phosphate, the abundance of family Xanthobacteraceae, mainly represented by the genus *Bradyrhizobium*, increased after bioremediation (Figure 5B). This genus has been found in oil wells located at crude oil-contaminated saline soils in the Yellow River Delta Natural Reserve, where it was related with the nitrogen metabolism. In other studies, *Bradyrhizobium* has been extensively characterized for its ability to completely denitrifies nitrate to dinitrogen, as an alternative form to respiration (Gao et al., 2022; Siqueira et al., 2017). Thus, it is reasonable to assume that the injection of nitrate can be related with the increase of this genus abundance. *Methylocystis* also increased in abundance after the bioremediation treatment in area 4.E25-BAN (Figure 5B). Previous studies have shown that members of this genus are stimulated with the addition of ethanol or acetate, thus promoting methanotrophic-mediated hydrocarbon degradation and suggesting that it may be a useful strategy to enhance bioremediation of polluted sites (You et al., 2021). Other works showed the capacity of *Methylocystis* to degrade anthracene (Im and Semrau, 2011). Genome analysis revealed the presence of several genes involved in the nitrogen metabolism in *Methylocystis* sp. strain SC2, such as N_2 fixation, ammonium transport, assimilatory nitrate/nitrite reduction and denitrification (Dam et al., 2012). However, genes *nar* or *nap* that encode nitrate reductase for dissimilatory nitrate reduction were not found (Dam et al., 2013; Dam et al., 2012). These results were corroborated by physiological studies, where the authors observed accumulation of nitrogen in the cells (Dam et al., 2013; Rodríguez et al., 2020), suggesting that nitrate could help the growth of *Methylocystis* but not as electron acceptor coupled to hydrocarbon oxidation. *Parachlamydia* abundance

decreased in area 4.E25-BAN and increased in area 7.E10-BAS. This genus has been found in oil well samples highly contaminated with PAH (Júlio et al., 2019; Magdy et al., 2022). Genus *Pedomicrobium* showed an increase in the relative abundance in area 4.E25-BAN at sampling times 12 and 24. Members belonging to this genus are mainly related to the nitrogen cycle (de la Cueva et al., 2016; Zhang et al., 2021); and they have been previously found in soils polluted with phenanthrene (Yi et al., 2022).

The abundance of *Bryobacter* spp. increased in area 7.E10-BAS at time 12. Previous studies have associated this genus with degradation of PAHs, such as fluoranthene (Bouhajja et al., 2017; Martirani-Von Abercron et al., 2016; Song et al., 2016).

On the other hand, areas contaminated with B20 showed highly similar microbial community composition before (time 0) and one year after (time 12) air injection and bioaugmentation but not following 3 months (time 24) after the reapplication of biostimulants (Figure 5B). As it was discussed above, this finding could be explained by two different facts, first, the microbiota in time 0 had a high level of resilience, second, the oxygen and nutrients depletion was very rapid (Figure S4A).

Contrary to the gasohol areas, the abundance of *Extensimonas* increased in areas 5.B20-ANM and 8.B20-BAA after the bioremediation treatment (Figure 5B), which could be explained by the aerobic stimulation performed in those areas in Phase 2. As discussed above, this genus is comprised of aerobic bacteria that can be related to degradation metabolism (Peng et al., 2020; Wang et al., 2021). In area 5.B20-ANM, despite the bioremediation treatment consisted of air injection and bioaugmentation with aerobic inoculum (obtained by indigenous microbiota enrichment in IALR), methanogenic archaea (i.e. *Methanobacterium*) were detected in high abundance one year after the treatment (Figure 5B). In soils, due to the local consumption of oxygen (Cozzarelli et al., 2010) or fluctuating conditions (Cravo-Laureau et al., 2011), aerobic and anaerobic microniches may be simultaneously found, where aerobic and anaerobic microbial functional communities may coexist (Cébron et al., 2022; Gieg et al., 2014). The presence of methanogens also could be related to the rapid consumption of oxygen by the enriched microbial population and

the absence of alternative electron acceptors, favoring the production of methane (Gieg et al., 2014). Furthermore, one year after the reactor operation starts (when time 12-soil sample was obtained) the availability of oxygen (either within the reactor or directly into the source zone) could be limitans, probably due to high microbial density and also contaminant concentration. Contrarily, in samples from time 24 i.e., just three months after the second source zone intervention, the amount of oxygen delivery was sufficient to microbial population degrade low concentrations of contaminants and, no methanogenic archaea were detected. Such aerobic condition, however, did not imply an increase in *Extensimonas* presumably by the hydrocarbon depletion.

Acidocella (Family Acetobacter) was also found in high abundance in both B20 impacted areas (area 5.B20-ANM and 8.B20-BAA), even after the first aerobic biostimulation. Eze et al. (2021) isolated microorganisms from a long-term petroleum contaminated soil, through successive enrichment cultures with diesel fuel as sole carbon and energy source. The enrichment culture obtained was composed mainly by *Acidocella*, which in the genomic analysis was shown to possess many of the genes for aromatic hydrocarbon degradation (Eze et al., 2021). These results suggested that this genus could potentially serve as inoculum for bioremediation of diesel impacted sites. The methanotroph *Methylocystis* was also found in both B20 areas before and after the bioremediation treatment. As discussed above, *Methylocystis* is related with degradation of anthracene and other PAHs (Dam et al., 2013; Dam et al., 2012; You et al., 2021). In area 8.B20-BAA, the abundance of genera *Burkholderia*, *Caballeronia* and *Paraburkholderia*, or BCP group, stood out in all sampling times (Figure 5B). BCP group has been associated with six from twelve hydrocarbon degradation pathways, including the ones for degradation of nitrotoluene, dioxin, xylene, benzoate, among others (Vera et al., 2022). *Mycobacterium* was enriched in area 8.B20-BAA after bioremediation. Some species from this genus are well known for their ability to degrade a wide range of high-molecular-weight PAHs, such as benzo[a]pyrene, pyrene, fluoranthene and phenanthrene (Hennessee and Li, 2016; Kim et al., 2018; Kim et al., 2015). *Bradyrhizobium* (Xanthobacteraceae) was also enriched in biodiesel/diesel areas

(Figure 5B). As it mentioned before, this genus has been previously found in hydrocarbon impacted areas and it has been related with nitrogen metabolisms (Gao et al., 2022; Siqueira et al., 2017). Additionally, co-occurrence networks showed that this genus strongly co-occurred with hydrocarbon-degrading organisms, such as *Burkholderia* (Yang et al., 2016). The same authors reported that some species from *Bradyrhizobium* genus harbor multiples genes for acyl-homoserine lactone or autoinducer quorum sensing molecules, suggesting that bacterial hydrocarbon degradation could be a quorum sensing-regulated process (Huang et al., 2013; Yang et al., 2016; Yergeau et al., 2014).

3.4. Microbial inoculum composition was distinct from in situ microbiome

Airlift reactors were installed in areas 5.B20-ANM (IALR) and 8.B20-BAA (EALR) in order to stimulate and increase the abundance of native microorganisms from the source zones by using an enrichment culture medium (See composition at section 2.4). Samples from the reactor were taken in the last campaign for microbial community analysis. Microbial families present in the reactor samples and in the source zones, in the respective sampling times, of areas 5.B20-ANM and 8.B20-BAA were compared (Figure S5). Microbial communities of both reactors were similar and a high abundance of anaerobic microorganisms (i.e. Clostridiaceae) was observed. For definition, members of the Clostridiaceae are obligate anaerobes but some species can grow under microaerophilic conditions, such as *Clostridium sensu stricto* (Wiegel et al., 2006). Besides, Clostridiaceae family is well known for being glucose fermenters (Wüst et al., 2011), and the culture medium used for the enrichment of native microorganisms contained molasses, which includes 5 to 10% of glucose (Palmonari et al., 2020). In area 8.B20-BAA, the microbial community present in the soil was similar to the reactor microbiota. Contrarily, the microbial community profile observed in area 5.B20-ANM was different from the one of the reactors. These findings reinforce the hypothesis that the soil microbiome in area 5.B20-ANM can be highly stable, showing resistance or resilience to perturbations (Griffiths et al., 2000; Shade et al., 2012; Van Elsas et al., 2012). In general, the effectiveness of reactor 8 was better than reactor 5 in enriching native microbiota from the source zone in the

areas. Bacterial genera as *Clostridium sensu stricto* and *Methylocystis* were only detected in high abundance after the reactor installation (Figure S5). As it was discussed before, *Methylocystis* has been previously associated with methanotrophic-mediated hydrocarbon degradation (You et al., 2021) and anthracene degradation (Im and Semrau, 2011).

3.5. Microbiome assembly processes are mostly driven by deterministic events

The Canonical Correspondence Analysis, performed to evaluate the influence of environmental variables on microbial community composition and structure, showed that no variables were statistically significantly correlated with microbial data. These results suggested that the microbial community assembly could be driven by stochastic events. However, the Bray-Curtis index based NST demonstrated that stochastic processes played a partial role in driving the microbiome assembly in almost all sampling times and areas (Figure S6). The magnitude of the stochasticity ranged from 20.6% to 44.8%. These results are in line with those obtained by Jiao and collaborators, who analyzed several long-term oil-contaminated fields and showed that deterministic processes exerted a primary influence on the structure of the abundant taxa (Jiao et al., 2017). However, they also demonstrated that the rare subcommunity was primarily influenced by stochastic processes. Here, area 5.B20-ANM at time zero showed a different behavior, with 87% of stochasticity. It is important to highlight that this area had not been intervened until then. Due to the high values of Shannon diversity index and observed species, we hypothesized that a high proportion of rare species (Figure S6-S7) could be one of the reasons associated with the high percentage of stochasticity playing a role in area 5.B20-ANM (Jiao et al., 2017). One of the most common stochastic processes is the dispersal limitation (Jiao et al., 2017). In this area, despite the air injection, limited hydrocarbon degradation was observed in soil and anaerobic methanogenic archaea were found. As mentioned above, a limited air flow distribution may likely have occurred in this area, promoting anaerobic niches in soil and consequent low degradation rates.

3.6. Microbial co-occurrence patterns are sensitive to perturbations

Co-occurrence network analysis was performed to explore the complexity of interactions within the microbial communities in areas polluted with different biofuel/fossil fuels blends (Figure 6). Through this analysis, previously unnoticed microbial co-occurrence patterns can uncover to decipher complex relationships among microbial species (Liu et al., 2021; Zhang et al., 2018). For this, SparCC correlations were calculated between microbial taxa at ASV level based on 16S rRNA amplicons sequencing. The co-occurrence networks of microbial communities from biofuel blend-impacted soils showed substantial differences in topological properties (Table 1). The network of microbial community associated with the biodiesel-impacted areas presented 1656 correlations, with an average degree of 3.457 and an average clustering coefficient of 0.206, while the microbial community network associated with the gasohol impacted areas was less complex, with 680 correlations, an average degree of 2.464, and clustering coefficient of 0.141. In both networks, the number of positive correlations was higher than the negative ones, but it is important to note that the gasohol-associated network presented the highest proportion of negative correlations (18.7%). Negative correlations suggest direct competition for resources among microbial species, or the result of resource partitioning (Fuhrman and Steele, 2008). Previous works showed that substrate complexity can increase positive interactions between microbial species, since cooperation of different metabolisms is needed in order to degrade complex molecules (Deng and Wang, 2016). As gasohol areas were amended with electron acceptors, microbial competition for these molecules is a possibility. Chen et al. (2008) evaluated BTEX degradation in gasohol and electron acceptor-amended microcosms and observed that electron donors obtained from the ethanol mineralization process induced a competition for the electron acceptors added, affecting BTEX degradation (Chen et al., 2008). Low concentration of BTEX and high proportion of less complex molecules could also be related with the high proportion of negative correlations (Deng and Wang, 2016). High average degree in the biodiesel network is related with a higher degree of heterogeneity, as it was observed in the compositional analysis (Figure 3 and Figure 4B). In the same way, a high value in the average clustering coefficient in the network of biodiesel-impacted areas is indicative that the number of inter-community

connections were lower than the number of intra-community connections, suggesting that links between soil microbes in similar niches were stronger than those in different niches (Ji et al., 2022; Ji et al., 2021). Moreover, the average path length was lower in the biodiesel network than in the gasohol network. Community networks with small path lengths are called 'small-world' networks (Watts and Strogatz, 1998) and they are related to the quick responses of an ecosystem to perturbations (Shu et al., 2018).

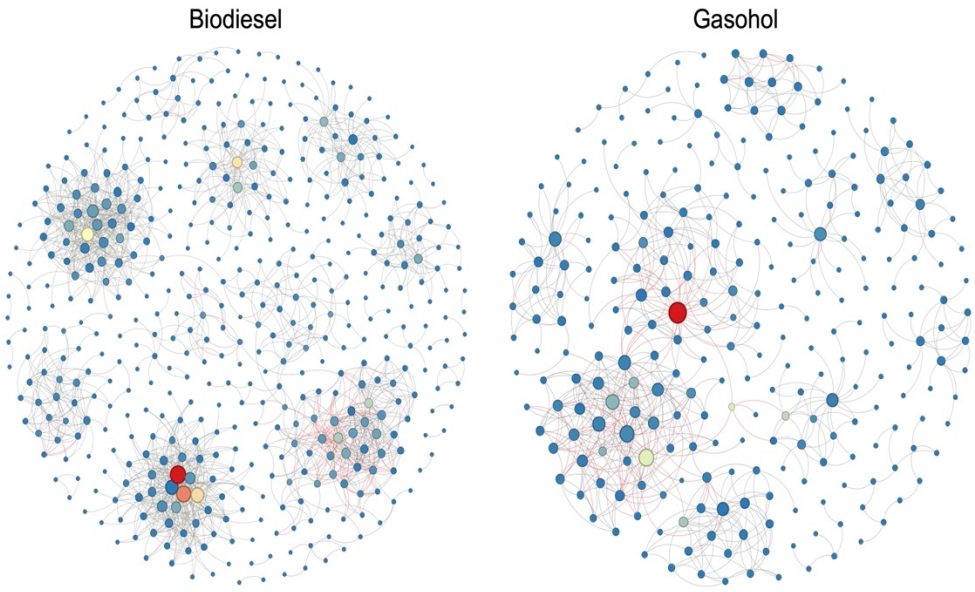


Figure 6. Co-occurrence correlations of soil bacterial and archaeal species in areas polluted with biodiesel and gasohol. Each node represents taxa at ASV level, and the size of node is proportional to the number of connections (Degree). The color of the nodes is based on the betweenness centrality, where darker colors indicated higher values. Black edges represent SparCC correlation >0.8 (positive correlations) and red edges <-0.8 (negative correlation) and statistically significant ($p < 0.01$).

Table 1. Correlations and topological properties of microbial networks from biodiesel and gasohol polluted areas.

Topological property of microbial networks	**Biodiesel-polluted area**	**Gasohol-polluted area**
[a] Number of nodes	479	276
[b] Number of edges	1656	680
[c] Positive edges	1530 (92.4%)	553 (81.3%)
[d] Negative edges	126 (7.6%)	127 (18.7%)
[e] Modularity	0.846	0.807
[f] Number of communities	28	11
[g] Network diameter	5	7

[h] Average path length	1.848	2.658
[i] Average degree	3.457	2.464
[j] Average clustering coefficient	0.206	0.141

[a] ASVs with at least one significant ($p < 0.01$) and correlation SparCC >0.8 or <-0.8
[b] Number of correlations calculated by SparCC analysis
[c] SparCC positive correlation (>0.6 with $p < 0.01$)
[d] SparCC negative correlation (<-0.6 with $p < 0.01$)
[e] Express how well the network is divided into communities/clusters (many edges within, and only a few between them).
[f] Group of nodes (clusters) highly connected
[g] Number of edges to quantify the longest distance between nodes in the network
[h] Average of all edges between all pairs of nodes.
[I] Arithmetic mean of the number of connections per node in the network.
[j] Express how likely the nodes are to form clusters.

Higher complexity and connectivity were observed in the biodiesel-polluted area community, showing that in addition to microbial composition and diversity, community interactions varied according to the biofuel blend. These results reinforced the hypothesis that the highest complexity observed in the microbiome associated with the biodiesel-polluted area is likely related with the presence of different chemical groups in the biodiesel molecular composition being necessary more different microbial species to degrade (Bücker, et al., 2011) . Thus, the network analysis performed suggests that the type of biofuel, along with other environmental factors, drives the community dynamics and co-occurrence of the associated microbiomes.

Co-occurrence network analysis in each area and sampling time was performed, in order to compare the community complexity and to identify the keystone species before and after the bioremediation treatments applied in each area in Phase 2 (Figure 7, Table S8). Statistical significance of the changes in the network topological characteristics (i.e. Betweenness Centrality, Closeness Centrality and Degree) in each area along the sampling times was analyzed using Student T-test (Figure S8). Results did not show a pattern of complexity by fuel or bioremediation treatment applied. However, it was possible to observe that the modularity index in all networks ranged from 0.45 to 0.65, suggesting modular structure in all of them (Newman, 2006). Also, after the application of the bioremediation treatment, negative correlations increased in all areas, which is interpretated as higher competition or less cooperation with contaminants concentration dropping. Previous studies suggest that substrates

with high chemical complexity promote cooperative interactions and reduce the competitiveness (Deng and Wang, 2016; Lindemann, 2020). We hypothesize that the high proportion of positive correlations before the bioremediation treatment could be due to the high concentration of BTEX in some areas (Figure 1A), when microorganisms cooperated more intensively to degrade the pollutants. In the same way, after the bioremediation treatment, the increase of the antagonistic correlations might be related with more competition interactions for the lower concentration of BTEX and higher intermediate product concentration (Gieg et al., 2014).

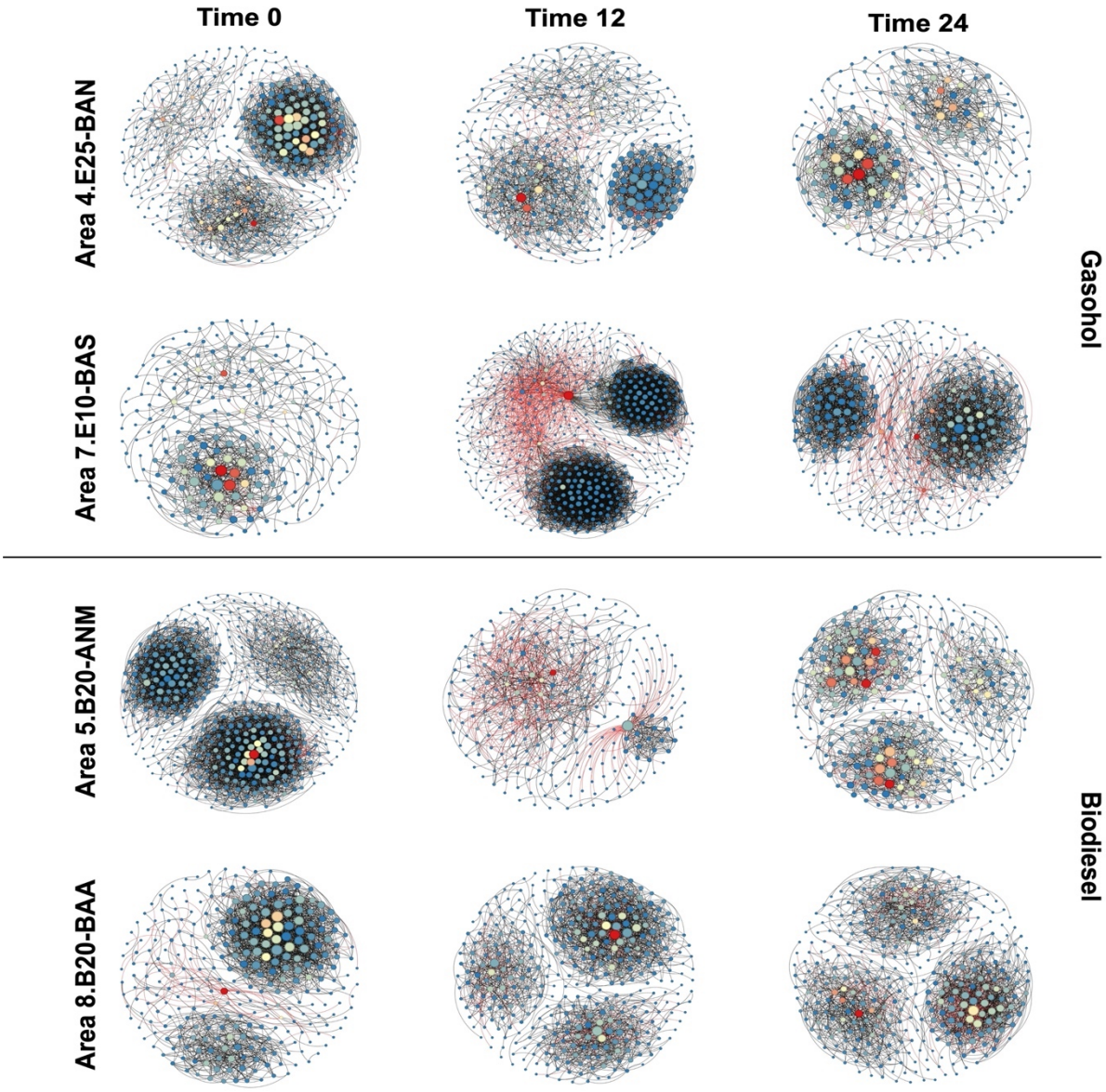


Figure 7. Co-occurrence correlations of soil bacterial and archaeal species from each polluted area. Each node represents taxa at ASV level, and the size of node is proportional to the number of connections (Degree). The color of the nodes is based on the betweenness centrality, where darker colors indicate higher values. Black edges represent Sparcc correlation >0.8 (positive correlations) and red edges <-0.8 (negative correlations) and statistically significant ($p < 0.01$). Time 0 (immediately before the first intervention in the source zone), time 12 (12 months after the first intervention in the source zone) and time 24 (three months after the second intervention in the source zone)

Compositional and diversity results altogether unraveled a resilient microbiome in 5.B20-ANM area. This site had not been submitted to a bioremediation treatment for twelve years. Co-occurrence network analysis of microbial community sampled at time 0 showed a highly connected and complex community (Figure 7). However, after the bioremediation treatment, that consisted in air injection and bioaugmentation of the indigenous microbiota in an IALR and coupled to groundwater recirculation throughout the source zone, the microbial community showed a big shift, and a simpler network was revealed. Betweenness centrality and degree were significantly lower after the treatment (Figure S8), suggesting a lesser connected network. These results were confirmed by the number of nodes and edges, which were reduced from 510 nodes and 4437 edges (edge/node ratio = 8.7) to 276 nodes and 807 edges (edge/node ratio = 2.9), almost three-times lower, demonstrating that the bioremediation treatment had a huge impact in the network complexity. Some theories claim that in case of perturbation, microbial interactions are the first community property to be affected, thus altering the functions of the ecosystem even before the species disappear (Valiente-Banuet et al., 2014). The alteration of the network structure could lead to a shift in the ecosystem functionality and to a reduction in their stability (resilience/resistant) in the long term (Tylianakis et al., 2010; Vacher et al., 2016). In highly connected networks, the loss of one species after a perturbation could more easily change the structure (Dunne et al., 2002; Sole and Montoya, 2001). On the other hand, in 5.B20-ANM area the high proportion of low and intermediate abundant microbial species could be related with the big shift in the network complexity before and after the source zone intervention (Figure S7). As it was discussed before, rare subcommunity could be more affected by stochastic processes (Jiao et al., 2017). Additionally, abundant and rare taxa assemblies are driven by different factors (Jiao et al., 2017).

Regarding pollutant concentrations (Figure 1), in 5.B20-ANM area some pollutants oscillated after the bioremediation treatment. Dong et al. (2021) analyzed the assembly mechanisms of soil community under increasing pyrene stress and observed that with the increase of pyrene concentration, the community network tended to be simplified. Opposite results were observed in this work since an increase in the network complexity was observed after the second intervention in the source zone (month 21) (Figure 7 and Table S8) probably due to nutrient amendments. The betweenness centrality and degree were significantly higher (time 12 vs time 24), suggesting a network more cohesive and connected, with more highly connected nodes located in the core of the network. The rare subcommunity decreased, suggesting that the treatment allowed to enrich some taxa, as it was observed in the compositional analysis (Figure 5).

Contrastingly, area 7.E10-BAS showed a simpler microbial network before the bioremediation treatment when compared to the network observed one year after the treatment. An increase was observed in the average degree, as well as a 10-fold increase in the number of correlations and 2-fold increase in the number of nodes. Also, a decrease in BTEX concentrations in soil was detected (Figure 1). These results were also congruent with the increase of microbial richness and diversity (Table S2 and Table S7), suggesting that injection of electron acceptors and nutrients in this area changed the microbial community composition, structure and interactions and was effective in the removal of >93% of the BTEX concentrations in soil (Figure 1A). The average clustering coefficient is indicative of the network complexity and strong interactions among the microbial taxa. In area 7.E10-BAS this topological property highly increased after the two electron acceptor injections (Table S8). De Vries et al. (2018) showed that higher clustering coefficient may be related with more dynamic and active community (de Vries et al., 2018).

In gasohol areas, the average clustering coefficient increased, and the average path length decreased after bioremediation treatments, indicating more compact networks and strong microbial interactions (Guo et al., 2022). A co-occurrence network analysis performed in an anaerobic reactor, showed that higher clustering

coefficients and lower average path lengths were related with high hydrolysis rate (Guo et al., 2022). Communities harboring a higher number of connections could yield a higher rate of hydrocarbon degradation (de Vries et al., 2018).

3.7. Keystones abundant species are potential hydrocarbon degraders

Keystone species have an important role in maintaining community structure and have a greater impact on the microbiome not only based on their relative abundance or total biomass (Banerjee et al., 2018; Banerjee et al., 2021; Ma et al., 2016). Besides the general co-occurrence network analysis, the topological parameters of the individual ASVs are indicative of their ecological roles by using the within-module connectivity (*Zi*-score) and among-module connectivity (*Pi*-score). Peripheral nodes (low *Zi*-score and *Pi*-score) are taxa that have a few links to other taxa within their modules (Guimera and Nunes Amaral, 2005). The peripheral nodes are also called as specialists that interact less with other taxa (Guimera and Nunes Amaral, 2005). In gasohol-polluted areas (4.E25-BAN and 7.E10-BAS), the proportion of peripheral or specialist nodes increased at time 12 (twelve months after the first intervention in the source zone) (Figure 8A and Figure S9). Connector nodes (low *Zi*-score and high *Pi*-score) are the nodes that have links with several modules (i.e., inter-module communication). With some exceptions, the connector node proportion increased at time 12 (one year after the first intervention in the source zone) and/or at time 24 (three months after the second intervention in the source zone), suggesting stronger inter-module communication or more connections between modules (Qian et al., 2020). Comparison between contaminated and uncontaminated permafrost showed that specialist taxa are more abundant in contaminated samples, while generalists are more abundant in uncontaminated samples (Yang et al., 2016). After the bioremediation treatment, in most cases, a decrease in the BTEX contamination was observed (Figure 1). We hypothesized that the bioremediation treatment and the reduction of the pollutants contributed to increase the generalist community. Biostimulation performed in the different areas likely favored the co-metabolism and/or syntrophic relationships within the community. In previous studies, it was observed that the addition of biostimulants enhanced the bioremediation via co-

metabolism (Couto et al., 2010; Feng et al., 2021). The module hubs (high *Zi*-score and low *Pi*-score) represent the ASVs with high number of links in their own modules. Only seven nodes were classified as module hubs, which were affiliated to *Mycobacterium, Acidibacter, Derxia*, Order Subgroup2 (Class Acidobacteriae), and families Oxalobacteraceae, Pedosphaeraceae and Comamonadaceae (Figure 8A). Finally, network hubs (high *Zi*-score and *Pi*-score) are the ASVs with links among modules. No network hubs were identified. Connectors, and module and network hubs represent generalists in the community (Guimera and Nunes Amaral, 2005). Generalists mean taxa that are highly connected with others among modules (network hubs), within modules (module hubs), and among different modules within a network (connectors) (Pan et al., 2021).

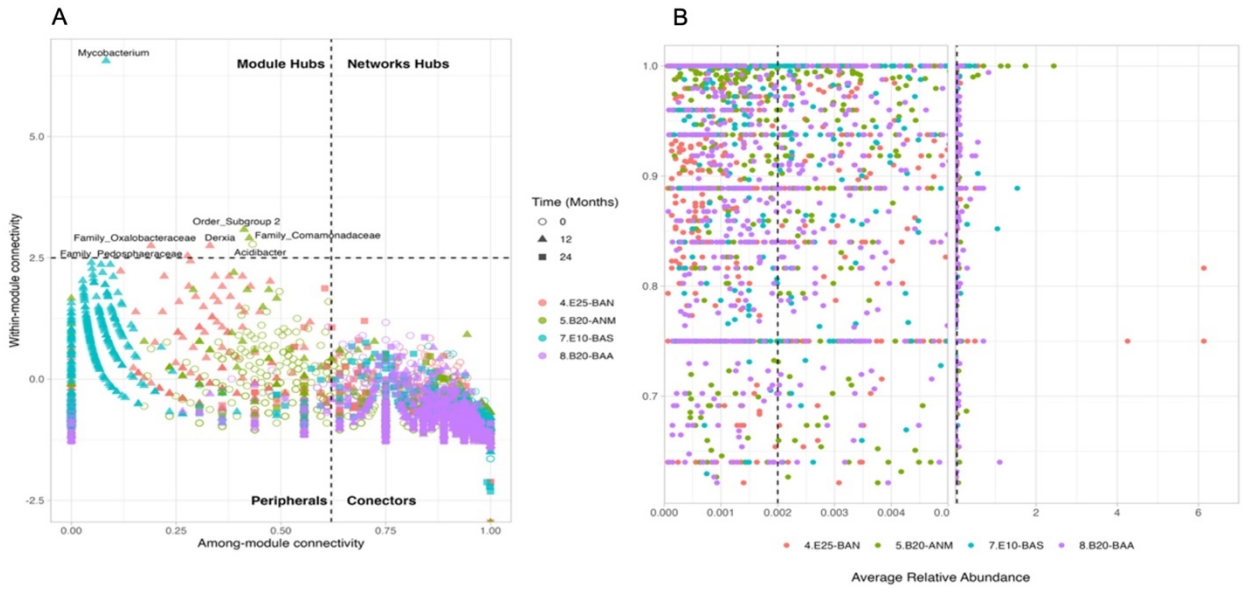


Figure 8. (A) Identification of keystone taxa based on Zi-Pi plot showing the distributions of ASVs based on their topological roles in the different areas and time samplings. Module hubs are identified as Zi-score ≥ 2.5 and Pi-score < 0.62; connectors are identified as Zi-score < 2.5 and Pi-score ≥ 0.62 (B) Average relative abundance of connectors. Dotted lines represent the limits between rare/intermediate (0.01%) and intermediate/abundant taxa (0.05%).

Six out of seven module hubs appeared after the first intervention in the source zone (time 12) in three out of four areas (4.E25-BAN, 7.E10-BAS and 5.B20-ANM), reinforcing the hypothesis that the bioremediation treatments improved microbial interactions. From the module hubs detected, only *Mycobacterium* belonged to the abundant subcommunity. As discussed above, *Mycobacterium* has been related with

degradation of a wide range of high-molecular-weight PAHs (Hennessee and Li, 2016; Kim et al., 2018; Kim et al., 2015). Jiao et al. (2017) investigated the conditionally rare taxa (CRT), species that are usually rare within a community but under some conditions become abundant, in several soils under long-term oil contamination (Jiao et al., 2017). The authors observed that Mycobacterium was a predominant CRT from Actinobacteriota phylum. Additionally, only 3.6% of generalist/keystone nodes were abundant species (average of relative abundance > 0.05%), suggesting that rare taxa can also play key roles in maintaining microbial networks (Figure 8B) (Jiao et al., 2017; Pan et al., 2021; Qian et al., 2020). These results are congruent with other studies (Dong et al., 2021; Jia et al., 2018; Jiao et al., 2017; Pan et al., 2021; Yang et al., 2016). Topological features of the rare and abundant sub-community networks have been compared previously. Results showed that in most cases the rare sub-community had low betweenness centrality, high closeness centrality and high degree values indicating that the rare taxa had a peripheral distribution (Barberán et al., 2012; Dong et al., 2021; Jiao et al., 2017; Yang et al., 2016). In contrast, our results showed that betweenness and degree values were not significantly different between rare and abundant nodes, while the closeness centrality values in abundant nodes were significantly higher than in rare nodes (Wilcoxon test, p-value < 0.05, Figure S10). These findings suggested higher intra-taxon association in the abundant sub-community and that these taxa are more often located in the network core.

Module hub *Acidibacter* was present in 5.B20-ANM area (Figure 8A) before the bioremediation treatment. This iron-reducing bacterium has been reported in petroleum-contaminated soils from the Daqing Oilfield as able to grow in total petroleum hydrocarbon concentrations ranging from 0.0250 to 0.4043 g/g (Feng et al., 2020). Members of this genus have also been found in phenanthrene-polluted coastal wetlands (Chi et al., 2021). and as dominant bacteria during rhizoremediation of diesel contaminated soil (Seo and Cho, 2021). Interestingly, in a microcosm-based study using compost to bioremediate diesel-contaminated soil, *Acidibacter*, among other bacteria, was negatively correlated with the residual diesel concentration, and positively correlated with the concentration of methane, suggesting that this microorganism contribute with diesel degradation and/or methane oxidation during

the bioremediation of the diesel-contaminated soil mesocosms (Yang et al., 2022). *Derxia* is a nitrogen-fixing bacterium, member of the *Alcaligenaceae* family, and has been reported as able to degrade hydrocarbons (John et al., 2011). Unclassified Acidobacteria members were shown to play hub and connector roles (Figure 8 and Figure S11) as rare and abundant taxa in all areas. Previous analyses of bacterial community structure and function in contaminated and uncontaminated soils have shown the phylum Acidobacterota as highly prevalent across hydrocarbon contaminated samples, with some taxa being suggested as indicator of soil quality (Gałązka et al., 2018; Li, 2017; Shahi et al., 2016). An unclassified Oxalobacteraceae member was found as module hub in 4.E25-BAN area after the bioremediation treatment (injection of electron acceptors such as nitrate). Members of this family have been identified as a key n-alkane degrader in crude-oil-contaminated sites in Nigerian soils (Wang et al., 2016). Several genera from this family are known for their ability to degrade aromatic hydrocarbons aerobically (Lee and Lee, 2001) and anaerobically (Kim et al., 2014) as well as for the capability to reduce nitrate under anaerobic conditions (Kim et al., 2014; Muller et al., 2006). Finally, a member of the Comamonadaceae family was also identified as module hub in 5.B20-ANM area. Nitrate biostimulation of a refinery sludge yielded an increase of the abundance of Comamonadaceae members among other bacteria. This consortium was enriched under aerobic and anaerobic conditions, showing ability to degrade alkanes, aromatic compounds and crude oil (Sarkar et al., 2020). Another study showed that Comamonadaceae members were amongst the first organisms that responded to different types of oil addition in freshwater microcosms (Butler, 2018).

Abundant connectors of microbial networks were also assessed (Figure S11). *Sulfuritalea*, *Candidatus Koribacter*, BCP group, members of Order Subgroup2 (Class Acidobacteriae) and *Bradyrhizobium* were keystone taxa in all areas. *Sulfuritalea* is a sulfur-oxidizing bacterium able to grow hetetrophically under aerobic conditions by using organic acids and aromatic compounds (Chen et al., 2019; Kojima and Fukui, 2011). This genus has been reported as the main responsible for benzene degradation in a full-scale petroleum refinery wastewater treatment plant (Kim et al., 2020). Co-occurrence of members of Rhizobiales order (i.e., *Afipia*, Family

Xanthobacteriaceae, *Methylocystis*, *Nordella*), including *Bradyrhizobium*, with other hydrocarbon-degrading bacteria, mainly *Burkholderia, Arthrobater* and *Rhodococcus* has been reported elsewhere (Yang et al., 2016). These genera were found as connectors in this study.

Nonetheless, the most abundant connector taxa were *Extensimonas, Methylocystis, Candidatus Koribacter* and *Bradyrhizobium* (Figure S11). As it was discussed above, *Methylocystis* is a methanotroph that can be stimulated with the addition of ethanol or acetate, to promote methanotrophic-mediated hydrocarbon degradation (You et al., 2021). A study of aerobic methanotrophs in an urban water cycle system showed that some methanotrophs could act as “primary producers” in methane-driven food webs (Lu et al., 2021).

Interestingly, *Clostridium sensu stricto* and methanogenic archaea, such as *Methanoregula, Methanocella* and *Methanobacterium*, were observed as keystone taxa, mainly in the biodiesel areas (5.B20-ANM and 8.B20-BAA). These findings suggested the presence of a syntrophic microbial network based on anaerobic degradation. Usually, Clostridium acts as primary fermentative bacteria able to degrade hydrocarbons with production of short chain fatty acids, alcohols together with CO_2 and H_2. A second group of fermentative bacteria can also participate (e.g., *Geobacter*). The syntrophic association can also include hydrogen- or acetate-consuming methanogenic archaea to produce methane as final step (McInerney et al., 2009; Morris et al., 2013).

Our results suggested that these abundant keystone taxa can be the core targets for biostimulation in future soil bioremediation treatments. The rare microbial biosphere, on the other hand, could increase functional redundancy and enhance the resilience or resistance for soil microbiomes impacted by biofuels/fossil fuels.

Conclusions

Altogether, results gathered herein broadened our understanding of microbial community composition, diversity, assembly processes and co-occurrence patterns in

soils impacted with distinct blends of biofuels/fossil fuels and submitted to different bioremediation treatments along a temporal gradient. The blend type and proportion were the main drivers of the soil microbiome composition and diversity. Biodiesel blend yielded more diverse and complex microbial communities and with high level of stochasticity when compared to gasohol contaminated areas. Deterministic processes were responsible for driving the assemblies after bioremediation treatments. Significant shifts in the microbiome structure were observed mainly in the gasohol impacted areas after bioremediation, with increased abundance of hydrocarbon degraders identified as keystone species in the network analysis. Microbial co-occurrence patterns were more sensitive to fuel blends and/or bioremediation treatments than microbial composition, shedding light about the pivotal role of microbial interactions in response to environmental stressors.

Acknowledges

The authors are grateful to Petrobras for their financial and technical support and authorization to publish this work. K.J.H. thanks the Colombian Department of Science - COLCIENCIAS for providing a doctoral scholarship (860/2020).

Funding

This work was supported by PETROBRAS.

Supplementary information

Table S1. Permutational multivariate analysis of variance (Adonis) test to evaluate the influence of the fuel blend on the microbial community composition based on the Bray-Curtis distance.

	df	SumOfSqs	R^2	F	*p*-value
Fuel Blend	1	1.0275	0.27222	3.7404	**0.002997****
Residual	10	2.7471	0.72778		
Total	11	3.7746	1.00		

* Values in bold denote statistical significance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

Table S2. Alpha diversity indexes.

Area	Time	Observed Species[a]	Chao1[b]	Shannon Index[c]	Simpson Index[d]
4.E25-BAN	0	277±51.3	333±74.7	2.59±0.775	0.714±0.161
	12	367±70.1	386±86.7	4.94±0.131	0.980±0.0014
	24	290±40.7	299±40.8	4.99±0.0634	0.989±0.00036
5.B20-ANM	0	347±49.9	356±54.5	4.22±0.277	0.957±0.00249
	12	284±90	290±93	4.14±0.600	0.94±0.04585
	24	329±13.8	331±15	4.8±0.132	0.979±0.00466
7.E10-BAN	0	171±31.5	171±31.5	1.49±0.229	0.551±0.134
	12	520±142	528±149	5.47±0.188	0.992±0.00088
	24	294±89.7	299±89.1	4.54±0.173	0.961±0.00283
8.B20-BAA	0	251±64.3	268±56.7	3.30±0.760	0.876±0.0786
	12	342±5.86	352±15.1	4.44±0.188	0.965±0.00811
	24	345±22.2	349±23.6	4.73±0.141	0.979±0.00406

[a] Number of ASVs observed (Richness)

[b] Species richness estimator

[c] >0; higher more diverse

[d] 0-1; 0 = most simple

Table S3. Shapiro-Wilk test for normal distribution of alpha diversity index

Diversity Index	Statistics	Df	Sig.
Shannon	0.92513	12	0.3992
Simpson	0.8714	12	0.753

Table S4. T-test analysis of the effect of the biofuel in the alpha diversity indexes.

Alpha Diversity Index	Statistic	df	*p*-value
Shannon	5.6392	5	**0.0024****
Simpson	5.5855	5	**0.0025****

* Values in bold denote statistical significance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

Table S5. Permutational multivariate analysis of variance (Adonis) test to evaluate the influence of the bioremediation treatment on the microbial community composition based on the Bray-Curtis distance

	4.E10-BAN					5.B20-ANM					7.E10-BAS					8.B20-BAA				
	df	SumOf Sqs	R^2	F	*p*-value	df	SumOf Sqs	R^2	F	*p*-value	df	SumOf Sqs	R^2	F	*p*-value	df	SumOf Sqs	R^2	F	*p*-value
Time	2	1.99	0.83	15.17	**0.004****	2	1.43	0.64	5.45	**0.002****	2	2.18	0.86	17.80	**0.004****	2	1.06	0.68	6.52	**0.003****
Residual	6	0.39	0.17			6	0.79	0.35			6	0.37	0.14			6	0.49	0.32		
Total	8	2.38	1.00			8	2.22	1.00			8	2.55	1.00			8	1.54	1.00		

* Values in bold denote statistical significance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

Table S6. Shapiro-Wilk test for normal distribution of alpha diversity index

	Shapiro-Wilk normality test		Levene's homogeneity of Variance test	
Areas	**Statistics**	**Sig.**	**Statistics**	**Sig.**
4.E25-BAN	0.83635	0.05259	2.8844	0.1325
5.B20-ANM	0.93575	0.5379	0.6162	0.571
7.E10-BAN	0.91381	0.3435	0.0491	0.9525
8.B20-BAA	0.9005	0.2549	0.9154	0.4498

Table S7. ANOVA and Tukey post-hoc test of the effects of bioremediation treatments on Shannon index

Areas	Anova			Tukey Post-Hoc Test	
	df	*F*	*p*-values	*p*-values	
				0 vs 12	12 vs 24
4.E25-BAN	2	27.201	**0.00098*** **	**0.00169** **	0.99237
5.B20-ANM	2	5.798	**0.0396***	0.3431	**0.0334***
7.E10-BAN	2	330.7	**7.27e-07*** **	**<0.001 *** **	**0.00323** **
8.B20-BAA	2	8.052	**0.02***	0.0530	0.7295

* Values in bold denote statistical significance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

Table S8. Correlations and topological properties of microbial networks from the biodiesel and gasohol polluted areas before and after the bioremediation treatment applied.

Topological property	4.E25-BAN			7.E10-BAS			5.B20-ANM			8.B20-BAA		
	0	**12**	**24**	**0**	**12**	**24**	**0**	**12**	**24**	**0**	**12**	**24**
# Nodes	414	367	236	235	535	335	510	276	313	293	373	362
# Edges	2641	1750	962	678	6082	2395	4437	807	1349	1739	2014	1971
Positive edges	2576 (97.5%)	1640 (93.7%)	933 (96.7%)	664 (97.9%)	5499 (90.4%)	2230 (93.1%)	4401 (99.2%)	521 (64.5%)	1253 (92.9%)	1660 (95.4%)	1873 (92.9%)	1759 (89.2%)
Negative edges	65 (2.5%)	110 (6.3%)	29 (3.3%)	14 (2.1%)	633 (9.6%)	165 (6.9%)	36 (0.8%)	286 (35.5%)	96 (7.1%)	79 (4.6%)	141 (7.1%)	212 (10.8%)
Modularity	0.496	0.604	0.558	0.507	0.572	0.538	0.587	0.532	0.642	0.445	0.618	0.643
Number of communities	3	4	6	6	6	6	4	5	4	3	3	4
Network diameter	7	8	6	6	9	7	8	8	7	8	7	7
Average path length	2.229	2.516	2.076	2.099	2.920	2.385	2.253	2.683	2.298	2.180	2.339	2.241
Average degree	6.379	4.768	4.076	2.885	11.368	7.149	8.7	2.924	4.31	5.935	5.299	5.445
Average clustering coefficient	0.078	0.082	0.089	0.058	0.166	0.116	0.097	0.075	0.083	0.093	0.084	0.094
Edge/node ratio	6.37	4.76	4.07	2.9	11.37	7.14	8.7	2.9	4.3	5.9	5.4	5.4

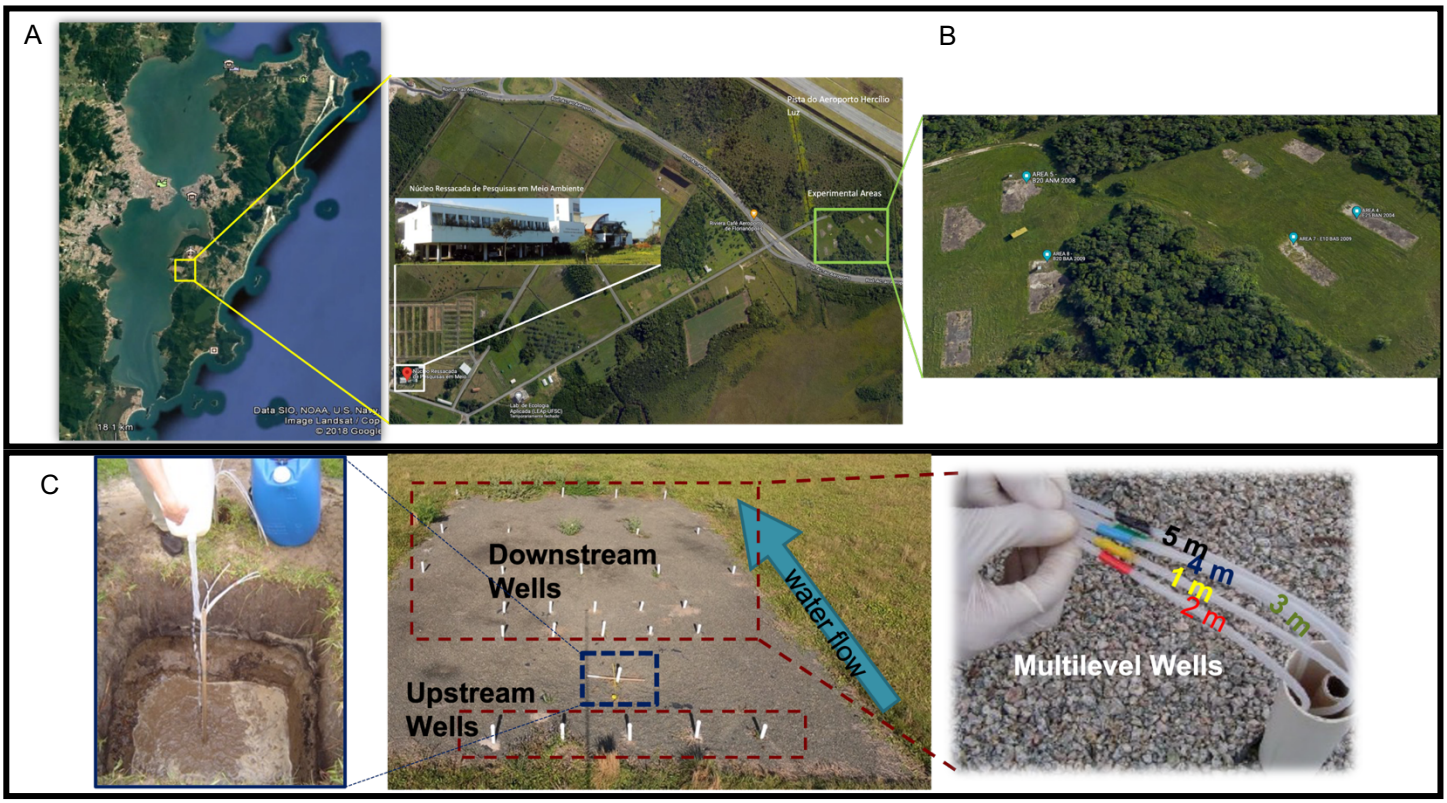


Figure S1. (A) Satellite view of the Florianopólis Island and the Ressacada Experimental Farm. (B) Satellite view of the experimental areas configuration. (C) From the left to the right: Fuels release, spatial monitoring wells distribution along water flow direction and identification of monitoring levels. Square dashed in blue indicates the source well

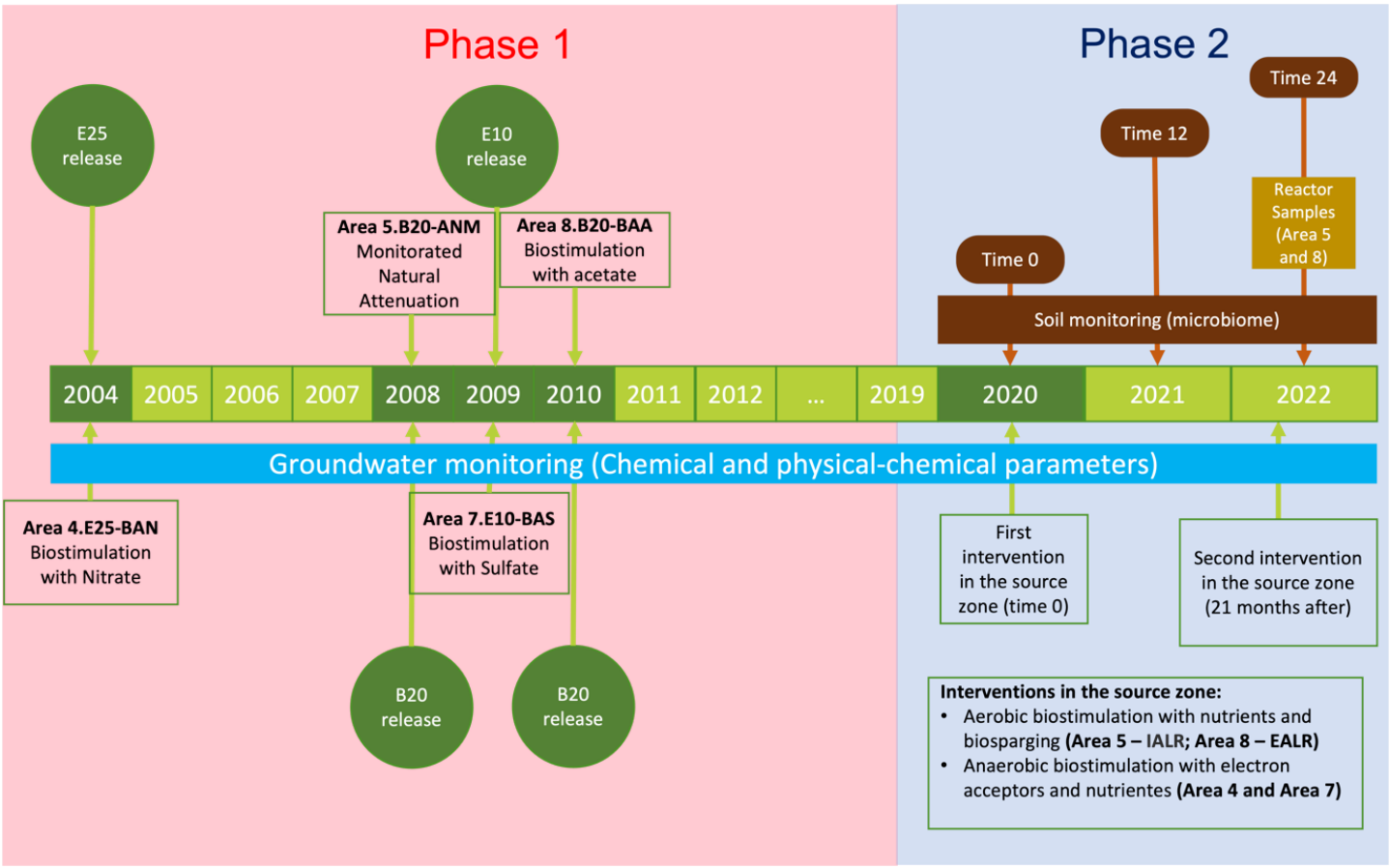


Figure S2. Timeline of the fuel releases and bioremediation treatments and details of the experimental areas.

Figure S3. Example of source zone monitoring well and soil sampling points.

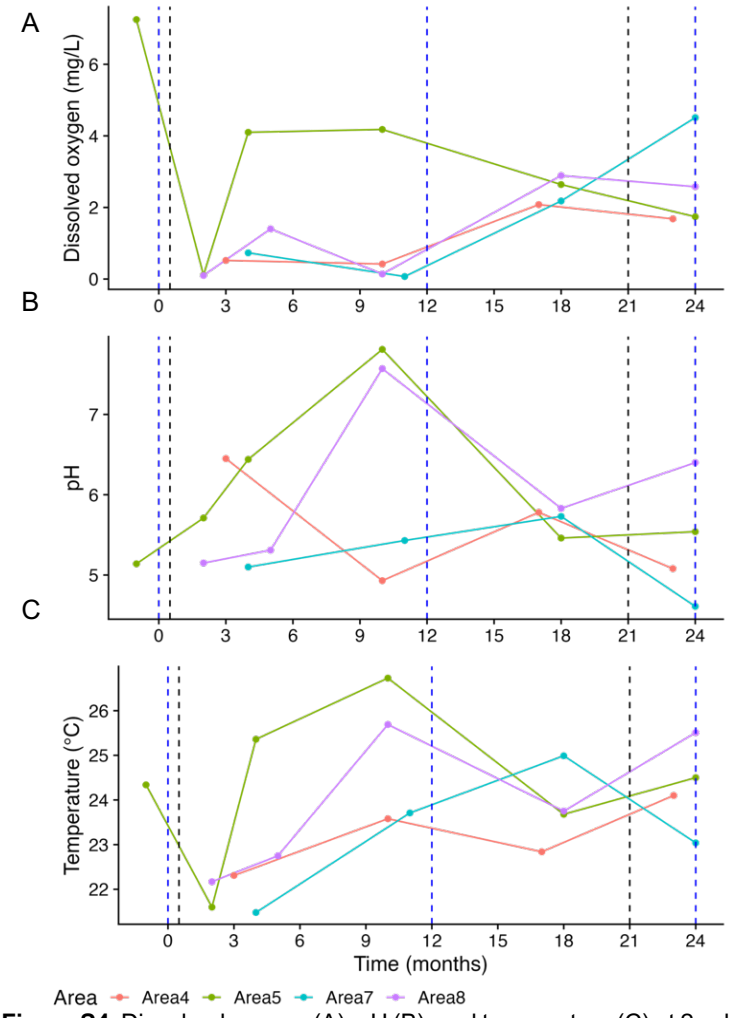


Figure S4. Dissolved oxygen (A); pH (B); and temperature (C) at 2 m below ground surface. Blue dashed lines represent soil microbiome sampling. Black dashed line represents bioremediation application.

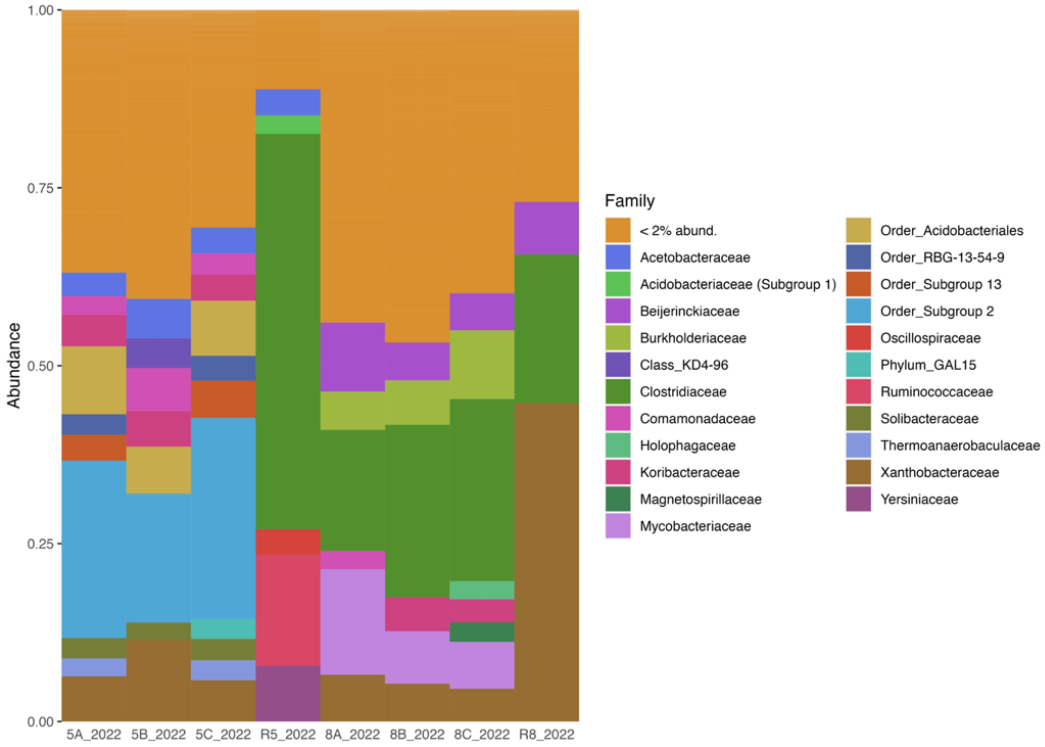


Figure S5. Relative abundances of microbial families in areas 5 and 8 and in the respective reactors, based on 16S rRNA gene sequencing. Families with <2% de abundance were grouped in one category (<2% abund). Samples named as R5 and R8 are from reactors in areas 5 and 8, respectively.

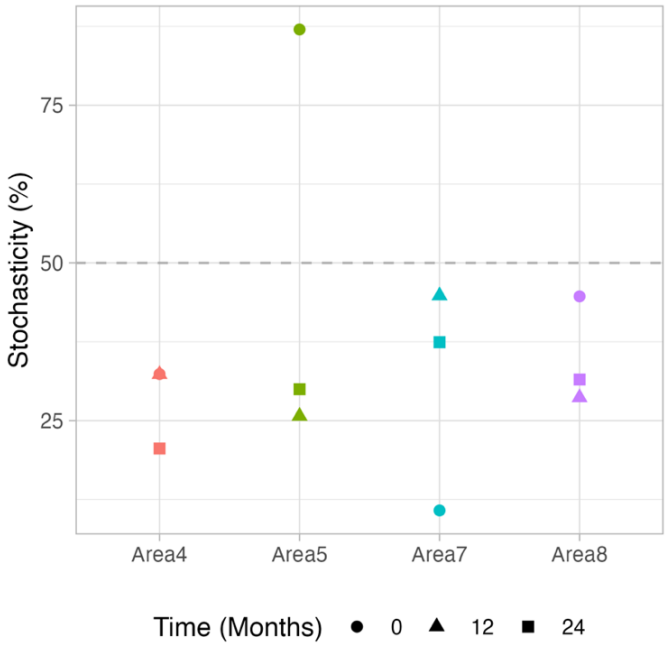


Figure S6. The magnitude of stochasticity quantified as the normalized stochasticity ratio in the different impacted areas and sampling times. The values were calculated based on the Jaccard dissimilarity.

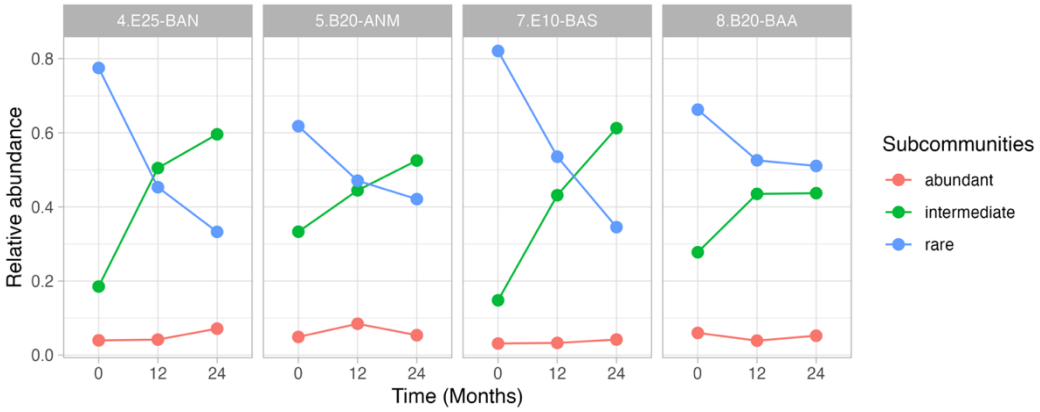


Figure S7. Relative abundance of rare (≤ 0.01%), intermediate (>0.01% & < 0.5%) and abundant (≥ 0.5%) sub-communities in each area over the time.

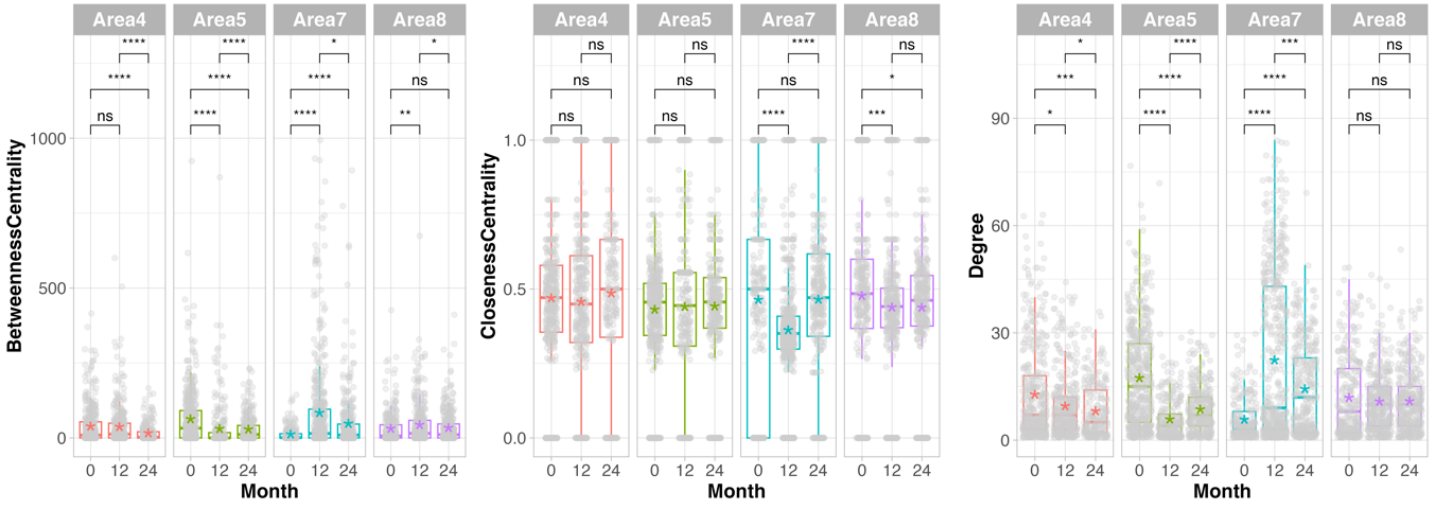


Figure S8. Unique node topological features, specifically, betweenness and closeness centrality and degree. Means comparison by Student t-test (*p*-value < 0.05)

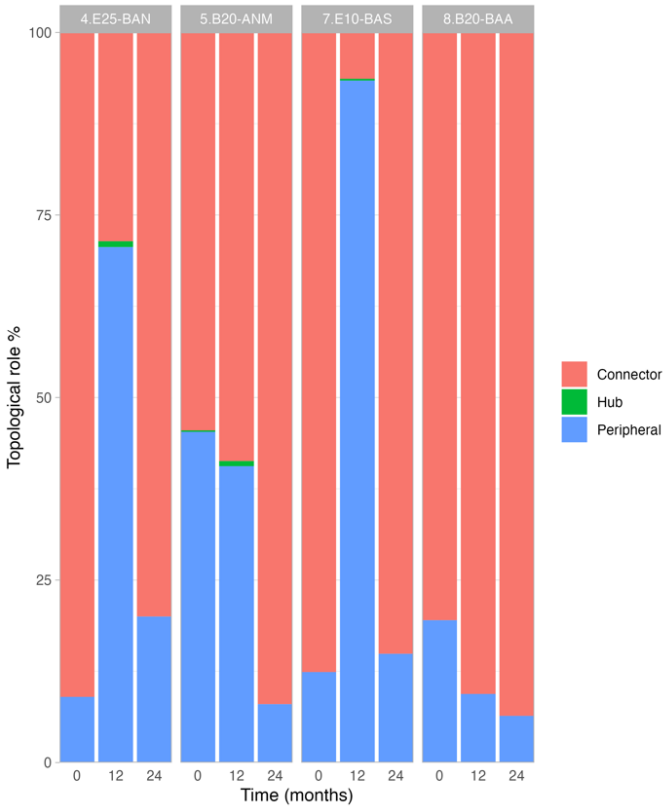


Figure S9. Percentage of nodes according with the ecological roles, connector, hub or peripherals in all the areas over the time

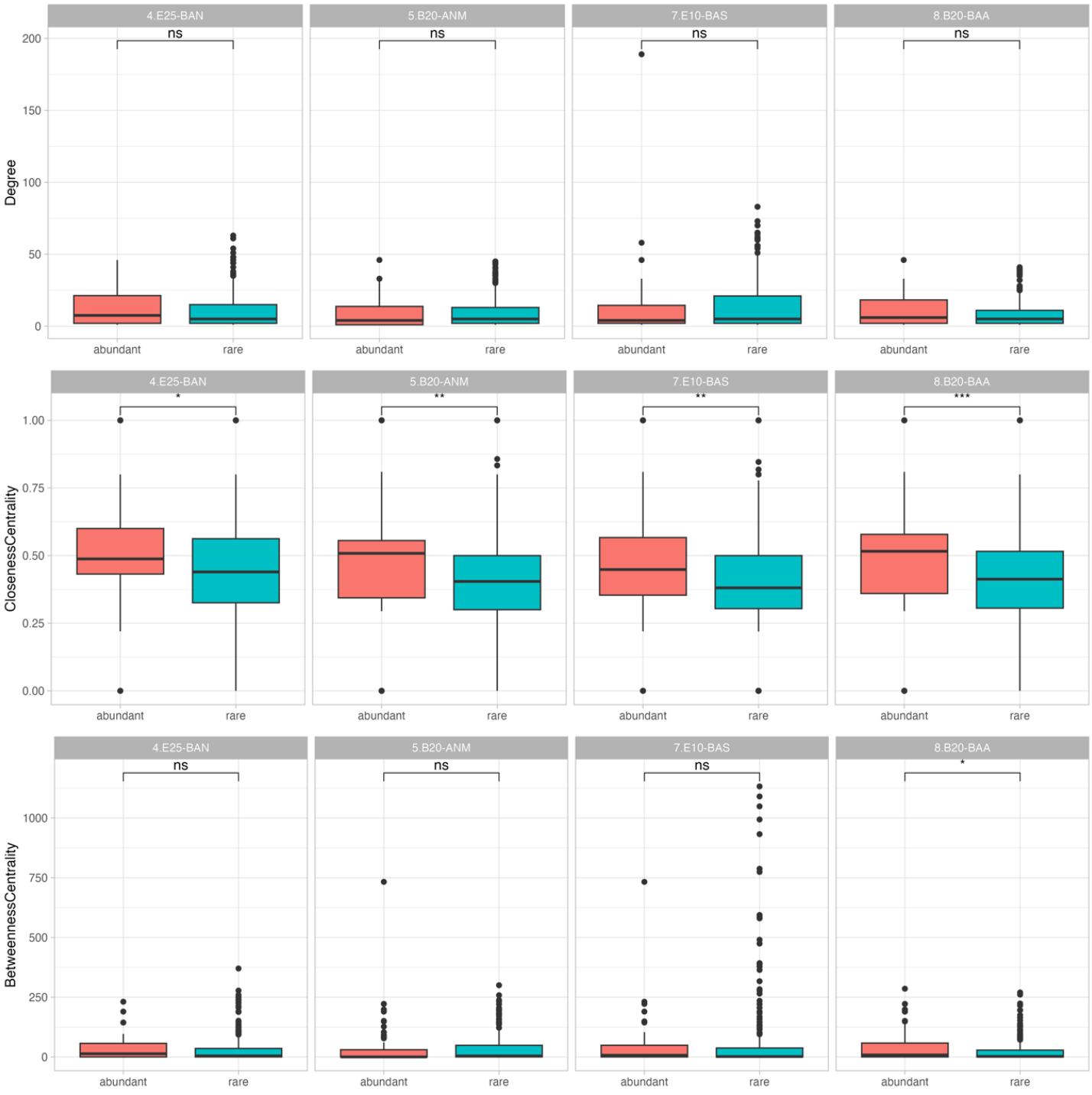


Figure S10. Unique node topological features of rare and abundant taxa in all the areas over the time, specifically the degree, betweenness and closeness centrality. Means comparison by Wilcoxon rank sum test (*p*-value < 0.05)

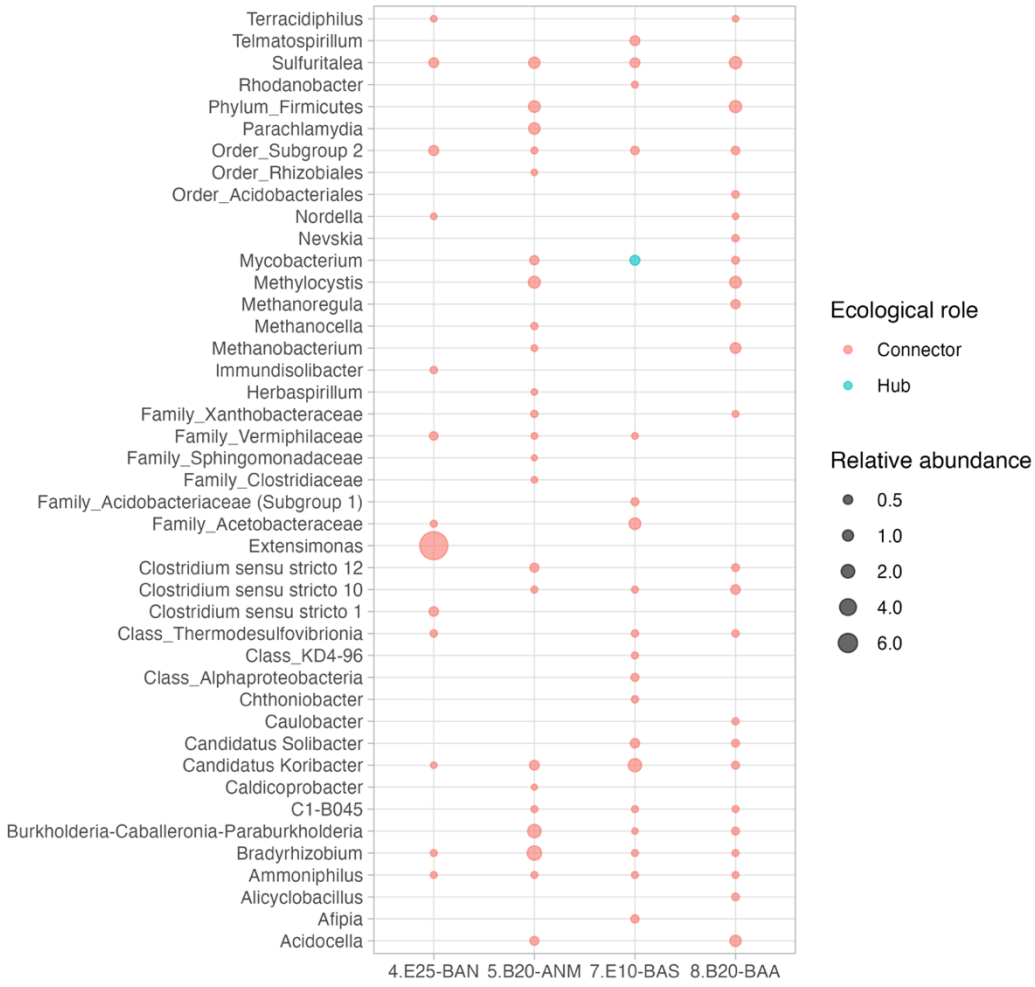


Figure S11. Relative abundance and taxonomic classification at genus level of taxa categorized as connectors and module hubs keystone.

References

(USEPA). USEPA. METHOD 8015C NONHALOGENATED ORGANICS USING GC/FID. 2000.

Al-Hawash AB, Dragh MA, Li S, Alhujaily A, Abbood HA, Zhang X, et al. Principles of microbial degradation of petroleum hydrocarbons in the environment. The Egyptian Journal of Aquatic Research 2018; 44: 71-76.

Alvarez PJ, Hunt CS. The effect of fuel alcohol on monoaromatic hydrocarbon biodegradation and natural attenuation. Revista Latinoamericana de Microbiologia 2002; 44: 83.

Anderson MJ. Permutational multivariate analysis of variance (PERMANOVA). Wiley statsref: statistics reference online 2014: 1-15.

Banerjee S, Schlaeppi K, van der Heijden MG. Keystone taxa as drivers of microbiome structure and functioning. Nature Reviews Microbiology 2018; 16: 567-576.

Banerjee S, Zhao C, Kirkby CA, Coggins S, Zhao S, Bissett A, et al. Microbial interkingdom associations across soil depths reveal network connectivity and keystone taxa linked to soil fine-fraction carbon content. Agriculture, Ecosystems & Environment 2021; 320: 107559.

Baniasadi M, Mousavi SM. A Comprehensive Review on the Bioremediation of Oil Spills. In: Kumar V, Kumar M, Prasad R, editors. Microbial Action on Hydrocarbons. Springer Singapore, Singapore, 2018, pp. 223-254.

Barberán A, Bates ST, Casamayor EO, Fierer N. Using network analysis to explore co-occurrence patterns in soil microbial communities. The ISME journal 2012; 6: 343-351.

Bastian M, Heymann S, Jacomy M. Gephi: an open source software for exploring and manipulating networks. Proceedings of the international AAAI conference on web and social media. 3, 2009, pp. 361-362.

Beals EW. Bray-Curtis ordination: an effective strategy for analysis of multivariate ecological data. Advances in ecological research. 14. Elsevier, 1984, pp. 1-55.

Berry D, Widder S. Deciphering microbial interactions and detecting keystone species with co-occurrence networks. Frontiers in microbiology 2014; 5: 219.

Beyter D, Tang P-Z, Becker S, Hoang T, Bilgin D, Lim YW, et al. Diversity, productivity, and stability of an industrial microbial ecosystem. Applied and environmental microbiology 2016; 82: 2494-2505.

Bouhajja E, McGuire M, Liles MR, Bataille G, Agathos SN, George IF. Identification of novel toluene monooxygenase genes in a hydrocarbon-polluted sediment using sequence- and function-based screening of metagenomic libraries. Applied Microbiology and Biotechnology 2017; 101: 797-808.

BRASIL. Resolução nº 420, de 28 de dezembro de 2009. In: Ambiente. MdMACNdM, editor. 420, Brasilia, 2009.

Briones A, Raskin L. Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. Current opinion in biotechnology 2003; 14: 270-276.

Bücker F, Santestevan NA, Roesch LF, Jacques RJS, Peralba MdCR, de Oliveira Camargo FA, et al. Impact of biodiesel on biodeterioration of stored Brazilian diesel oil. International Biodeterioration & Biodegradation 2011; 65: 172-178.

Butler TM. Microbial Community Response to Light and Heavy Crude Oil in Freshwater Systems. Michigan Technological University, 2018.

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. Nature methods 2016; 13: 581.

Canabarro NI, Silva-Ortiz P, Nogueira L, Cantarella H, Maciel-Filho R, Souza GM. Sustainability assessment of ethanol and biodiesel production in Argentina, Brazil, Colombia, and Guatemala. Renewable and Sustainable Energy Reviews 2023; 171: 113019.

Cébron A, Borreca A, Beguiristain T, Biache C, Faure P. Taxonomic and functional trait-based approaches suggest that aerobic and anaerobic soil microorganisms allow the natural attenuation of oil from natural seeps. Scientific Reports 2022; 12: 7245.

Chen C, Ming J, Yoza BA, Liang J, Li QX, Guo H, et al. Characterization of aerobic granular sludge used for the treatment of petroleum wastewater. Bioresource technology 2019; 271: 353-359.

Chen M, Xu P, Zeng G, Yang C, Huang D, Zhang J. Bioremediation of soils contaminated with polycyclic aromatic hydrocarbons, petroleum, pesticides, chlorophenols and heavy metals by composting: Applications, microbes and future research needs. Biotechnology Advances 2015; 33: 745-755.

Chen YD, Barker JF, Gui L. A strategy for aromatic hydrocarbon bioremediation under anaerobic conditions and the impacts of ethanol: A microcosm study. Journal of Contaminant Hydrology 2008; 96: 17-31.

Chi Z, Hou L, Li H, Wu H, Yan B. Indigenous bacterial community and function in phenanthrene-polluted coastal wetlands: Potential for phenanthrene degradation and relation with soil properties. Environmental Research 2021; 199: 111357.

Corseuil HX, Hunt CS, Dos Santos RCF, Alvarez PJ. The influence of the gasoline oxygenate ethanol on aerobic and anaerobic BTX biodegradation. Water Research 1998; 32: 2065-2072.

Corseuil HX, Monier AL, Fernandes M, Schneider MR, Nunes CC, do Rosario M, et al. BTEX plume dynamics following an ethanol blend release: geochemical footprint and thermodynamic constraints on natural attenuation. Environmental Science & Technology 2011a; 45: 3422-3429.

Corseuil HX, Monier AL, Gomes AP, Chiaranda HS, do Rosario M, Alvarez PJ. Biodegradation of soybean and castor oil biodiesel: implications on the natural attenuation of monoaromatic hydrocarbons in groundwater. Groundwater Monitoring & Remediation 2011b; 31: 111-118.

Costa AHR, Nunes CC, Corseuil HX. Biorremediação de águas subterrâneas impactadas por gasolina e etanol com o uso de nitrato. Engenharia Sanitária e Ambiental 2009; 14: 265-274.

Couto MNPFS, Monteiro E, Vasconcelos MTSD. Mesocosm trials of bioremediation of contaminated soil of a petroleum refinery: comparison of natural attenuation, biostimulation and bioaugmentation. Environmental Science and Pollution Research 2010; 17: 1339-1346.

Cozzarelli IM, Bekins BA, Eganhouse RP, Warren E, Essaid HI. In situ measurements of volatile aromatic hydrocarbon biodegradation rates in groundwater. Journal of contaminant hydrology 2010; 111: 48-64.

Cravo-Laureau C, Hernandez-Raquet G, Vitte I, Jézéquel R, Bellet V, Godon J-J, et al. Role of environmental fluctuations and microbial diversity in degradation of hydrocarbons in contaminated sludge. Research in microbiology 2011; 162: 888-895.

Cyplik P, Schmidt M, Szulc A, Marecik R, Lisiecki P, Heipieper HJ, et al. Relative quantitative PCR to assess bacterial community dynamics during biodegradation of diesel and biodiesel fuels under various aeration conditions. Bioresource Technology 2011; 102: 4347-4352.

Da Silva ML, Ruiz-Aguilar GM, Alvarez PJ. Enhanced anaerobic biodegradation of BTEX-ethanol mixtures in aquifer columns amended with sulfate, chelated ferric iron or nitrate. Biodegradation 2005; 16: 105-114.

Da Silva MLB, Alvarez PJJ. Enhanced Anaerobic Biodegradation of Benzene-Toluene-Ethylbenzene-Xylene-Ethanol Mixtures in Bioaugmented Aquifer Columns. Applied and Environmental Microbiology 2004; 70: 4720.

da Silva MLB, Corseuil HX. Groundwater microbial analysis to assess enhanced BTEX biodegradation by nitrate injection at a gasohol-contaminated site. International Biodeterioration & Biodegradation 2012; 67: 21-27.

Dam B, Dam S, Blom J, Liesack W. Genome Analysis Coupled with Physiological Studies Reveals a Diverse Nitrogen Metabolism in Methylocystis sp. Strain SC2. PLOS ONE 2013; 8: e74767.

Dam B, Dam S, Kube M, Reinhardt R, Liesack W. Complete genome sequence of Methylocystis sp. strain SC2, an aerobic methanotroph with high-affinity methane oxidation potential. Am Soc Microbiol, 2012.

de la Cueva SC, Rodríguez CH, Cruz NOS, Contreras JAR, Miranda JL. Changes in Bacterial Populations During Bioremediation of Soil Contaminated with Petroleum Hydrocarbons. Water, Air, & Soil Pollution 2016; 227: 91.

de Vries FT, Griffiths RI, Bailey M, Craig H, Girlanda M, Gweon HS, et al. Soil bacterial networks are less stable under drought than fungal networks. Nature communications 2018; 9: 3033.

Deng Y-J, Wang SY. Synergistic growth in bacteria depends on substrate complexity. Journal of Microbiology 2016; 54: 23-30.

Dong Y, Wu S, Deng Y, Wang S, Fan H, Li X, et al. Distinct functions and assembly mechanisms of soil abundant and rare bacterial taxa under increasing pyrene stresses. Frontiers in Microbiology 2021; 12: 689762.

Dörr de Quadros P, Cerqueira VS, Cazarolli JC, Peralba MdCR, Camargo FAO, Giongo A, et al. Oily sludge stimulates microbial activity and changes microbial structure in a landfarming soil. International Biodeterioration & Biodegradation 2016; 115: 90-101.

Dunne JA, Williams RJ, Martinez ND. Network structure and biodiversity loss in food webs: robustness increases with connectance. Ecology letters 2002; 5: 558-567.
Eiler A, Heinrich F, Bertilsson S. Coherent dynamics and association networks among lake bacterioplankton taxa. The ISME journal 2012; 6: 330-342.
Elazhari-Ali A, Singh AK, Davenport RJ, Head IM, Werner D. Biofuel components change the ecology of bacterial volatile petroleum hydrocarbon degradation in aerobic sandy soil. Environmental Pollution 2013; 173: 125-132.
Eze MO, Hose GC, George SC, Daniel R. Diversity and metagenome analysis of a hydrocarbon-degrading bacterial consortium from asphalt lakes located in Wietze, Germany. AMB Express 2021; 11: 89.
Falkowski PG, Fenchel T, Delong EF. The microbial engines that drive Earth's biogeochemical cycles. science 2008; 320: 1034-1039.
Faust K, Sathirapongsasuti JF, Izard J, Segata N, Gevers D, Raes J, et al. Microbial co-occurrence relationships in the human microbiome. PLoS computational biology 2012; 8: e1002606.
Feng L, Jiang X, Huang Y, Wen D, Fu T, Fu R. Petroleum hydrocarbon-contaminated soil bioremediation assisted by isolated bacterial consortium and sophorolipid. Environmental Pollution 2021; 273: 116476.
Feng X, Liu Z, Jia X, Lu W. Distribution of Bacterial Communities in Petroleum-Contaminated Soils from the Dagang Oilfield, China. Transactions of Tianjin University 2020; 26: 22-32.
Fowler SJ, Toth CR, Gieg LM. Community structure in methanogenic enrichments provides insight into syntrophic interactions in hydrocarbon-impacted environments. Frontiers in microbiology 2016; 7: 562.
Friedman J, Alm EJ. Inferring correlation networks from genomic survey data. PLoS computational biology 2012; 8: e1002687.
Fuhrman JA, Steele JA. Community structure of marine bacterioplankton: patterns, networks, and relationships to function. Aquatic Microbial Ecology 2008; 53: 69-81.
Gałązka A, Grządziel J, Gałązka R, Ukalska-Jaruga A, Strzelecka J, Smreczak B. Genetic and functional diversity of bacterial microbiome in soils with long term impacts of petroleum hydrocarbons. Frontiers in Microbiology 2018; 9: 1923.
Gao Y, Yuan L, Du J, Wang H, Yang X, Duan L, et al. Bacterial community profile of the crude oil-contaminated saline soil in the Yellow River Delta Natural Reserve, China. Chemosphere 2022; 289: 133207.
Gauchotte-Lindsay C, Aspray TJ, Knapp M, Ijaz UZ. Systems biology approach to elucidation of contaminant biodegradation in complex samples–integration of high-resolution analytical and molecular tools. Faraday discussions 2019; 218: 481-504.
Gieg LM, Fowler SJ, Berdugo-Clavijo C. Syntrophic biodegradation of hydrocarbon contaminants. Current Opinion in Biotechnology 2014; 27: 21-29.
Greenblum S, Turnbaugh PJ, Borenstein E. Metagenomic systems biology of the human gut microbiome reveals topological shifts associated with obesity and inflammatory bowel disease. Proceedings of the National Academy of Sciences 2012; 109: 594-599.
Griffiths B, Ritz K, Bardgett RD, Cook R, Christensen S, Ekelund F, et al. Ecosystem response of pasture soil communities to fumigation-induced microbial diversity reductions: an examination of the biodiversity–ecosystem function relationship. Oikos 2000; 90: 279-294.
Guimera R, Nunes Amaral LA. Functional cartography of complex metabolic networks. nature 2005; 433: 895-900.
Guittar J, Shade A, Litchman E. Trait-based community assembly and succession of the infant gut microbiome. Nature communications 2019; 10: 512.
Guo B, Zhang L, Sun H, Gao M, Yu N, Zhang Q, et al. Microbial co-occurrence network topological properties link with reactor parameters and reveal importance of low-abundance genera. npj Biofilms and Microbiomes 2022; 8: 3.
Hallam SJ, McCutcheon JP. Microbes don't play solitaire: how cooperation trumps isolation in the microbial world. Environmental microbiology reports 2015; 7: 26-28.
Haritash A, Kaushik C. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. Journal of hazardous materials 2009; 169: 1-15.
Heermann SE, Powers SE. Modeling the partitioning of BTEX in water-reformulated gasoline systems containing ethanol. Journal of Contaminant Hydrology 1998; 34: 315-341.

Hennessee CT, Li QX. Effects of Polycyclic Aromatic Hydrocarbon Mixtures on Degradation, Gene Expression, and Metabolite Production in Four Mycobacterium Species. Applied and Environmental Microbiology 2016; 82: 3357-3369.

Hidalgo KJ, Teramoto EH, Soriano AU, Valoni E, Baessa MP, Richnow HH, et al. Taxonomic and functional diversity of the microbiome in a jet fuel contaminated site as revealed by combined application of in situ microcosms with metagenomic analysis. Science of The Total Environment 2019: 135152.

Huang Y, Zeng Y, Yu Z, Zhang J, Feng H, Lin X. In silico and experimental methods revealed highly diverse bacteria with quorum sensing and aromatics biodegradation systems–a potential broad application on bioremediation. Bioresource Technology 2013; 148: 311-316.

Im J, Semrau JD. Pollutant degradation by a Methylocystis strain SB2 grown on ethanol: bioremediation via facultative methanotrophy. FEMS Microbiology Letters 2011; 318: 137-142.

Islam TH, Ghosh B, Magnet MMH, Fatema K, Akter S, Khan MAR, et al. Isolation and identification of petroleum degrading bacteria from oil contaminated soil & water and assessment of their potentiality in bioremediation. Journal of Environmental Science, toxocology and Food Technology 2013; 5: 55 - 58.

Ji L, Shen F, Liu Y, Yang Y, Wang J, Purahong W, et al. Contrasting altitudinal patterns and co-occurrence networks of soil bacterial and fungal communities along soil depths in the cold-temperate montane forests of China. Catena 2022; 209: 105844.

Ji L, Yang Y, Yang L. Seasonal variations in soil fungal communities and co-occurrence networks along an altitudinal gradient in the cold temperate zone of China: A case study on Oakley Mountain. Catena 2021; 204: 105448.

Jia X, Dini-Andreote F, Salles JF. Community assembly processes of the microbial rare biosphere. Trends in microbiology 2018; 26: 738-747.

Jiao S, Chen W, Wei G. Biogeography and ecological diversity patterns of rare and abundant bacteria in oil-contaminated soils. Molecular ecology 2017; 26: 5305-5317.

Jin H, Hao J, Chang X, Zhang J, Qi J, Lü L, et al. Zonation and assessment of frozen-ground conditions for engineering geology along the China–Russia crude oil pipeline route from Mo'he to Daqing, Northeastern China. Cold Regions Science and Technology 2010; 64: 213-225.

John R, Itah A, Essien J, Ikpe D. Fate of nitrogen-fixing bacteria in crude oil contaminated wetland ultisol. Bulletin of environmental contamination and toxicology 2011; 87: 343-353.

Júlio ADL, de Cássia Mourão Silva U, Medeiros JD, Morais DK, dos Santos VL. Metataxonomic analyses reveal differences in aquifer bacterial community as a function of creosote contamination and its potential for contaminant remediation. Scientific Reports 2019; 9: 11731.

Kim D-W, Lee K, Lee D-H, Cha C-J. Comparative genomic analysis of pyrene-degrading Mycobacterium species: Genomic islands and ring-hydroxylating dioxygenases involved in pyrene degradation. Journal of Microbiology 2018; 56: 798-804.

Kim E, Yulisa A, Kim S, Hwang S. Monitoring microbial community structure and variations in a full-scale petroleum refinery wastewater treatment plant. Bioresource Technology 2020; 306: 123178.

Kim S-J, Kweon O, Sutherland JB, Kim H-L, Jones RC, Burback BL, et al. Dynamic Response of Mycobacterium vanbaalenii PYR-1 to BP Deepwater Horizon Crude Oil. Applied and Environmental Microbiology 2015; 81: 4263-4276.

Kim S-J, Park S-J, Jung M-Y, Kim J-G, Madsen EL, Rhee S-K. An uncultivated nitrate-reducing member of the genus Herminiimonas degrades toluene. Applied and environmental microbiology 2014; 80: 3233-3243.

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Research 2012; 41: e1-e1.

Kojima H, Fukui M. Sulfuritalea hydrogenivorans gen. nov., sp. nov., a facultative autotroph isolated from a freshwater lake. International journal of systematic and evolutionary microbiology 2011; 61: 1651-1655.

Koshlaf E, Ball AS. Soil bioremediation approaches for petroleum hydrocarbon polluted environments. AIMS microbiology 2017; 3: 25.

Lee S-K, Lee S. Isolation and characterization of a thermotolerant bacterium Ralstonia sp. strain PHS1 that degrades benzene, toluene, ethylbenzene, and o-xylene. Applied microbiology and biotechnology 2001; 56: 270-275.

Leharne S. Risk assessment and remediation of NAPL contaminated soil and groundwater. ChemTexts 2021; 8: 5.
Li X. [Influence of oil pollution on soil microbial community diversity]. Sheng wu gong cheng xue bao = Chinese journal of biotechnology 2017; 33: 968-975.
Lindemann SR. A piece of the pie: engineering microbiomes by exploiting division of labor in complex polysaccharide consumption. Current Opinion in Chemical Engineering 2020; 30: 96-102.
Liu L, Yang J, Yu Z, Wilkinson DM. The biogeography of abundant and rare bacterioplankton in the lakes and reservoirs of China. The ISME journal 2015; 9: 2068-2077.
Liu X, Yang T, Shi Y, Zhu Y, He M, Zhao Y, et al. Strong partitioning of soil bacterial community composition and co-occurrence networks along a small-scale elevational gradient on Zijin Mountain. Soil Ecology Letters 2021; 3: 290-302.
Logares R, Audic S, Bass D, Bittner L, Boutte C, Christen R, et al. Patterns of rare and abundant marine microbial eukaryotes. Current Biology 2014; 24: 813-821.
Louca S, Polz MF, Mazel F, Albright MBN, Huber JA, O'Connor MI, et al. Function and functional redundancy in microbial systems. Nature Ecology & Evolution 2018; 2: 936-943.
Lovley DR. Cleaning up with genomics: applying molecular biology to bioremediation. Nature Reviews Microbiology 2003; 1: 35.
Lu L, Li X, Li Z, Chen Y, Sabio y García CA, Yang J, et al. Aerobic methanotrophs in an urban water cycle system: Community structure and network interaction pattern. Science of The Total Environment 2021; 772: 145045.
Luisa W, Letícia T, Francielle B, Raquel D, Patricia DdQ, Kateryna Z, et al. Culture-independent analysis of bacterial diversity during bioremediation of soil contaminated with a diesel-biodiesel blend (B10) S. Journal of Bioremediation and Biodegradation 2015; 6.
Ma B, Wang H, Dsouza M, Lou J, He Y, Dai Z, et al. Geographic patterns of co-occurrence network topological features for soil microbiota at continental scale in eastern China. The ISME journal 2016; 10: 1891-1901.
Magdy MM, Gaber Y, Sebak M, Azmy AF, AbdelGhani S. Different metabolic pathways involved in anthracene biodegradation by Brevibacillus, Pseudomonas and Methylocystis Species. Beni-Suef University Journal of Basic and Applied Sciences 2022; 11: 4.
Martirani-Von Abercron S-M, Pacheco D, Benito-Santano P, Marín P, Marqués S. Polycyclic aromatic hydrocarbon-induced changes in bacterial community structure under anoxic nitrate reducing conditions. Frontiers in microbiology 2016; 7: 1775.
McInerney MJ, Sieber JR, Gunsalus RP. Syntrophy in anaerobic global carbon cycles. Current opinion in biotechnology 2009; 20: 623-632.
McMurdie PJ, Holmes S. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLOS ONE 2013; 8: e61217.
Morris BE, Henneberger R, Huber H, Moissl-Eichinger C. Microbial syntrophy: interaction for the common good. FEMS microbiology reviews 2013; 37: 384-406.
Muller D, Simeonova DD, Riegel P, Mangenot S, Koechler S, Lievremont D, et al. Herminiimonas arsenicoxydans sp. nov., a metalloresistant bacterium. International journal of systematic and evolutionary microbiology 2006; 56: 1765-1769.
Müller JB, Ramos DT, Larose C, Fernandes M, Lazzarin HSC, Vogel TM, et al. Combined iron and sulfate reduction biostimulation as a novel approach to enhance BTEX and PAH source-zone biodegradation in biodiesel blend-contaminated groundwater. Journal of hazardous materials 2017; 326: 229-236.
Newman ME. Modularity and community structure in networks. Proceedings of the national academy of sciences 2006; 103: 8577-8582.
Ng YF, Ge L, Chan WK, Tan SN, Yong JWH, Tan TTY. An environmentally friendly approach to treat oil spill: Investigating the biodegradation of petrodiesel in the presence of different biodiesels. Fuel 2015; 139: 523-528.
Ning D, Deng Y, Tiedje JM, Zhou J. A general framework for quantitatively assessing ecological stochasticity. Proceedings of the National Academy of Sciences 2019; 116: 16892-16898.
Norris RD. In-situ bioremediation of soils and ground water contaminated with petroleum hydrocarbons. Handbook of bioremediation. CRC Press, 2017, pp. 17-38.
OECD/FAO. Agricultural Outlook. 2023.

Okoh E, Yelebe ZR, Oruabena B, Nelson ES, Indiamaowei OP. Clean-up of crude oil-contaminated soils: bioremediation option. International Journal of Environmental Science and Technology 2020; 17: 1185-1198.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'hara R, et al. Package 'vegan'. Community ecology package, version 2013; 2: 1-295.

Olesen JM, Bascompte J, Dupont YL, Jordano P. The modularity of pollination networks. Proceedings of the National Academy of Sciences 2007; 104: 19891-19896.

Ossai IC, Ahmed A, Hassan A, Hamid FS. Remediation of soil and water contaminated with petroleum hydrocarbon: A review. Environmental Technology & Innovation 2020; 17: 100526.

Palmonari A, Cavallini D, Sniffen C, Fernandes L, Holder P, Fagioli L, et al. Characterization of molasses chemical composition. Journal of dairy science 2020; 103: 6244-6249.

Pan Z, Chen Y, Zhou M, McAllister TA, Guan LL. Microbial interaction-driven community differences as revealed by network analysis. Computational and Structural Biotechnology Journal 2021; 19: 6000-6008.

Peng Q, Sheng M, Yang Z, Ni H, Li Q, Li Y, et al. Extensimonas perlucida sp. nov., a Novel Bacterium Isolated from Sludge. Current Microbiology 2020; 77: 1316-1320.

Philp JC, Atlas RM. Bioremediation of contaminated soils and aquifers. Bioremediation: Applied Microbial Solutions for Real-World Environmental Cleanup 2005: 139-236.

Poudel R, Jumpponen A, Schlatter DC, Paulitz T, Gardener BM, Kinkel LL, et al. Microbiome networks: a systems framework for identifying candidate microbial assemblages for disease management. Phytopathology 2016; 106: 1083-1096.

Qian X, Li S, Wu B, Wang Y, Ji N, Yao H, et al. Mainland and island populations of Mussaenda kwangtungensis differ in their phyllosphere fungal community composition and network structure. Scientific Reports 2020; 10: 952.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic acids research 2012; 41: D590-D596.

R Development Core Team. R: A language and environment for statistical computing. 2021.

Rama F, Ramos DT, Müller JB, Corseuil HX, Miotliński K. Flow field dynamics and high ethanol content in gasohol blends enhance BTEX migration and biodegradation in groundwater. Journal of contaminant hydrology 2019; 222: 17-30.

Ramos DT. Bioestimulação de processos metanogênicos com acetato de amônio para degradação acelerada de hidrocarbonetos de petróleo em águas subterrâneas contaminadas com diesel B20. Engenharia Ambiental. Doutor. Universidade Federal de Santa Catarina, Florianóplis, 2013.

Ramos DT, da Silva MLB, Chiaranda HS, Alvarez PJ, Corseuil HX. Biostimulation of anaerobic BTEX biodegradation under fermentative methanogenic conditions at source-zone groundwater contaminated with a biodiesel blend (B20). Biodegradation 2013; 24: 333-341.

Ramos DT, da Silva MLB, Nossa CW, Alvarez PJJ, Corseuil HX. Assessment of microbial communities associated with fermentative–methanogenic biodegradation of aromatic hydrocarbons in groundwater contaminated with a biodiesel blend (B20). Biodegradation 2014; 25: 681-691.

Ramos SM. Análise comparativa da influência do etanol em diferentes processos de remediação de águas subterrâneas impactadas por gasolina. 2012.

Ramos SM, Nunes CC, Corseuil HX. Biorremediação de águas subterrâneas impactadas por gasolina E10 com o uso de sulfato, 2010.

Ren X, Zeng G, Tang L, Wang J, Wan J, Liu Y, et al. Sorption, transport and biodegradation – An insight into bioavailability of persistent organic pollutants in soil. Science of The Total Environment 2018; 610-611: 1154-1163.

Rodríguez Y, Firmino PIM, Pérez V, Lebrero R, Muñoz R. Biogas valorization via continuous polyhydroxybutyrate production by Methylocystis hirsuta in a bubble column bioreactor. Waste Management 2020; 113: 395-403.

Sarkar J, Saha A, Roy A, Bose H, Pal S, Sar P, et al. Development of nitrate stimulated hydrocarbon degrading microbial consortia from refinery sludge as potent bioaugmenting agent for enhanced bioremediation of petroleum contaminated waste. World Journal of Microbiology and Biotechnology 2020; 36: 156.

Satapanajaru T, Chokejaroenrat C, Sakulthaew C, Yoo-Iam M. Remediation and restoration of petroleum hydrocarbon containing alcohol-contaminated soil by persulfate oxidation activated with soil minerals. Water, Air, & Soil Pollution 2017; 228: 1-15.

Seo Y, Cho K-S. Effects of plant and soil amendment on remediation performance and methane mitigation in petroleum-contaminated soil. 2021.

Shade A, Peter H, Allison SD, Baho DL, Berga M, Bürgmann H, et al. Fundamentals of microbial community resistance and resilience. Frontiers in microbiology 2012; 3: 417.

Shahi A, Aydin S, Ince B, Ince O. Reconstruction of bacterial community structure and variation for enhanced petroleum hydrocarbons degradation through biostimulation of oil contaminated soil. Chemical Engineering Journal 2016; 306: 60-66.

Shu D, Zhang B, He Y, Wei G. Abundant and rare microbial sub-communities in anammox granules present contrasting assemblage patterns and metabolic functions in response to inorganic carbon stresses. Bioresource Technology 2018; 265: 299-309.

Siqueira AF, Minamisawa K, Sánchez C. Anaerobic Reduction of Nitrate to Nitrous Oxide Is Lower in Bradyrhizobium japonicum than in Bradyrhizobium. Microbes and Environments 2017; 32:398-401

Sole RV, Montoya M. Complexity and fragility in ecological networks. Proceedings of the Royal Society of London. Series B: Biological Sciences 2001; 268: 2039-2045.

Song M, Jiang L, Zhang D, Luo C, Wang Y, Yu Z, et al. Bacteria capable of degrading anthracene, phenanthrene, and fluoranthene as revealed by DNA based stable-isotope probing in a forest soil. Journal of Hazardous Materials 2016; 308: 50-57.

Steele JA, Countway PD, Xia L, Vigil PD, Beman JM, Kim DY, et al. Marine bacterial, archaeal and protistan association networks reveal ecological linkages. The ISME journal 2011; 5: 1414-1425.

Stegen JC, Lin X, Fredrickson JK, Chen X, Kennedy DW, Murray CJ, et al. Quantifying community assembly processes and identifying features that impose them. The ISME journal 2013; 7: 2069-2079.

Steiner LV, Ramos DT, Liedke AMR, Serbent MP, Corseuil HX. Ethanol content in different gasohol blend spills influences the decision-making on remediation technologies. Journal of environmental management 2018; 212: 8-16.

Stepanova AY, Gladkov EA, Osipova ES, Gladkova OV, Tereshonok DV. Bioremediation of Soil from Petroleum Contamination. Processes 2022; 10: 1224.

Sun Y, Chen W, Wang Y, Guo J, Zhang H, Hu X. Nutrient depletion is the main limiting factor in the crude oil bioaugmentation process. Journal of Environmental Sciences 2021; 100: 317-327.

Tahhan RA, Abu-Ateih RY. Biodegradation of petroleum industry oily-sludge using Jordanian oil refinery contaminated soil. International Biodeterioration & Biodegradation 2009; 63: 1054-1060.

Tylianakis JM, Laliberté E, Nielsen A, Bascompte J. Conservation of species interaction networks. Biological conservation 2010; 143: 2270-2279.

Vacher C, Tamaddoni-Nezhad A, Kamenova S, Peyrard N, Moalic Y, Sabbadin R, et al. Learning ecological networks from next-generation sequencing data. Advances in ecological research. 54. Elsevier, 2016, pp. 1-39.

Valiente-Banuet A, Aizen M, Alcántara J, Arroyo J, Cocucci A, Galetti M, et al. Beyond species loss: extinction of interactions in a changing world. Functional Ecology, 2015, vol. 29, p. 299-307 2014.

Van Elsas JD, Chiurazzi M, Mallon CA, Elhottovā D, Krištůfek V, Salles JF. Microbial diversity determines the invasion of soil by a bacterial pathogen. Proceedings of the National Academy of Sciences 2012; 109: 1159-1164.

Vera A, Wilson FP, Cupples AM. Predicted functional genes for the biodegradation of xenobiotics in groundwater and sediment at two contaminated naval sites. Applied Microbiology and Biotechnology 2022; 106: 835-853.

Wang M, Garrido-Sanz D, Sansegundo-Lobato P, Redondo-Nieto M, Conlon R, Martin M, et al. Soil Microbiome Structure and Function in Ecopiles Used to Remediate Petroleum-Contaminated Soil. Frontiers in Environmental Science 2021; 9.

Wang X, Zhao X, Li H, Jia J, Liu Y, Ejenavi O, et al. Separating and characterizing functional alkane degraders from crude-oil-contaminated sites via magnetic nanoparticle-mediated isolation. Research in Microbiology 2016; 167: 731-744.

Watts DJ, Strogatz SH. Collective dynamics of 'small-world'networks. nature 1998; 393: 440-442.

Wiegel J, Tanner R, Rainey FA. An Introduction to the Family Clostridiaceae. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E, editors. The Prokaryotes: Volume 4: Bacteria: Firmicutes, Cyanobacteria. Springer US, New York, NY, 2006, pp. 654-678.

Wu M, Li W, Dick WA, Ye X, Chen K, Kost D, et al. Bioremediation of hydrocarbon degradation in a petroleum-contaminated soil and microbial population and activity determination. Chemosphere 2017; 169: 124-130.

Wüst PK, Horn MA, Drake HL. Clostridiaceae and Enterobacteriaceae as active fermenters in earthworm gut content. The ISME journal 2011; 5: 92-106.

Yang H, Lee J, Cho K-S. Dynamics of functional genes and bacterial community during bioremediation of diesel-contaminated soil amended with compost. 2022.

Yang S, Wen X, Shi Y, Liebner S, Jin H, Perfumo A. Hydrocarbon degraders establish at the costs of microbial richness, abundance and keystone taxa after crude oil contamination in permafrost environments. Scientific Reports 2016; 6: 37473.

Yergeau E, Sanschagrin S, Maynard C, St-Arnaud M, Greer CW. Microbial expression profiles in the rhizosphere of willows depend on soil contamination. The ISME journal 2014; 8: 344-358.

Yi M, Zhang L, Qin C, Lu P, Bai H, Han X, et al. Temporal changes of microbial community structure and nitrogen cycling processes during the aerobic degradation of phenanthrene. Chemosphere 2022; 286: 131709.

You J, Chen H, Xu L, Zhao J, Ye J, Zhang S, et al. Anodic-potential-tuned bioanode for efficient gaseous toluene removal in an MFC. Electrochimica Acta 2021; 375: 137992.

Zaan BMvd, Saia FT, Stams AJM, Plugge CM, Vos WMd, Smidt H, et al. Anaerobic benzene degradation under denitrifying conditions: Peptococcaceae as dominant benzene degraders and evidence for a syntrophic process. Environmental Microbiology 2012; 14: 1171-1181.

Zhang B, Zhang J, Liu Y, Shi P, Wei G. Co-occurrence patterns of soybean rhizosphere microbiome at a continental scale. Soil Biology and Biochemistry 2018; 118: 178-186.

Zhang W-Y, Fang M-X, Zhang W-W, Xiao C, Zhang X-Q, Yu Z-P, et al. Extensimonas vulgaris gen. nov., sp. nov., a member of the family Comamonadaceae. International Journal of Systematic and Evolutionary Microbiology 2013; 63: 2062-2068.

Zhang Z, Wei Z, Guo W, Wei Y, Luo J, Song C, et al. Two types nitrogen source supply adjusted interaction patterns of bacterial community to affect humifaction process of rice straw composting. Bioresource Technology 2021; 332: 125129.

Zhao D, Liu C, Liu L, Zhang Y, Liu Q, Wu W-M. Selection of functional consortium for crude oil-contaminated soil remediation. International Biodeterioration & Biodegradation 2011; 65: 1244-1248.

Chapter III - Manuscript 4: Functional redundancy as key microbial strategy to cope with pollution in biofuel impacted soils

Fuel/biofuel blend-affected soils stimulated by different bioremediation treatments harbor microbial taxonomic diversity and functional redundancy as key strategy to cope with pollution

K.J. Hidalgo[a,b5], L.G. Cueva A.J. Giachini[c] , M.R. Schneider[c], A.U. Soriano[d], M.P. Baessa[d], L.F. Martins[d], and V.M. Oliveira[a]

[a] Microbial Resources Division, Research Center for Chemistry, Biology and Agriculture (CPQBA), University of Campinas – UNICAMP, Av. Alexandre Cazellato, 999, ZIP 13148-218, Paulínia, SP, Brazil,

[b] Graduate Program in Genetics and Molecular Biology, Institute of Biology, University of Campinas (UNICAMP), Rua Monteiro Lobato 255, Cidade Universitária ZIP 13083-862, Campinas, SP, Brazil.

[c] Núcleo Ressacada de Pesquisas Em Meio Ambiente (REMA) - Department of Microbiology, Federal University of Santa Catarina (UFSC), Campus Universitário Sul da Ilha - Rua José Olímpio da Silva, 1326 - Bairro Tapera, 88049-500 Florianópolis, SC, Brazil

[d] PETROBRAS/ R&D Center (CENPES), CENPES Expansão, Av. Horácio Macedo, s/ número. ZIP 21941-915 Cidade Universitária, Ilha do Fundão, Rio de Janeiro, Brazil.

[5] Corresponding author: K.J. Hidalgo
E-mail: khidalgo@javeriana.edu.co
Telephone: +55 19 98172 1510

Abstract

As a result of climate and energetic crises, as well as the environmental implications of fossil fuel consumption, the use of biofuels has expanded in recent decades. In Brazil, biodiesel is used in blends with diesel (also known as B12 – 12% Biodiesel / 88% Diesel) and ethanol in blends with gasoline (gasohol) (also known as E27 – 27% ethanol / 63% gasoline). The growing use of such blends increases the risk of biofuel contamination in soil, surface, and groundwater. In addition to the identification and quantification of the pollutants to assess the effectiveness of the bioremediation treatments in biofuel/fossil fuel blend impacted soils, elucidating changes in the specific functional profiles related to hydrocarbon degradation brings also important information about the direct impact of these approaches about the responses of the main actors of the bioremediation, the microorganisms. Such knowledge is scarce and may be of pivotal relevance to design customized bioremediation protocols, contributing to optimize and accelerate the pollutant removal and recovery of impacted areas. This study aimed to extensively describe the dynamics of hydrocarbon degradation functional profiles of four areas deliberated polluted with E10, E25 or B20 and subjected to different bioremediation treatments (i.e., injection of anaerobic electron acceptors, biosparging, or bioaugmentation). Metagenomic shotgun sequencing results indicated that long-term polluted areas that have never been submitted to any bioremediation treatment had a specific functional profile different from the treated areas. Interestingly, the same hydrocarbon degradation genes were enriched in all areas regardless the type of bioremediation applied. However, the taxa associated with these genes were different between the areas under different blend contamination and/or bioremediation treatment, demonstrating the importance of the functional redundancy to maintain the soil ecosystem functioning. Finally, several of the keystone species in the community are hydrocarbon degraders, showing that these taxa can be targets for biostimulation in future soil remediation processes. Altogether, these findings contribute to the understanding of the effects of bioremediation treatments over the functional genetic potential of the microbiome in biofuel/petrofuel impacted soils, helping to define the most appropriate technique according to the type of contamination.

Keywords: Biodiesel, ethanol, hydrocarbon degradation, biostimulation, bioaugmentation

1. INTRODUCTION

Due to pipeline ruptures, underground storage tank leakages, and transport incidents, the use of fossil fuels has resulted in numerous accidental spills over the past few decades (Baniasadi and Mousavi, 2018; Islam et al., 2013). Due to their toxicity, mutagenicity, and carcinogenicity, petroleum hydrocarbons discharged into the environment can have significant negative effects on ecosystems and human health (Baniasadi and Mousavi, 2018; Tahhan and Abu-Ateih, 2009). Energy challenges related to environmental sustainability and supply crises have made it more desirable to switch from fossil fuels to cost-benefit, clean, and renewable energy sources like ethanol and biodiesel. Currently, biofuels are used as blends in combination with other fuels. In the case of Brazil, ethanol is combined with gasoline (27% ethanol, known as E27), and biodiesel is blended with diesel (12% biodiesel, known as B12) (Canabarro et al., 2023).

Because of the increased use of biofuel/fossil fuel blends, environmental contamination due to these blends is likely to increase. Bioremediation treatments are well recognized as a low-cost method for treating hydrocarbon-polluted areas (Baniasadi and Mousavi, 2018; Ng et al., 2015; Norris, 2017; Zhao et al., 2011) and several laboratory and field experiments have shown that biological treatments are effective in the recovery of damaged soils (Chen et al., 2015; Stepanova et al., 2022; Wu et al., 2017). These treatments include the injection of nutrients or electron acceptors (i.e., biostimulation), the addition of external microorganisms (i.e., bioaugmentation), and/or the injection of air (i.e., biosparging), among others (Koshlaf and Ball, 2017; Okoh et al., 2020). Although the number of studies on bioremediation of biofuel/petrofuel pollution have increased (Chen et al., 2008; Cyplik et al., 2011; Ng et al., 2015; Rama et al., 2019), a significant number of them have solely evaluated the effect of biofuels on the dynamics, rates, and efficiency of petroleum hydrocarbon degradation (Alvarez and Hunt, 2002; Cyplik et al., 2011; Rama et al., 2019). Despite the relevance of the microbial metabolism in the pollutant removal, the degrading microbial community behavior has been investigated only in a few examples (Müller et al., 2017; Satapanajaru et al., 2017) (Hidalgo et al., submitted).

While early studies focused on the assessment of how the presence of ethanol and biodiesel can disturb the rate and dynamics of degradation of petroleum hydrocarbons

(i.e., BTEX, alkanes, PAHs) (Chen et al., 2008; Corseuil et al., 2011; Costa et al., 2009; da Silva and Corseuil, 2012; Rama et al., 2019; Ramos et al., 2014; Steiner et al., 2018), a more recent study of our group focused on the understanding of how the soil microbial community composition, structure and co-occurrence patterns respond to different biofuel/fossil fuel blend contaminations and distinct bioremediation treatments (Hidalgo et al., submitted). We showed that the microbial community composition and structure varied according to the type of biofuel and the blend proportion. Furthermore, we demonstrated that soils never submitted to any active bioremediation approach can house a more diverse and resilient microbial community, with lower abundances of microbial populations specialized in hydrocarbon degradation, resulting in slower decontamination. Additionally, network analysis revealed that microbial interactions were more sensitive to perturbations such as contaminations and/or bioremediation treatments when compared to community structure or composition. However, it is still unclear how these perturbations can affect the potential metabolic profiles.

Several laboratory and field experiments have shown that bioremediation treatments are effective in the recovery of damaged soils (Chen et al., 2015; Norris, 2017; Stepanova et al., 2022; Wu et al., 2017). These treatments include the injection of nutrients or electron acceptors (i.e. biostimulation), the addition of external microorganisms (i.e. bioaugmentation), or the injection of air (i.e. biosparging), among others (Koshlaf and Ball, 2017; Okoh et al., 2020). All of them enable modulation of the microbial community through the enrichment and/or selection of microorganisms with functional potential to degrade the contaminants, thus improving the degradation rates. However, there are no reports in the literature on the assessment of how bioremediation approaches influence microbial community functional profiles in biofuel/fossil fuel blend-affected areas.

In the current work, we assessed the potential functional profiles of the microbiome in soils affected by different biofuel/fossil fuel blend contaminations, before and after being submitted to distinct bioremediation treatments. For this, metagenomic shotgun sequencing was used, allowing to unveil the composition, taxonomy and dynamics of hydrocarbon degradation functional profiles in long term-impacted soils. We hypothesized that (i) similar to what we previously reported for microbial community composition and

taxonomic diversity on these polluted areas, the specific functional profiles are influenced by the type of biofuels; (ii) different BTEX degradation genes and related metabolisms are enriched, depending on the bioremediation treatment; and (iii) the taxonomic groups involved in BTEX degradation are keystone species supporting microbial networks and community homeostasis. This study sheds light on central questions regarding the specific metabolisms and main actors involved in aerobic and anaerobic hydrocarbon degradation in *in situ* bioremediation processes.

2. MATERIAL AND METHODS

2.1. Site description, field setup, sampling and previous molecular biology procedures

The study site is located at Ressacada Experimental Farm owned by the Federal University from Santa Catarina (UFSC), in Florianópolis, Santa Catarina state, Brazil. Four areas ranging from 330 to 549 m^2 were intentionally polluted with biofuel/fossil fuel blends, named gasohol (i.e., areas 4.E25-BAN and 7.E10-BAS) and biodiesel plus diesel (i.e., 5.B20-BAA and 8.B20-BAA). Setup and more details of the areas were detailed described in our previous work (Figures 1-2, section 2.1) (Hidalgo et al., submitted). Briefly, the phase 1 of the project consisted in controlled releases of biofuel/petrofuel blends (100 L) in four experimental areas throughout time (2004 – 2010). The source zones were established into a one-square-meter pit deep enough to reach the water table (1.0 – 1.6 m deep) (see Figure 1c and Figure 2 in Hidalgo et al., submitted). Different bioremediation treatments were applied in each area at the dissolved phase (Table 1). The results of that first phase have been published elsewhere (Costa et al., 2009; da Silva and Corseuil, 2012; Ramos, 2013; Ramos et al., 2013; Ramos et al., 2014; Ramos et al., 2010).

The second phase (2020 – 2022) consisted in the application of bioremediation treatments focusing on the source zones in order to define protocols able to reduce hydrocarbon concentrations to levels below those established by Brazilian legal standards (BRASIL, 2009) (results not published yet). The present work comprised the functional characterization of the soil microbiome in the source zone of those four experimental areas (Table 1).

Table 2. Summary of study areas and bioremediation treatments.

Areas	Blends released	Setup year	Bioremediation treatment at dissolved phase	References (results from the Phase 1)	Bioremediation treatment at source zone (this work)
4.E25-BAN	Ethanol 25%/ Gasoline 75%	2004	Biostimulation with nitrate	(Costa et al., 2009; da Silva and Corseuil, 2012)	Biostimulation with anaerobic electron acceptors such as niacin, nitrate and phosphate
7.E10-BAS	Ethanol 10%/ Gasoline 90%	2009	Biostimulation with sulfate	(Ramos et al., 2010)	Biostimulation with ammonium acetate and acid mine drainage
5.B20-ANM	Biodiesel 20% / Diesel 80%	2008	Monitored natural attenuation	(Ramos, 2013; Ramos et al., 2013)	Biostimulation by air injection, and bioaugmentation by installation of internal loop airlift reactor (IALR)
8.B20-BAA	Biodiesel 20% / Diesel 80%	2010	Biostimulation with acetate	(Ramos et al., 2013; Ramos et al., 2014)	Biostimulation by air injection, and bioaugmentation by installation of external loop airlift reactor (EALR)

Soil samples at source zones were taken in triplicate for the microbiome assessment at three different times, time 0 (immediately before the bioremediation treatment at the source zone), time 12 (twelve months after the bioremediation at source zone) and time 24 (3 months after a second bioremediation treatment at source zone) (Table S1). This last treatment was performed due to the appearance of odors and increase in some hydrocarbon concentrations. Additionally, samples from reactors installed in 5.B20-ANM and 8.B20-BAA areas (Table 1) were also taken on time 24. These reactors were installed in the biodiesel/diesel areas with the aim of stimulate and modulate the native microbiota. Briefly, a culture media was added into the source zone in each

area. After 30 days, the enriched microbial community (inoculum) was pumped to the reactor where similar culture media was used, in order to bioaugmented this microbiota and recirculating to the source zone again. The procedures for soil sampling for further physicochemical analyses and DNA extraction are described in detail in Hidalgo et al. (submitted 2023). DNA extracts obtained were used as templates for Multiple Displacement Amplification (MDA) with GenomiPhi™ V2 DNA amplification kit (Cytiva). °Amplifications were performed according to the manufacturer's instructions at 30°C for 2 h with subsequent inactivation at 65°C for 10 min. The reactions were carried out by triplicate for each sample, and it was used as negative control tubes without template DNA, for monitored contamination. The amplifications triplicates were pooled and purified using PCR OneStep inhibitor Removal Kit® (Zymo Research, Irvine, CA).

2.2. Shotgun sequencing and bioinformatic analyses

Library preparation was performed using the Illumina® DNA prep kit and metagenomic sequencing were performed by NGS – Soluções Genômicas (Piracicaba, São Paulo Brazil) in the Illumina NovaSeq 550 2x100 bp platform, following the manufacturer's guidelines. The sequences were deposited at the National Center of Biotechnology Information (NCBI) database under the accession numbers SAMN36438915-SAMN36438952.

Quality control of raw reads was done using FastQC v0.11.9 (Andrews, 2010). Trimmomatic v0.39 (Bolger et al., 2014) was used to remove the low-quality reads (Phred Score $\leq$ 30). Clean reads were used to assess the coverage of metagenomic datasets with NonPareil v3.4 (Rodriguez-R et al., 2018). For each time point and area, datasets were co-assembled into scaffolds using metaSPAdes v3.15.5 (Nurk et al., 2017) and the k-mers 21, 29, 39, 59 and 79. Statistics from assembled metagenomes was analyzed using MetaQUAST v5.0.2 (Mikheenko et al., 2016). The scaffolds were submitted to Prodigal v2.6.3 (Hyatt et al., 2010) for gene prediction. Diamond v2.0.9.147 was used for functional annotation, aligning the predicted genes against the latest publicly available version of KEGG (Kanehisa et al., 2017; Kanehisa and Goto, 2000; Kanehisa et al., 2016) and AnHyDeg (Callaghan and Wawrik, 2016) databases. For specific functional analysis, a set of genes involved in aerobic and anaerobic hydrocarbon degradation and related

metabolisms, such as methanogenesis and reduction of sulfate and nitrate, were filtered (Table S2). For taxonomic annotation, Kraken2 v2.1.2 (Wood et al., 2019) with the GTDB (release 207.2) (Chaumeil et al., 2019) was used. Bowtie2 v2.3.5.1 (Langmead and Salzberg, 2012) was used to map the reads against the co-assembled metagenomes and calculating relative abundances of the genes and taxonomies found. All the steps of this metagenomica pipeline presented here at https://github.com/khidalgo85/Metagenoma_Total_Shotgun.

For recovery of metagenome-assembled genomes (MAGs), co-assembly with all the samples (38 metagenomes) was performed using metaSpades v3.15.5 (Nurk et al., 2017). Co-assembled metagenomes (only scaffolds longer than 1000 bp were used) was submitted to an in-house binning pipeline that consists in six trials with four binning tools, taxonomic and functional annotation, and, finally, relative abundance quantification. The pipeline includes three runs with Metabat2 v2.2.15(Kang et al., 2019) changing the minimum length of the contig/scaffold (`--minContig 1500`, `--minContig 2500`, `--minContig 3000`), MaxBin2 v2.2.7 (Wu et al., 2015), CONCOCT v1.1.0 (Alneberg et al., 2014) and BinSanity v0.5.4 (Graham et al., 2017) with the default settings. The functional annotation was performed as described above. MAGs refinement was performed by the bin_refinement module of metaWRAP v1.3.2(Uritskiy et al., 2018). In order to improve MAGs quality, metagenomic sequence reads were mapped to each bin, and then, reassembled with metaSpades via the reassemble_bins module of metaWRAP. The quality profile (completeness and contamination) was determined by CheckM v1.1.3 (Parks et al., 2015). Drep v3.4.2 (Olm et al., 2017) was used to dereplicate MAGs with an average nucleotide identity (ANI) higher than 95%.

According to the quality profile obtained by CheckM, the refined MAGs were divided into medium-quality MAGs (Completeness > 50% and contamination < 10%) and high-quality MAGs (Completeness > 90% and contamination < 5%) based on “Minimum information about metagenome-assembled genomes” (MIMAG)(Bowers et al., 2017).

For the taxonomic affiliation, GTDB-tk v2.3.0 (GTDB release 207.2) (Chaumeil et al., 2019) was used. CoverM v0.6.1 (https://github.com/wwood/CoverM, accessed on 30 April 2023) was employed to assess the relative abundance of the genomes in the samples.

The genomes obtained were submitted to PhyloPhlan v3.0.60 (Segata et al., 2013) for phylogenetic reconstruction. Finally, the tree obtained was plotted with iTOL v6 (Letunic and Bork, 2019). The complete binning workflow are found at https://github.com/khidalgo85/Binning.

The *mcr*A gene sequences from MAGs and those recovered from the Genbank database were aligned with Muscle (Edgar, 2004), and the best substitution model was determined with the function Find Best DNA/Protein Models implemented in the MEGA-X software (Kumar et al., 2016). The phylogenetic reconstruction was performed using the Maximum Likelihood (ML) method and the General Time Reversible model with Gamma distribution (+G) and Invariable sites (+I). The support of nodes was estimated by bootstrapping with 1,000 replications. The phylogenetic analysis resulting from MEGA X was exported in Netwick format and customized with the web-based iTOL tool (http://itol.embl.de) (Letunic and Bork, 2019).

2.3. Statistical Analyses

Principal Correspondence Analysis was performed to compare the specific functional gene profiles of the biodiesel/diesel vs. gasohol polluted areas before the bioremediation treatment. Then, permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2014) was applied to test the significance of the clusters in PCoA. For this analysis, adonis function from the vegan (Oksanen et al., 2013) R packages were used, including the pollutant blend as an independent variable with default parameters (Bray-Curtis' dissimilarity matrix (Beals, 1984) and 999 permutations). The relationship between the specific functional gene profile and the time of sampling was analyzed using a PCoA in vegan package. A PERMANOVA using Bray-Curtis distances was applied to analyze the differences in the specific gene composition between the sampling times (before and after the bioremediation treatment applications).

3. RESULTS

3.1. Metagenomic sequencing and gene prediction

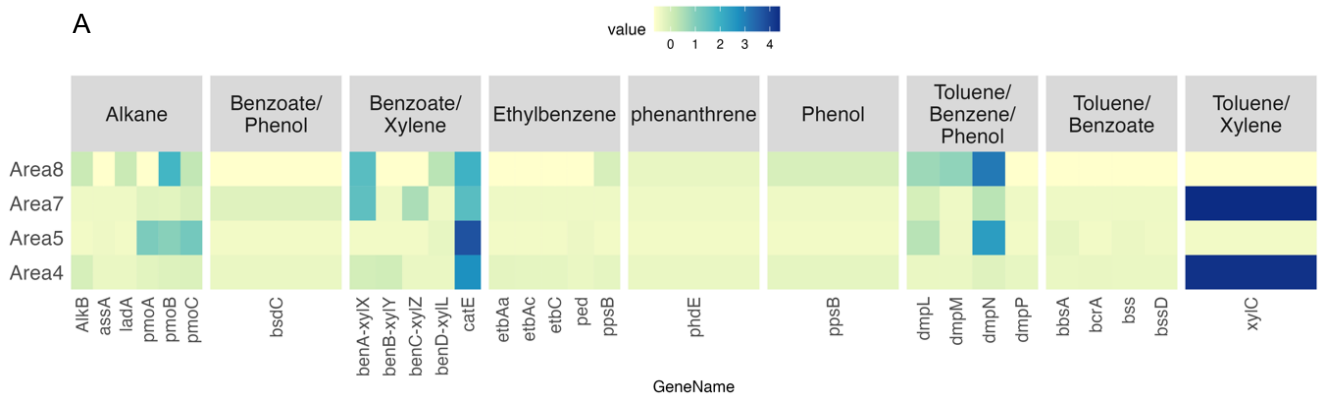
A total of 38 metagenomes were obtained in our study, being 36 from soil samples collected in the source-zone of four areas (4.E25-BAN, 7.E10-BAS, 5.B20-ANM and

8.B20-BAA) along three time points: immediately before the second bioremediation treatment (time 0), 12 months after the second bioremediation treatment (time 12) and three months after the third bioremediation treatment (time 24); and 2 reactor samples from time 24 (installed in areas 5.B20-ANM and 8.B20-BAA) (Table S1). The metagenome sizes ranged from 14 to 24 million reads and an average read length ~101 bp. After quality trimming, metagenome sizes were 13.6 to 23.3 millions of reads and ~88 bp of average read length (Table S3). The estimated coverages based on the read redundancy value calculated by Nonpareil (Rodriguez-R et al., 2018) ranged from 0.75 to 0.99, indicating that the sequencing depth of our samples were sufficient to cover most taxonomic diversity (Table S3). Sequence diversity Nonpareil index (alpha diversity derived from Nonpareil curves) ranged from 11.5 to 18.9 (Table S3). According to the Nonpareil tool authors, it is expected a Nonpareil index for soils from 20 to 22 (http://enve-omics.ce.gatech.edu/nonpareil/faq, accessed on July 3rd), suggesting that the contamination negatively impacted the diversity of soils under study. Coassembly of the metagenomes by area and time point allowed to recover, in total for the fourteen coassemblies (twelve from four areas and three sampling times, and two from the bioreactor samples), over one million contigs of at least 500 bp in length each and ~5.1 million predicted genes (Figure S1). A total of 5,162 ORFs were annotated as specific gene-like (aerobic and anaerobic hydrocarbon degradation and related metabolisms – e.g. sulfate and nitrate reduction and methanogenesis genes) sequences in the 38 metagenomes (ranging from 16 to 860 ORFs by sample) (Figure S2). The total relative abundance of specific genes (Table S2) in the datasets were in the range of the 0.001-2.0% (Figure S2).

3.2. Hydrocarbon degradation functional profiles are influenced by previous bioremediation treatments applied in the field

A total of 225 target genes were searched across all datasets (Table S2). These specific genes included aerobic and anaerobic aromatic and aliphatic hydrocarbon degradation genes (n=168), as well as genes related with anaerobic respiration pathways, such as reduction of nitrate and sulfate, and methanogenesis (n=57), based on KEGG (Kanehisa et al., 2017; Kanehisa and Goto, 2000; Kanehisa et al., 2016) and AnHyDeg (Callaghan and Wawrik, 2016) databases.

Specific functional profiles for the samples before the second bioremediation treatment (time 0) were assessed (Figure 1). Only 28 hydrocarbon degradation genes (16% of total hydrocarbon degradation genes, Table S2) were found in all areas, which were mainly related with degradation of alkanes and monoaromatics such as benzene, toluene and phenol (Figure 1A). Genes for degradation of benzaldehyde and catechol from aerobic degradation of monoaromatics and benzoyl-CoA for anaerobic pathway were found in higher abundance. Methane/ammonia monooxygenase enzyme encoded by the *pmo* genes were found in high abundance in areas polluted with biodiesel. Regarding the related metabolisms (Figure 1B), only genes for the methanogenesis metabolism were found in area 5.B20-ANM (blue). The functional profiles from areas 4.E25-BAN and 7.E10-BAS showed mainly the presence of nitrate reduction genes. In Phase 1, these two areas were biostimulated with anaerobic electron acceptors in the dissolved phase (Table 1).



B

Figure 1. A) Heat map showing the relative abundance of genes involved in degradation of hydrocarbons in the metagenomes (time 0); B) Treemap showing the relative abundance of the related metabolisms. Table S3 contains the list of the specific genes.

A permutational ANOVA (PERMANOVA) and ordination analyses were performed in order to test if the specific functional profiles of the microbial communities associated to gasohol and biodiesel/diesel polluted areas were different, considering the relative abundance of the target genes (Table S2). Results showed significant (*F=3.63, P=0.003*) differences between the functional profiles of the gasohol and biodiesel/diesel areas, explaining more than 26% of the variance. However, the PERMANOVA results could have been affected by non-homogeneous dispersion of the data (*betadisper*, *F=15.87, P=0.003)*. Nevertheless, the ordination analysis (Figure 2) indicates that the areas firstly actively bioremediated had a specific functional profile different from the one of the monitored natural attenuation area (5.B20-ANM).

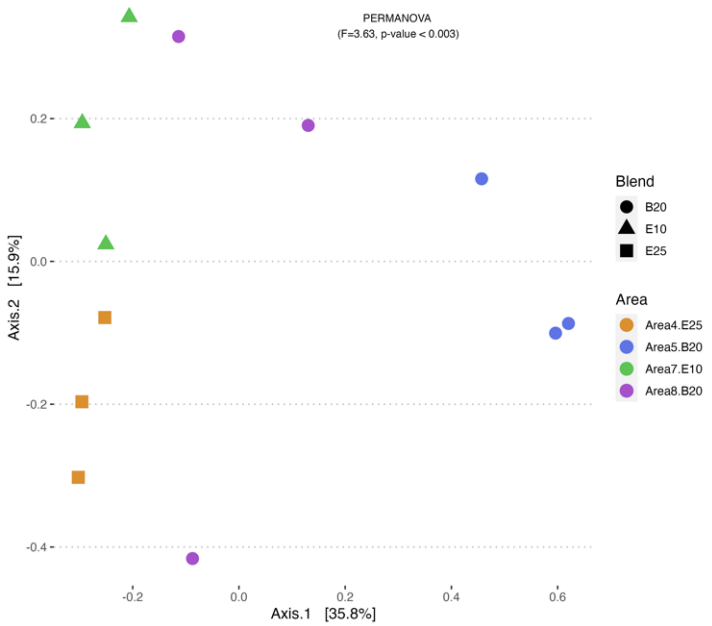


Figure 2. Biofuel effects on the functional specific profiles' composition. Principal Coordinates Analyses (PCoA) using Bray-Curtis distance by fuel contaminant before the bioremediation approach

3.3. Specific functional profiles were enriched after bioremediation treatments

In order to assess the effect of the bioremediation treatments in the hydrocarbon degradation profiles, the number of the target genes present was calculated in each area at time 0 (immediately before the application of bioremediation treatment at source zone), time 12 (twelve months after the application bioremediation treatment at source zone) and time 24 (three months after the reapplication bioremediation treatment at source zone). Additionally, the sum of the relative abundance of the specific genes was evaluated (Figure 3A). Also, the percentage of completeness of the metabolic pathways (number of present genes divided by the number of total genes of each metabolic pathway) was calculated for each area and time (Figure 3B). The abundance and number of hydrocarbon degradation genes increased 12 months after the bioremediation at source zone in all areas (Figure 3A). As expected, the completeness of anaerobic and aerobic degradation pathways was also increased (Figure 3B). In addition, after bioremediation, the related metabolisms such as methanogenesis, sulfate and nitrate reduction became complete or almost complete (Figure 3B). In the specific case of area 5.B20-ANM, the number of genes increased more than twice, but the relative abundance was lower

compared with all other areas, even after the bioremediation treatment at source zone. Nevertheless, three months after the reapplication of the bioremediation treatment at the source zone (time 24), area 5.B20-ANM showed the greatest number of specific degradation genes and respective relative abundance.

Similar results were shown by the PERMANOVA ($P<0.05$) and ordination analyses (Figure S3), where a shift in the specific functional profiles was observed after the bioremediation treatment at source zone (time 12), mainly in areas 4.E25-BAN and 7.E10-BAS, biostimulated with electron acceptors, and in area 8.B20-BAA, submitted to biostimulation with air and bioaugmentation through EALR reactor. Contrarily, area 5.B20-ANM did not show different specific functional profiles between time 0 and time 12 (Figures S2-S3).

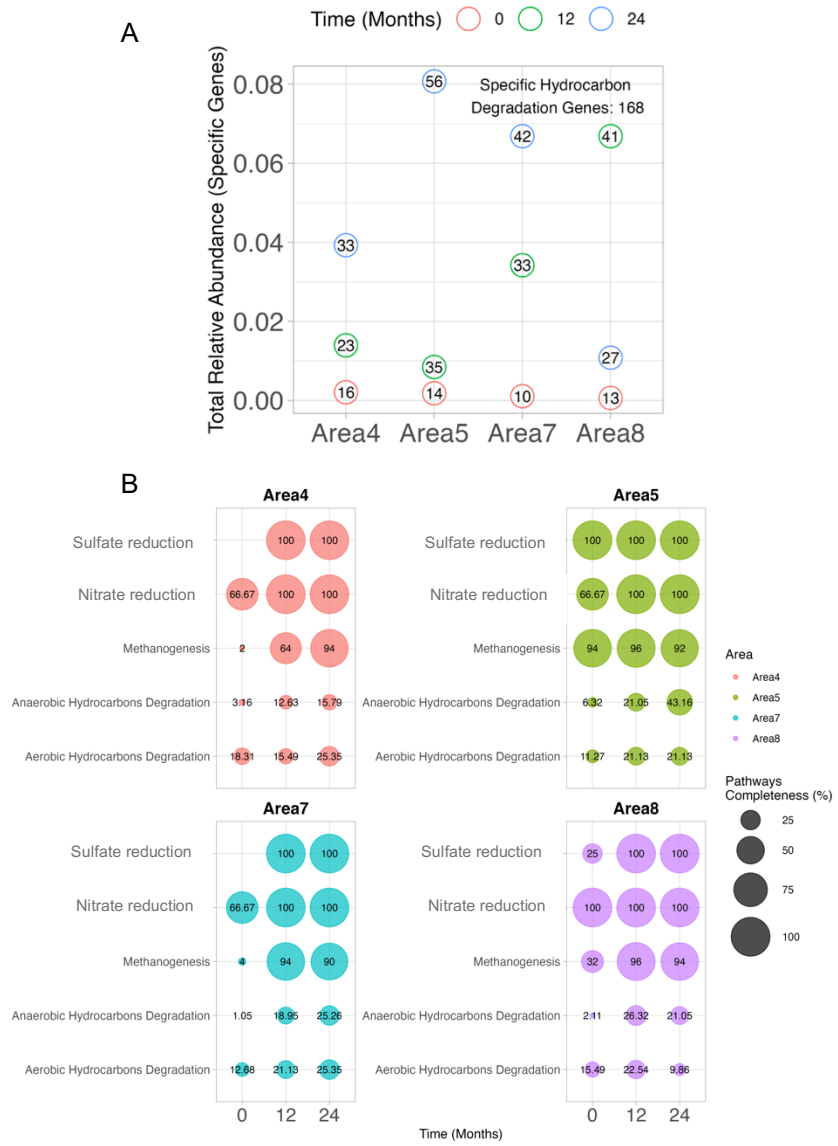


Figure 3. A) Relative abundance of specific hydrocarbon degradation genes (see Table S3) in relation to the total abundance of annotated genes in each area at times 0 and 12 (before and twelve months after the bioremediation treatment at source zone), and 24 (3 months after the reapplication of the bioremediation treatment at source zone) (bubble colors). The numbers inside the bubbles represent the number of specific genes in each area and time. B) Specific pathway completeness (number of genes found/ total number of genes in the metabolic pathway)

An assessment of the specific functional potential of the metagenomes was based on the distribution of the KEGG metabolic pathways in the areas before and after bioremediation treatments at source zone. Different aerobic and anaerobic hydrocarbon degradation pathways were enriched or not after the application of the bioremediation treatments in the areas under study (Figure S4). As it was shown above, in area 5.B20-ANM, the hydrocarbon degradation metabolisms increased only after the reapplication of

the bioremediation treatment at source zone (time 24). On the other hand, in area 8.B20-BAA, the number and abundance of all pathways decreased at time 24. The biostimulation with electron acceptors (4.E25-BAN and 7.E10-BAS) was able to enrich aerobic and anaerobic metabolisms, such as the ones for the degradation of alkanes, benzoate, monoaromatics such as BTEX, p-cresol and p-cymene, and di-aromatics like naphthalene.

3.4. Relationships between BTEX concentrations and hydrocarbon degradation genes

The influence of the BTEX concentrations on the hydrocarbon degradation gene abundance in the samples was explored by performing a canonical correlation analysis (CCA) (Figure 4). The four BTEX compounds (benzene, toluene, ethylbenzene and xylene) were significantly related to changes in hydrocarbon degradation gene abundances based on forward model selection ($p < 0.05$) and explained 33.56% of the variation. There was also a large proportion of variance that could not be explained, indicating that unmeasured biotic and abiotic environmental factors also play important roles in shaping hydrocarbon degradation gene content. Axis 1 accounted for 27.57% of the explained variance and axis 2 for 19.99%. BTEX compounds had a strong positive correlation with the first axis, and they contributed equally to the sample distribution. Samples from area 5.B20-ANM before the bioremediation treatment at the source zone (time 0) and after 12 months (time 12) were positively correlated with the BTEX concentrations, as well as samples from area 7.E10-BAS before the treatment, especially with toluene concentration. The other samples and most of the genes were ordered in relation to low BTEX concentrations, suggesting that the high relative abundance of the degradation genes may positively influence BTEX removal. On the other hand, few genes related with anaerobic degradation were related with the samples with high BTEX concentrations, suggesting that the limited number of genes did not confer metabolic potential for BTEX removal in these samples.

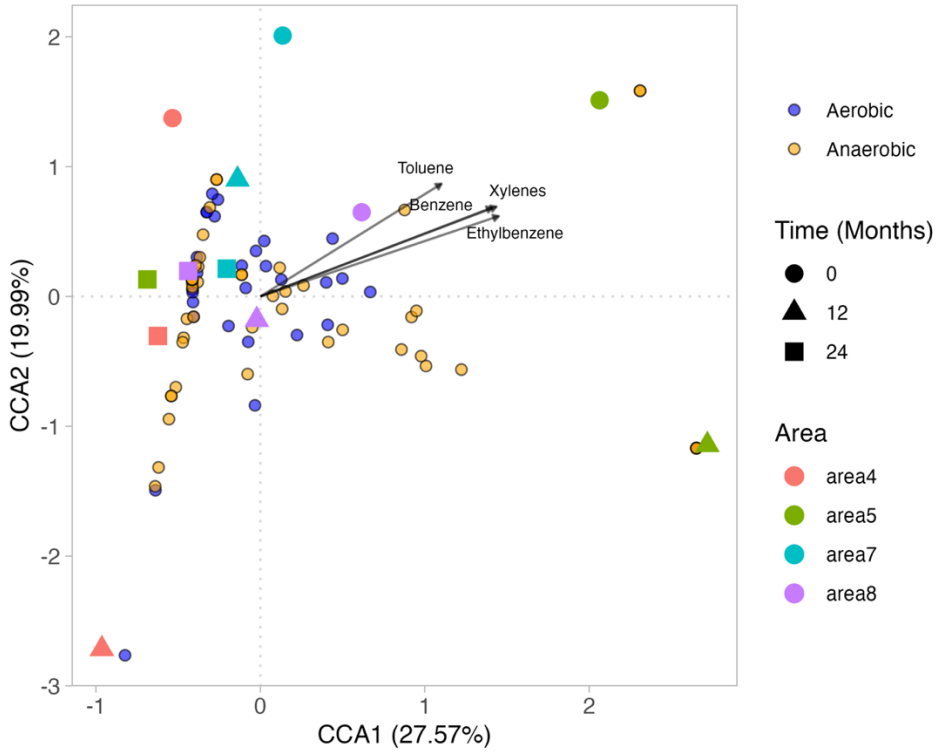


Figure 4. Canonical correlation analysis (CCA) of hydrocarbons degradation genes and BTEX concentration in the areas. Small blue and yellow points represent the genes from aerobic and anaerobic metabolisms, respectively.

3.5. Bioremediation treatments enriched different degrading taxa and the same catabolic genes showing functional redundancy

In order to identify which were the key microorganisms involved in hydrocarbon degradation in the polluted areas, individual genes of the peripheral (activation mechanism) and central pathways were taxonomically assigned.

Our results showed that the main genes of the degradation pathways were enriched in all areas after the bioremediation treatment at source zone (time 12) (Figure 5). In areas 4.E25-BAN and 8.B20-BAA, no enrichment was observed at time 24 (three months after the reapplication of the bioremediation treatment at source zone). This is in accordance with the results of BTEX concentrations in soil and groundwater from the source zones (Table S4), where the removal of these compounds was almost complete after the second bioremediation treatment.

Toluene Anaerobic Degradation (fumarate addition)

The toluene anaerobic degradation pathway first involves the fumarate addition as an activation mechanism, catalyzed by the enzyme benzylsuccinate synthase (encoded by *bss*ABCD genes) (Hermuth et al., 2002; Kuntze et al., 2011; von Netzer et al., 2016) (Figure 5). Additionally, methylation of benzene to toluene by an unknown enzyme has been proposed (Ulrich et al., 2005). For that reason, depending on the circumstances, the *bss*ABCD genes could also be used as indicators of the benzene methylation activation mechanism. In areas polluted with biodiesel/diesel blend (5.B20-ANM and 8.B20-BAA), the most important family involved with the fumarate addition stage was Geobacteraceae (Figure 5). In area 5, Desulfobacteraceae also had a relevant role in the toluene conversion. In the gasohol area 4.E25-BAN, Peptococcaceae and Ruminococcaceae were the only known families assigned to the functional genes. While in 7.E10-BAS area, besides Peptococcaceae, Geobacteraceae together with the archaea Methanobacteriaceae were involved in the step of fumarate addition in the toluene metabolism at time 24, to produce benzylsuccinate. Genes *bbs*EF and *bbs*H, of the intermediate metabolism, were only found after the bioremediation treatment in area 7.E10-BAS, and were assigned to the families Rubrobacteraceae and Peptococcaceae, respectively. Genes *bbs*AB, encoding the benzoylsuccinyl-CoA thiolase subunits A and B, which catalyzes the conversion of the benzoylsuccionyl-CoA to benzoyl-CoA (von Netzer et al., 2016), the central intermediate of the monoaromatic hydrocarbon anaerobic degradation, were found mainly in area 5.B20-ANM and had their abundance increased after the bioremediation treatment. Desulfobacteriaceae and Geobacteriaceae were also the most important families in this last stage of the toluene anaerobic degradation.

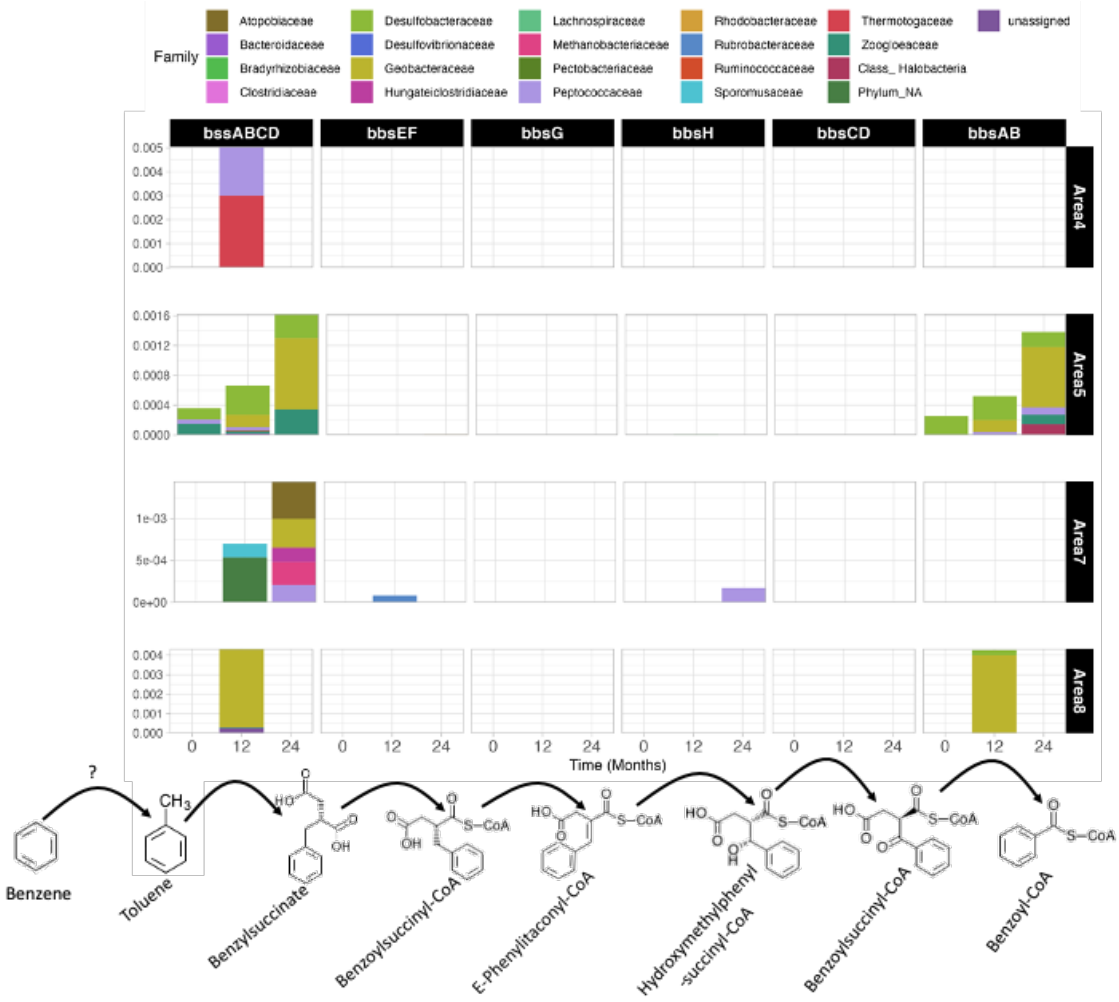


Figure 5. Relative abundance of genes encoding enzymes involved in toluene peripheral metabolism (fumarate addition) identified in each area before and after the bioremediation treatment and classified at family level.

Benzene Anaerobic Degradation (carboxylation)

For benzene, in addition to methylation, other two activation mechanisms have been proposed: i) conversion to benzoate through carboxylation via putative anaerobic benzene carboxylase (Kunapuli et al., 2008; Luo et al., 2014); and ii) hydroxylation to phenol by an oxygen-independent enzyme (Zhang et al., 2013).

In the benzene carboxylation reaction, the benzoate is ligated to acetyl-CoA by benzoate-CoA ligase, encoded by *bad*A gene, and 4-hydroxybenzoate-CoA ligase, encoded by *hba*A gene (Figure S5). These genes were found in all areas only after the bioremediation treatment. The most important families assigned to these genes were: Desulfobacteraceae, Ktedonosporobacteraceae Bradyrhizobiaceae, Comamonadaceae,

Syntrophaceae, Alcaligenaceae and Geobacteraceae, among others. Interestingly, the diversity of families involved in the conversion of benzoate increased at time 24 (three months after the reapplication of the bioremediation treatment at source zone).

Benzene Anaerobic Degradation (hydroxylation)

The third benzene activation mechanism is the hydroxylation to phenol (Figure S6). In this reaction, phenol is converted to 4-hydroxybenzoate by 4-hydroxybenzoate decarboxylase subunits C and D, encoded by genes *bsd*C and *bsd*D (Espinoza-Tofalos et al., 2020). These genes were found mainly in areas 7.E10-BAS and 8.B20-BAA. The most relevant family in this conversion was Clostridiaceae, followed by Bacillaceae. The next step in the pathway is the conversion to 4-hydroxybenzoyl-CoA by the 4-hydroxybenzoate-CoA ligase (*hba*A gene). This gene was found in areas 4.E25-BAN and 5.B20-ANM, at time 24, harbored by members of families Bacillaceae, Pseudonocardiaceae and Comamonadaceae. Finally, the main intermediate benzoyl-CoA is produced by the reaction catalyzed by 4-hydroxybenzoyl-CoA reductase subunits gamma, alpha and beta (*hcr*CAB or also named *hba*BCD) (Durante-Rodríguez et al., 2018). The abundance of these genes increased in almost all areas after the treatment, especially at time 24 (three months after reapplication of bioremediation treatment at source zone). Geobacteraceae, Peptococcaceae and Streptomycetaceae were the families mostly affiliated to these genes.

Ethylbenzene Anaerobic Degradation

In the ethylbenzene degradation peripheral pathway (Figure S7), the first stage consists in the hydroxylation to 1-phenylethanol by the ethylbenzene hydroxylase subunits alpha, beta and gamma encoded by genes *ebd*A, *ebd*B and *ebd*C, respectively (Durante-Rodríguez et al., 2018). These genes were found in low abundance in area 5.B20-ANM at time 24, and were assigned to families such as Rhodocyclaceae, Thermoguttaceae and Sulfuricellaceae, among others. In the next stage, acetophenone is produced by the action of the (S)-1-phenylethanol dehydrogenase (*ped*) (Durante-Rodríguez et al., 2018). This gene had its relative abundance increased in areas 4.E25-BAN, 7.E10-BAS and 8.B20-BAA after the bioremediation treatment at source zone. Several families were involved in

this reaction, such as Bacillaceae, Hymenobacteraceae, Lactobacillaceae and Pseudomonadaceae, as well as members of the phylum Cyanobacteria, among others. Further, the enzyme acethophenone carboxylase (*apc*ABCD genes) catalyzes the conversion to benzoylacetate (Weidenweber et al., 2017). The abundance of these genes increased at time 24 in almost all areas, and they were affiliated to families such as Panibacillaceae, Methylobacteriaceae, Pseudonocardiaceae and Rhodobacteraceae, among others.

Benzoyl-CoA Anaerobic Degradation (central pathway)

The peripheral pathways converge to the main intermediate benzoyl-CoA, which will be subsequent dearomatized and finally oxidized by a β-oxidation-like reaction (Figure 6) (von Netzer et al., 2016). In all areas, the increase in the abundance of *bcr*ABCD/*bam*BC genes, responsible for the benzoyl-CoA dearomatization, was observed at time 12. Desulfobacteraceae, Rhodospirillaceae, Peptococaceae and Syntrophomonadaceae were the most relevant families assigned to these genes in areas 5.B20-ANM, 7.E10-BAS and 8.B20-BAA, while in area 4.E25-BAN were Bradyrhizobiaceae, Comamonadaceae and Zooglaceae. In the next stage, a β-oxidation-like reaction cleaves the ring until the production of acetyl-CoA (von Netzer et al., 2016). These genes were found in low abundance. In area 5, after the reapplication of the bioremediation treatment (time 24), the abundance of gene *oah*, that encodes the enzyme 6-oxocyclohex-1-ene-carbonyl-CoA hydrolase, increased, and were harbored mainly by members of the families Desulfobacteraceae, Geobacteraceae and Hyphomicrobiaceae.

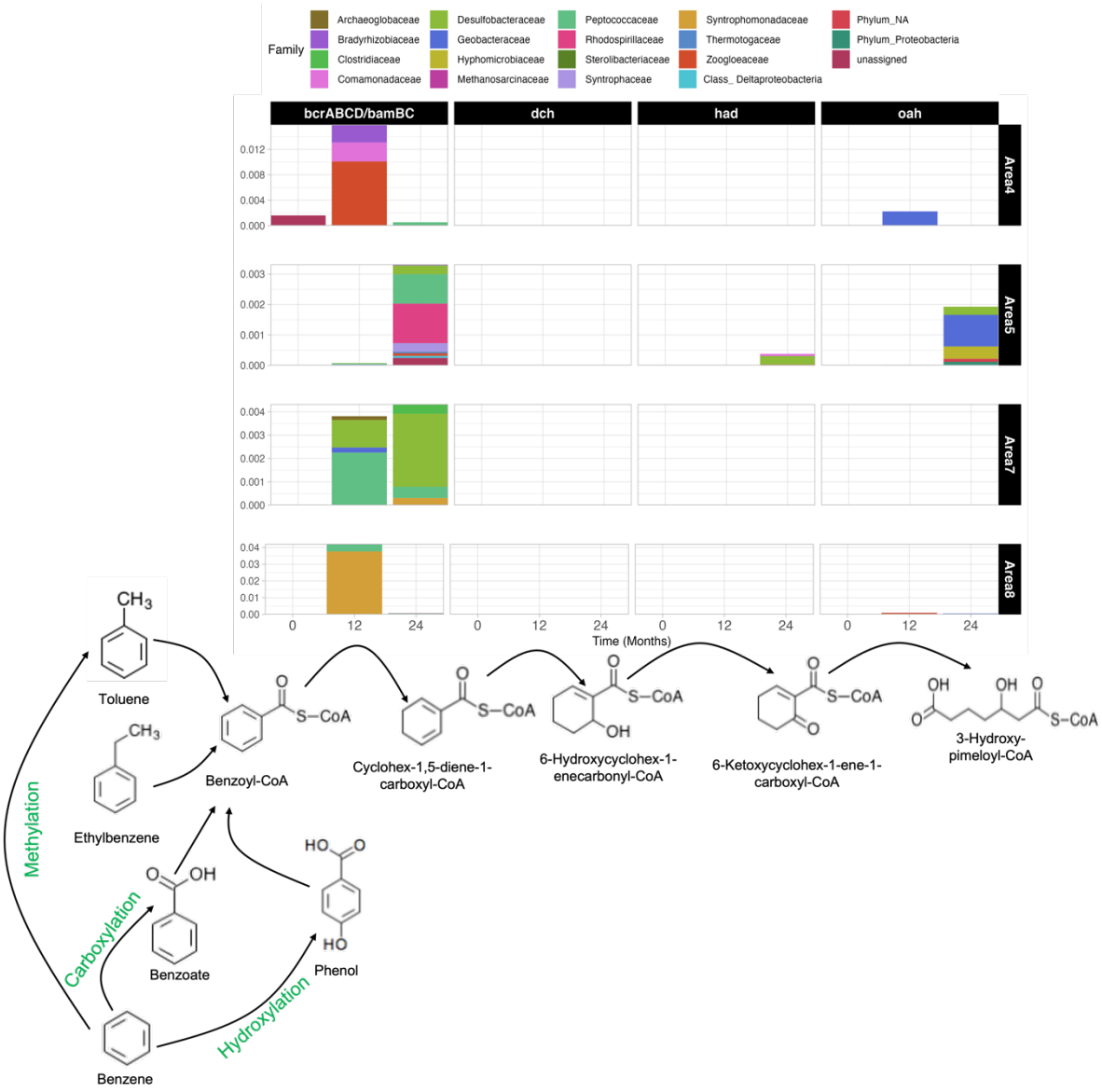


Figure 6. Relative abundance of genes encoding enzymes involved in benzoyl-CoA dearomatization and ring cleavage β-oxidation-like reactions identified in each area before and after the bioremediation treatment and classified at family level.

Aerobic Degradation of Monoaromatics

Phenol/toluene 2-monooxygenase (NADH), encoded by genes *dmp*KLMPNOP, is able to metabolize benzene to phenol and then to catechol, a central intermediate of aerobic aromatic hydrocarbon degradation (Figure 7) (Durán et al., 2019). This enzyme is also able to catalyze the conversion of toluene to 2-hydroxytoluene and then to 3-methylcatechol. These genes, assigned to families Bacillaceae and Alicyclobacillaceae, were found in all areas. However, their relative abundance decreased after the

bioremediation treatment. Gene *xyl*C codifies for the benzaldehyde dehydrogenase that converts the benzaldehyde (intermediate product of the toluene aerobic degradation) to benzoate (Li et al., 2020). This gene was affiliated to members of family Pseudomonadaceae, and it was found only in the gasohol areas before bioremediation. The benzoate is converted to cis-1,2-dihydroxyclohexa-3,5-diene-1-carboxylate by the benzoate/toluate 1,2-dioxygenase enzyme, encoded by genes *ben*ABC-*xyl*XYZ (Li et al., 2020). These genes were affiliated mainly to Oceanospirillaceae and Burkholderiaceae families, and their abundance decreased after bioremediation. Finally, catechol is produced by the enzyme dehydroxycyclohexadiene carboxylate dehydrogenase, encoded by gene *ben*D-*xyl*L (Li et al., 2020), which was found in low abundance after the treatment in area 4.E25-BAN and before in area 8.B20-BAA.

The dearomatization of catechol can be catalyzed by the di-oxygenase enzymes catechol 1,2-dioxygenase (gene *cat*A) or catechol 2,3-dioxygenase (gene *cat*E), producing cis-cis-muconate or 2-hydroxymuconate semialdehyde, respectively (Li et al., 2020). Gene *cat*E was more abundant in all areas, being mostly affiliated to Bacillaceae.

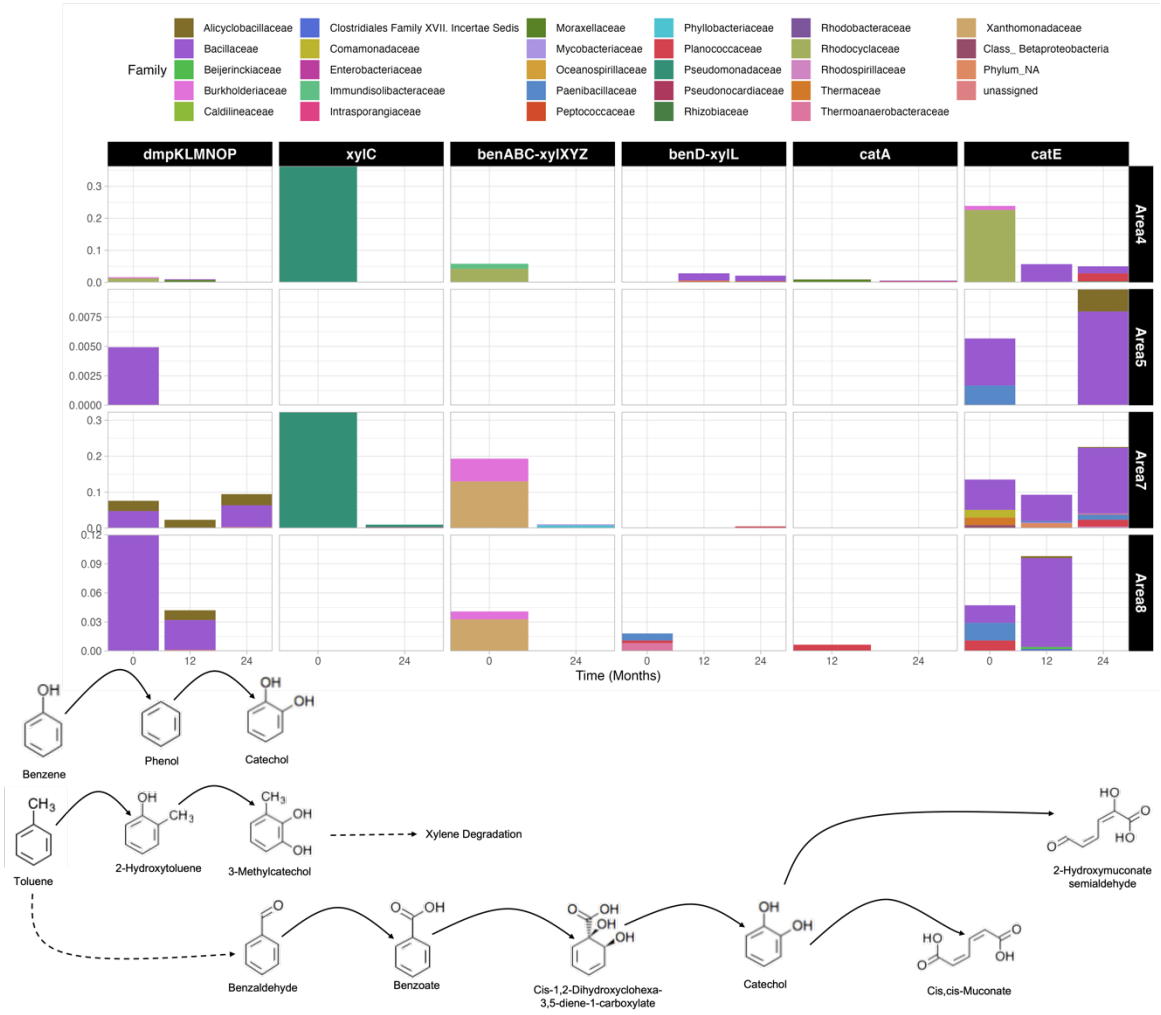


Figure 7. Relative abundance of genes encoding enzymes involved in monoaromatic hydrocarbon aerobic metabolism identified in each area before and after the bioremediation treatment and taxonomically assigned at family level.

Alkane Anaerobic Degradation

Fumarate addition is also known as an activation mechanism for anaerobic alkane degradation, carried out by the enzyme alkylsuccinate synthase. This enzyme is encoded by *ass*ABCD genes (von Netzer et al., 2016), which increased in all areas after the bioremediation treatment at source zone (time 12 and 24) (Figure S8). The main families assigned to this metabolism were Peptococcaceae, Clostridiaceae, Flavobacteraceae and Desulfomicrobiaceae. However, the families enriched by the biostimulation were different in each area and time.

Alkane Aerobic Degradation

Two enzymes responsible for alkane aerobic metabolism were found in the areas under study (Figure S9). The first one, alkane 1-monooxygenase, is encoded by gene *alk*B and performs the initial hydroxylation of C5-C12n-alkanes, oxidizing them to 1-alkonols (Van Beilen et al., 2003; Wang et al., 2021). Rhodocyclaceae, Legionellaceae and Chitinophagaceae were the families assigned to the aerobic degradation of alkanes in area 4.E25-BAN, while in area 7.E10-BAS, after bioremediation at source zone (time 12), the gene *alk*B was assigned to family Moraxellaceae. The second one, methane/ammonia monooxygenase, encoded by genes *pmo*ABC, are able to metabolize short-chain alkanes (C1-C5) (Wang et al., 2021). These genes were found in all areas before and after the bioremediation treatment at source zone. Members of phylum Thaumarchaeota, and from families Nitrosphaeraceae, Methylocystaceae and Methlycoccaceae, were the most important taxa affiliated to *pmo*ABC genes.

3.6. Specific functional profiles of the microbiome enriched in bioreactors

Specific functional profiles of the microbiome enriched in the reactors were assessed (Figure 8). Anaerobic genes were more abundant in both reactors than the aerobic ones. The pathways were more complete in area 8.B20-BAA' reactor, where benzene carboxylation peripheral pathway and benzoyl-CoA central pathway were enriched, showing complete functional potential to degrade benzene anaerobically. While in reactor 5, anaerobic alkane degradation via fumarate addition and aerobic toluene via benzaldehyde were the most enriched metabolisms. Additionally, in reactor 5, genes related with the degradation of p-cymene and p-cresol, two poly-substituted monoaromatic hydrocarbons, were found (Figure 8A). Regarding related metabolisms, methanogenesis was the most abundant metabolism in reactor 8. In reactor 5, in addition to methanogenesis, genes related to nitrate and sulfate reduction were also found (Figure 8B). As expected, the families affiliated to the specific functional profiles in the reactors were mainly methanogenic archaea, such as Methanobacteraceae and Methanosarcinaceae (Figure 8C). Clostridiaceae and Bradyrhizobiaceae were the bacteria more important in the reactors.

Figure 8. Specific functional profiles (Table S3) found in reactors airlift reactors installed in areas 5.B20-ANM and 8.B20-BAA. A) Voronoi chart showing the hydrocarbon degradation genes found; B) Sunburst chat showing the annotated genes of the related metabolisms (i.e., methanogenesis, nitrate and sulfate reduction); C) Families affiliated to the specific functional profiles.

3.7. Metabolic potential in MAGs

Co-assembly strategy and the four binning tools (i.e., MetaBat2, MaxBin, CONCOCT, BinSanity) yielded 90 bins, with 22 classified as high-quality genomes (Table S5). A general overview of the MAGs obtained and their associated information related to

completeness, contamination, taxonomic classification, and distribution are shown in Figure 9. Their relative abundance at phylum level is shown in Figure S10.

From the 90 MAGs yielded, 29 were assigned to the Archaea Domain, belonging to Methanobacteriota (6), Halobacteriota (12) and Thermoproteota (11). The bacterial MAGs were affiliated to the phyla Chlamydiota (5), Firmicutes (32), Proteobacteria (6), Desulfobacterota (2), Acidobacterota (3), Bacteroidota (8), Bdellovibrionota (1), Chloroflexota (1), Cyanobacteria (1), Myxococcota (1) and Nitrospirota (1) (Table S5). A total of 56 MAGs were recovered from the biodiesel polluted areas, 33 of them were unique in these areas. On the other hand, 37 MAGs were found in the gasohol polluted areas, 17 of them were exclusive. In general, MAGs from the biodiesel-polluted areas were mainly classified as archaea. Only three MAGs were unique in the air lift reactors, all of them classified as members of the obligate anaerobic order Oscillospirales.

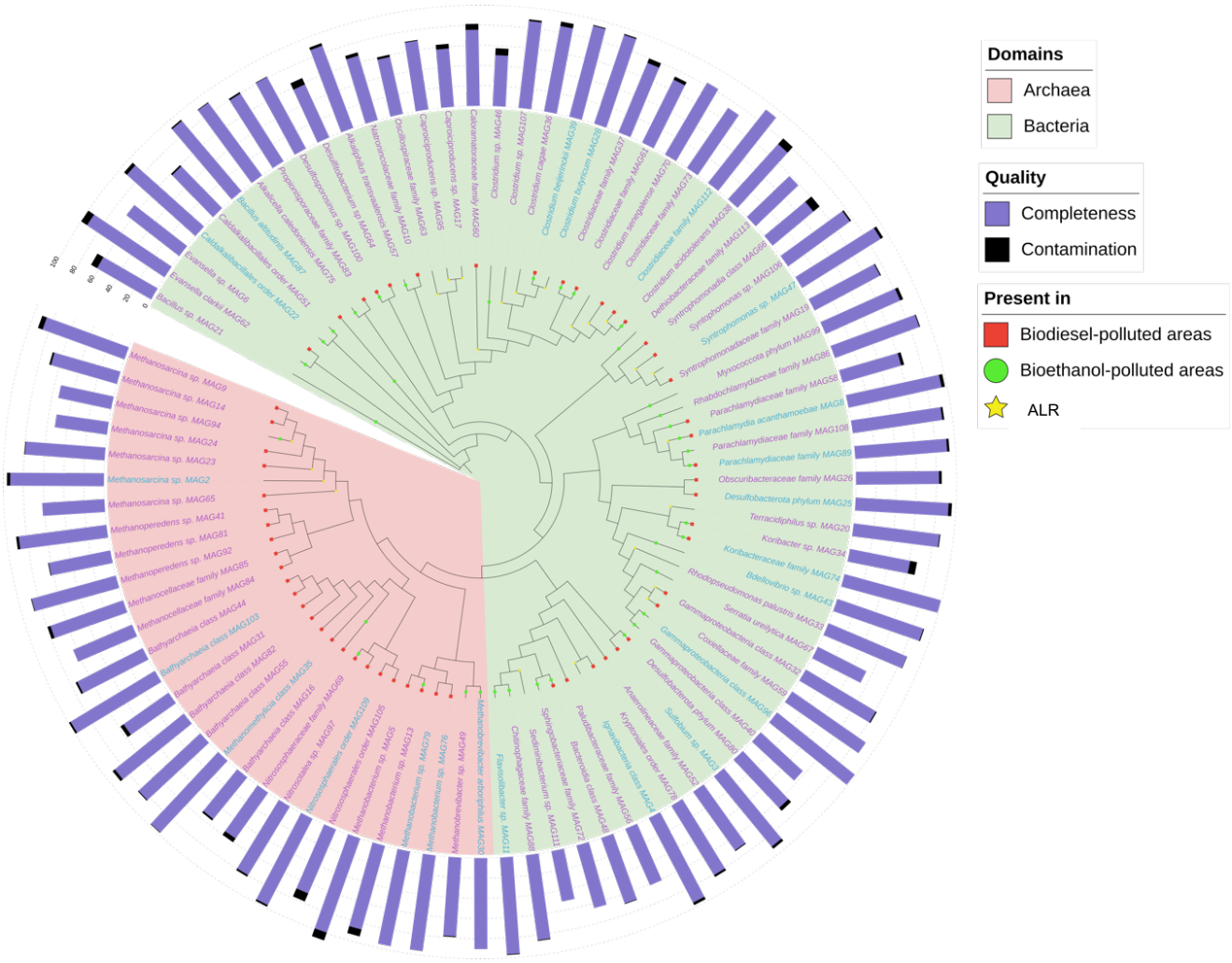


Figure 9. Phylogenetic tree of the 90 MAGs recovered from the biofuel/fossil fuel blend affected areas and the air lift reactors (ALR). The tree is decorated with colored backgrounds corresponding to domains, the labels represent the quality, being purple medium-quality and light blue high-quality genomes. The symbols in the branches correspond to the areas where they were found. Finally, bars indicate completeness and contamination values.

Functional annotations were performed in the archaeal MAGs in order to find the *mcr*A gene that encodes the methyl-coenzyme M reductase alpha subunit. Here, *mcr*A genes from four MAGs assigned to archaea were recovered for further phylogenetic analysis. A phylogenetic tree was constructed using these *mcr*A sequences and approximately 280 curated *mcr*A sequences recovered from PhyMet2 (Burdukiewicz et al., 2018). In addition to the *mcr*A sequences found, four *mcr*-like sequences belonging to NM1 (Candidatus Methanoliparia) and NM3 new lineages were included (Borrel et al., 2019). According to the phylogenetic analysis (Figure S11), none of the MAG *mcr*A sequences clustered with the NM1 or NM3 lineages.

4. DISCUSSION

Functional profiles of microbiomes from petrofuel-contaminated soils have been previously characterized (Bao et al., 2017; Pacwa-Płociniczak et al., 2016; Terrón-González et al., 2016). However, no literature reports focusing on the microbial functions in biofuel/fuel blend-polluted soils and their dynamics after bioremediation treatments at field-scale were found. Currently, it is consensus the main role that microorganisms play in biodegradation of petroleum hydrocarbons and the importance of bioremediation treatments for cleaning contaminated environments (Ite and Ibok, 2019). Thus, assessing the microbial communities in addition to the functional roles related to hydrocarbon degradation in the affected areas is crucial to evaluate the bioremediation efficiency and optimize protocols.

Herein, shotgun metagenomic analysis revealed that before the bioremediation treatment at the source zone, specific degradation functional profiles in long-term biofuel/fuel polluted areas encompassed a small array of hydrocarbon degradation genes. The presence of degradation genes of the main intermediate of BTEX aerobic and anaerobic degradation, such as benzaldehyde dehydrogenase (*xyl*C), catechol 2,3-dioxygenase (*cat*E), benzoate/toluate 1,2-dioxygenase (benABCD-xylXYZL) and phenol/toluene 2-monooxygenase (*dmp*LMNP), indicate the ability of the microbial community to potentially degrade BTEX and phenol compounds (El-Naas et al., 2014; Rabus et al., 2016) Similar results were obtained by Wu and colleagues (2023). They identified the potential of a bacterial consortium to degrade BTEX compounds by the presence of aerobic genes related with the metabolism of benzene, toluene and xylenes (Wu et al., 2023). Aerobic genes were more abundant than the anaerobic ones in all areas under study, since aerobic degradation is energetically more advantageous than the anaerobic one (Wartell et al., 2021). Alkane degradation potential was observed mainly in the biodiesel areas, since the diesel oil is composed of approximately 80-85% of saturated hydrocarbons such as alkanes and cycloalkanes (Bücker et al., 2018), while the gasoline has up to 35% of alkanes (Humans et al., 1989). The potential of alkane degradation was attributed to the presence of *pmo* genes that code for methane/ammonia monooxygenases. Besides methane oxidation, these monooxygenases are also related

with the degradation of short-chain alkanes (C2-C4) (Shao and Wang, 2013; van Beilen and Funhoff, 2007).

On the other hand, analysis of the related metabolisms (i.e., methanogenesis, nitrate and sulfate reduction) showed that methanogenesis was more important in the biodiesel areas (5.B20-ANM and 8.B20-BAA). Methanogenesis takes place in the absence of external electron acceptors such as oxygen, nitrate, sulfate or Fe(III) (Jiménez et al., 2016). Considering that no bioremediation treatment was applied in area 5.B20-ANM immediately after the blend release, electron acceptors could have been totally depleted during hydrocarbon degradation, further favoring the methanogenesis process. On the other hand, area 8.B20-BAA was firstly biostimulated with acetate (bioremediation treatment at the dissolved phase immediately after the blend release), in order to promote methanogenesis (Müller et al., 2017; Zhang and Lo, 2015). In addition, statistical and ordination analyses showed that specific functional profiles were different between the areas where a bioremediation treatment had been applied at the dissolved phase (areas 4.E25, 7.E10 and 8.B20) and the monitored natural attenuation area 5.B20. These results suggest that active bioremediation treatments cause shifts in the functional potential of the microbial communities. Similar results have been observed in previous studies, which showed the increased abundance of specific hydrocarbon degradation genes after the bioremediation treatment (Gielnik et al., 2021; Techtmann and Hazen, 2016).

After the bioremediation treatment at the source zone (time 12) and its reapplication (time 24), an increase in the number and relative abundance of hydrocarbon degradation genes was observed, suggesting that the bioremediation treatments were successful and able to influence the microbial and functional composition (Gielnik et al., 2021; Techtmann and Hazen, 2016). However, area 5.B20-ANM had a slightly different behavior, mainly at time 12 (twelve months after the bioremediation treatment at the source zone).As we discussed above and in our previous work (Hidalgo et al., 2023 submitted), this area has not been subjected to active bioremediation in the dissolved phase immediately after the release of the blend, allowing to thrive a microbial community likely adapted to the pollutants and that reached an equilibrium state. Additionally, this area presented the highest alpha diversity values at time 0, suggesting that the microbiome is highly resilient

(Griffiths et al., 2000; Shade et al., 2012; Van Elsas et al., 2012). This could have impacted the pollutant removal in this area, as shown by the BTEX concentrations in the source zone, since no decrease was observed.

Detailed analysis of metabolisms enriched in the areas after the bioremediation treatments at the source zones revealed functional potential to degrade aerobically and anaerobically aliphatic, mono-, di- and polyaromatic hydrocarbons, showing a wide repertoire of genes for degradation of petroleum hydrocarbons. Similar results were obtained by Zafra and collaborators (2016), where an increase in the abundance of genes related with aromatic hydrocarbon degradation and intermediate degradation pathways was observed after biostimulation of polycyclic aromatic hydrocarbon polluted soil, favoring hydrocarbon mineralization (Zafra et al., 2016). Other metagenomic studies have showed the increase of microbial degraders and functional genes after the application of bioremediation treatments, demonstrating the power of these techniques to enhance the pollutant degradation (Gao et al., 2021; Jung et al., 2016; Roy et al., 2018; Yergeau et al., 2012; Zafra et al., 2016).

Although different bioremediation treatments were applied at the source zone of the areas under study, overall, the same degradation pathways were enriched. Nevertheless, the enriched genes belonged to distinct taxa, showing that different microorganisms in the gasohol and biodiesel/diesel areas are responsible for the degradation of hydrocarbons, thus suggesting functional redundancy for contaminant removal independently of the pollutant and bioremediation type. Similar results had been observed in oil spill associated microbiomes (Bell et al., 2016; Morris et al., 2018; Mugge et al., 2021). Results suggested that anaerobic degradation of toluene and benzene in the areas under study are likely performed by members of the families Peptococcaceae, Geobacteraceae and Desulfobacteraceae, which are well-known for aromatic degradation (Hidalgo et al., 2020; Hidalgo et al., 2019). A previous work of our group based on metagenomics suggested the participation of these families in the degradation of toluene via fumarate addition in in-situ microcosms amended with toluene (Hidalgo et al., 2019). Herein, Clostridiaceae members were shown to be very relevant in the benzene/phenol degradation via activation by hydroxylation. Family Clostridiaceae comprises fermentative organisms able

to use hydrocarbons to produce acetate or formate and have been suggested to be involved in oil degradation (Sherry et al., 2013). Kunapuli and colleagues (2007) identified a Clostridiaceae member as responsible for the anaerobic benzene oxidation (Kunapuli et al., 2007). Peptococcaceae and Clostridiaceae members have been related with the degradation of benzene in syntrophy with sulfate reducers (Kleinsteuber et al., 2008; Steffi et al., 2010) and/or methanogenic archaea (Gieg et al., 2008; Starke et al., 2016). In a previous work of our group, Comamonadaceae members were very abundant in the gasohol biostimulated areas (Hidalgo et al., 2023 submitted), and herein they were associated to benzene and ethylbenzene degradation. Members of this family have been reported as benzene degraders coupled to nitrate (Xiong et al., 2012) and sulphate reduction (Aburto-Medina and Ball, 2015; Xiong et al., 2012). It has been proposed that Comamonadaceae species probably perform aerobic activation reactions and then complete mineralization of benzene coupled to nitrate reduction (Liu, 2015). On the other hand, ethylbenzene degradation genes belonged to different families, such as Paenibacillaceae and Pseudomonadaceae, among others. Family Paenibacillaceae has been more frequently related with PAH degradation due to the ability to produce biosurfactants (Mesbaiah et al., 2016; Timmusk et al., 2021). However, a genome from a *Paenibacillus* strain was shown to contain the ethylbenzene besides the PAH and atrazine degradation pathways (Chaudhry et al., 2013). Pseudomonadaceae members have been widely described as BTEX degraders (Bacosa et al., 2021; Dueholm et al., 2014; Khodaei et al., 2017). Benzoyl-CoA is the central intermediate of the anaerobic monoaromatic degradation and the main genes related to its transformation are *bcr*ABCD/*bam*BC (von Netzer et al., 2016). These and the genes downstream in the pathway were mostly assigned to Peptococcaceae, Clostridiaceae, Geobacteriaceae and Desulfobacteriaceae families, showing that members of these families are able to degrade monoaromatic hydrocarbons up to acetate (Hidalgo et al., 2020; Hidalgo et al., 2019).

The aerobic degradation of BTEX produces predominantly catechol via phenol or benzaldehyde (Peters et al., 2007). This metabolism was mostly affiliated to Bacillaceae family in all areas. The capability of Bacillaceae members to degrade different types of hydrocarbons has been reported and discussed in several papers (Kaida et al., 2018;

Margesin and Schinner, 2001; Perfumo et al., 2007), as well as their ability to produce biosurfactants (Mohanty et al., 2013).

The anaerobic alkane degradation pathway comprises fumarate addition by the enzyme alkane-succinate synthase, encoded by genes *ass*AD (von Netzer et al., 2016). This metabolism was enriched in all areas and associated mostly to Peptococcaceae and Clostridiaceae families, which are well-known degraders (Aburto-Medina and Ball, 2015; Zaan et al., 2012). On the other hand, the aerobic metabolism includes two types of enzymes, the alkane 1-monooxygenase and methane/ammonia monooxygenases, encoded by the genes *alk*B and *pmo*ABC, respectively. Gene *alk*B is widely used as indicator of potential microbial degradation (Bacosa et al., 2021; Gielnik et al., 2021; Khomarbaghi et al., 2019; Pacwa-Płociniczak et al., 2016; Richardson et al., 2015). Several studies have demonstrated the increase of *alk*B gene abundance after biostimulation (Dong et al., 2015; Gielnik et al., 2021; Khomarbaghi et al., 2019; Shahi et al., 2016) or bioaugmentation (Pacwa-Płociniczak et al., 2016) treatments. The first enzyme was mostly affiliated to phylum Thaumarchaeota, an archaeal group able to oxidize ammonia (Ke et al., 2014; Pester et al., 2011). *pmo*ABC genes are able to metabolize short-chain alkanes (C1-C5) (Wang et al., 2021).

The characterization of the functional potential of the microbiome from the airlift reactors (ALRs) installed in the biodiesel areas, internal and external loop reactors in areas 5.B20-ANM and 8.B20-BAA, respectively, revealed higher abundance and number of genes related with anaerobic degradation, despite the aeration of the reactors. The high relative abundance of members of Clostridiaceae family, well-known as glucose fermenters, and the presence of molasse in composition of the ALR culture medium, suggest anaerobic fermentation of glucose and other organic compounds to produce CO2 and hydrogen (Masset et al., 2012), which can be utilized by methanogenic archaea (Gieg et al., 2014).

The co-assembly strategy using four binning tools yielded 68 medium- and 22 high-quality MAGs. The 90 MAGs recovered were then selected for this study and they comprised a broad phylogenetic range of archaeal and bacterial phyla. According to the read mapping, 61 MAGs were unique in the biodiesel-, gasohol-contaminated areas or

ALR samples. Gasohol samples produced a higher number of MAGs in comparison with biodiesel areas, probably due to their lower microbial diversity (Hidalgo et al., 2023 submitted), which usually results in higher quality MAGs (Papudeshi et al., 2017). The archaeal MAGs selected for annotation included methanogenic lineages *Nitrosotalea* sp. MAG97 was only found in the biodiesel polluted areas. Several members of the Thermoproteota (previous Thaumarchaeota) phylum are known as ammonia-oxidizers (Kozlowski et al., 2016). Nitrosotalea devanaterra was cultivated and detected as an abundant lineage in many terrestrial ammonia oxidizing communities (Gubry-Rangin et al., 2011; Lehtovirta-Morley et al., 2011). The functional annotation showed that this MAG contains the genes *pmo*A and *pmo*B (data not shown). As discussed above, these genes have been related with short-chain alkane degradation (Wang et al., 2021). Several recent studies of the methyl-coenzyme M reductase complex (Mcr) in MAGs have shown that divergent *mcr*-like genes are involved in methane/alkane metabolism. NM3 lineage has been related with methyl-dependent hydrogenotrophic methanogenesis with the potential of using methanol and methanethiol (Borrel et al., 2019). While NM1 belongs to Ca. Methanoliparia and has been reported to be capable of both methane production and short-chain alkane degradation (Borrel et al., 2019; Laso-Pérez et al., 2019). The *mcr*A sequences recovered from the four archaeal MAGs were not closely related with NM1 or NM3 lineages (Figure S11), indicating that archaeal MAGs related with methanogenesis identified in this work are not involved with hydrocarbon degradation.

Conclusions

Functional assessment of metagenomes from biofuel/fossil fuel blend polluted soils allowed us to gain knowledge about the prevailing microbial metabolisms likely responsible for hydrocarbon degradation and the changes triggered by the distinct bioremediation treatments applied. Overall, the blend type did not influence the specific functional profiles in the microbiomes analyzed. Bioremediation treatments applied even many years after the contaminant release were shown to be a key driver of the functional profile. In addition, functional redundancy seems to be a microbial strategy used to cope with pollutant removal in a changing environment, since similar hydrocarbon pathways, harbored by distinct taxa, were enriched in soils submitted to different bioremediation

treatments. Some of the keystone species, identified in previous network analysis of the same samples, were related with hydrocarbon degradation metabolisms, highlighting their importance for bioremediation of biofuel/fossil fuel impacted areas. These species can be key targets for the design and optimization of future bioremediation strategies.

Supplementary information

Table S1. Phase 2 analyzed samples summary

Area	Before bioremediation treatment at sour zonee (t0)	Twelve months after (time 12)	Three months after reaplication (time 24)	Total samples
4.E25-BAN	3	3	3	9
7.E10-BAS	3	3	3	9
5.B20-ANM	3	3	3 (soil) 1 (reactor)	10
8.B20-BAA	3	3	3 (soil) 1 (reactor)	10

Table S2. Specific genes dataset

KO	Gene Name	Function	Pathway	Metabolism
K00496	AlkB	alkane 1-monooxygenase	Alkane	Aerobic
K20938	ladA	long-chain alkane monooxygenase	Alkane	Aerobic
K10944	pmoA	methane/ammonia monooxygenase subunit A	Alkane	Aerobic
K10945	pmoB	methane/ammonia monooxygenase subunit B	Alkane	Aerobic
K10946	pmoC	methane/ammonia monooxygenase subunit C	Alkane	Aerobic
K15760	tmoA	Toluene monooxygenase system protein A	Toluene	Aerobic
K15761	tmoB	Toluene monooxygenase system protein B	Toluene	Aerobic
K15762	tmoC	Toluene monooxygenase ferrodoxin subunit	Toluene	Aerobic
K15763	tmoD	Toluene monooxygenase system protein D	Toluene	Aerobic
K15764	tmoE	Toluene monooxygenase system protein E	Toluene	Aerobic
K15765	tmoF	Toluene monooxygenase electron transfer component	Toluene	Aerobic
K05797	pchF	4-cresol dehydrogenase (Hydroxylating) flavoprotein subunit	Toluene	Aerobic
K20200	pchC	4-cresol dehydrogenase (Hydroxylating) cytochrome subunit	Toluene	Aerobic
K20199	pchA	4-hydroxybenzaldehyde dehydrogenase (NADP+)	Toluene	Aerobic

K15757	xylM	Toluene methyl-monooxygenase	Toluene/ Xylene	Aerobic
K15758	xylA	Toluene methyl-monooxygenase electron transfer	Toluene/ Xylene	Aerobic
K00141	xylC	benzaldehyde dehydrogenase (NAD)	Toluene/ Xylene	Aerobic
K03380	E1.14.13.7	phenol 2-monooxygenase (NADPH)	Toluene/ Phenol	Aerobic
K03268	todC1	benzene/Toluene/chlorobenzene dioxygenase subunit alpha	Toluene/ Benzene	Aerobic
K16268	todC2	benzene/Toluene/chlorobenzene dioxygenase subunit beta	Toluene/ Benzene	Aerobic
K18089	todB	benzene/Toluene/chlorobenzene dioxygenase ferrodoxin component	Toluene/ Benzene	Aerobic
K18090	todA	benzene/Toluene/chlorobenzene dioxygenase ferrodoxin reductase component	Toluene/ Benzene	Aerobic
K16269	todD	cis-1,2-dihydrobenzene-1,2-diol/chlorobenzene dihydrodiol dehydrogenase	Toluene/ Benzene	Aerobic
K16249	dmpK	phenol/Toluene 2-monooxygenase	Toluene/ Benzene/ Phenol	Aerobic
K16243	dmpL	phenol/Toluene 2-monooxygenase (NADH) P1/A1	Toluene/ Benzene/ Phenol	Aerobic
K16244	dmpM	phenol/Toluene 2-monooxygenase (NADH) P2/A2	Toluene/ Benzene/ Phenol	Aerobic
K16242	dmpN	phenol/Toluene 2-monooxygenase (NADH) P3/A3	Toluene/ Benzene/ Phenol	Aerobic
K16245	dmpO	phenol/Toluene 2-monooxygenase (NADH) P4/A4	Toluene/ Benzene/ Phenol	Aerobic
K16246	dmpP	phenol/Toluene 2-monooxygenase (NADH) P5/A5	Toluene/ Benzene/ Phenol	Aerobic
K05549	benA-xylX	Benzoate/toluate 1,2-dioxygenase subunit alpha	Benzoate/ Xylene	Aerobic

K05550	benB-xylY	Benzoate/toluate 1,2-dioxygenase subunit beta	Benzoate/ Xylene	Aerobic
K05784	benC-xylZ	Benzoate/toluate 1,2-dioxygenase reductase component	Benzoate/ Xylene	Aerobic
K05782	benD-xylL	dihydroxycyclohexadiene carboxylate dehydrogenase	Benzoate/ Xylene	Aerobic
K03381	catA	catechol 1,2-dioxygenase	Benzoate	Aerobic
K00446	catE	catechol 2,3-dioxygenase	Benzoate/ Xylene	Aerobic
K14748	etbAa	Ethylbenzene dioxygenase subunit alpha	Ethylbenzene	Aerobic
K14749	etbAb	Ethylbenzene dioxygenase subunit beta	Ethylbenzene	Aerobic
K14750	etbAc	Ethylbenzene dioxygenase ferrodoxin component	Ethylbenzene	Aerobic
K14751	etbC	2,3-dihydroxyEthylbenzene 1,2-dioxygenase	Ethylbenzene	Aerobic
K18092	etbD	2-hydroxy-6-oxo-octa-2,4-dienoate hydrolase	Ethylbenzene	Aerobic
K14579	nahAc	Naphthalene 1,2-dioxygenase subunit alpha	Ethylbenzene/ PAH/ Naphthalene	Aerobic
K14580	nahAd	Naphthalene 1,2-dioxygenase subunit beta	Ethylbenzene/ PAH/ Naphthalene	Aerobic
K14578	nahAb	Naphthalene 1,2-dioxygenase ferredoxin component	Ethylbenzene/ PAH/ Naphthalene	Aerobic
K14581	nahAa	Naphthalene 1,2-dioxygenase ferredoxin reductase component	Ethylbenzene/ PAH/ Naphthalene	Aerobic
K14481	styA	styrene monooxygenase	styrene	Aerobic

K14482	styB	styrene monooxygenase reductase component	styrene	Aerobic
K07540	bss	benzylsuccinate synthase	Toluene/ Benzoate	Anaerobic
K07543	bbsE	benzylsuccinate CoA-transferase BbsE subunit	Toluene/ Benzoate	Anaerobic
K07544	bbsF	benzylsuccinate CoA-transferase BbsF subunit	Toluene/ Benzoate	Anaerobic
K07545	bbsG	(R)-benzylsuccinyl-CoA dehydrogenase	Toluene/ Benzoate	Anaerobic
K07546	bbsH	(E)-benzylidenesuccinyl-CoA hydratase	Toluene/ Benzoate	Anaerobic
K07547	bbsC	(2S)-[(R)-hydroxy(phenyl)methyl]-succinyl-CoA dehydrogenase BbsC subunit	Toluene/ Benzoate	Anaerobic
K07548	bbsD	(2S)-[(R)-hydroxy(phenyl)methyl]-succinyl-CoA dehydrogenase BbsD subunit	Toluene/ Benzoate	Anaerobic
K07549	bbsA	benzoylsuccinyl-CoA thiolase BbsA subunit	Toluene/ Benzoate	Anaerobic
K07550	bbsB	benzoylsuccinyl-CoA thiolase BbsB subunit	Toluene/ Benzoate	Anaerobic
K04112	bcrC	benzoyl-CoA reductase subunit C	Toluene/ Benzoate	Anaerobic
K04113	bcrB	benzoyl-CoA reductase subunit B	Toluene/ Benzoate	Anaerobic
K04114	bcrA	benzoyl-CoA reductase subunit A	Toluene/ Benzoate	Anaerobic
K04115	bcrD	benzoyl-CoA reductase subunit D	Toluene/ Benzoate	Anaerobic
K19515	bamB	benzoyl-CoA reductase subunit BamB	Toluene/ Benzoate	Anaerobic
K19516	bamC	benzoyl-CoA reductase subunit BamC	Toluene/ Benzoate	Anaerobic

K07437	dch	cyclohexa-1,5-dienecarbonyl-CoA hydratase	Toluene/ Benzoate	Anaerobic
K07538	had	6-hydroxycyclohex-1-ene-1-carbonyl-CoA dehydrogenase	Toluene/ Benzoate	Anaerobic
K07539	oah	6-oxocyclohex-1-ene-carbonyl-CoA hydrolase	Toluene/ Benzoate	Anaerobic
K04110	badA	Benzoate-CoA ligase	Benzoate	Anaerobic
K04105	hbaA	4-hydroxyBenzoate-CoA ligase	Benzoate/ Phenol	Anaerobic
K04107	hbaB	4-hydroxybenzoyl-CoA reductase subunit gamma	Benzoate/ Phenol	Anaerobic
K04108	hbaC	4-hydroxybenzoyl-CoA reductase subunit alpha	Benzoate/ Phenol	Anaerobic
K04109	hbaD	4-hydroxybenzoyl-CoA reductase subunit beta	Benzoate/ Phenol	Anaerobic
K01612	bsdC	vanillate/4-hydroxyBenzoate decarboxylase subunit C	Benzoate/ Phenol	Anaerobic
K21759	bsdD	vanillate/4-hydroxyBenzoate decarboxylase subunit D	Phenol	Anaerobic
K10700	ebdA	Ethylbenzene hydroxylase subunit alpha	Ethylbenzene	Anaerobic
K17048	ebdB	Ethylbenzene hydroxylase subunit beta	Ethylbenzene	Anaerobic
K17049	ebdC	Ethylbenzene hydroxylase subunit gamma	Ethylbenzene	Anaerobic
K14746	ped	(S)-1-phenylethanol dehydrogenase	Ethylbenzene	Anaerobic
K10701	apc	acetophenone carboxylase	Ethylbenzene	Anaerobic
K14747	bal	benzoylacetate-CoA ligase	Ethylbenzene	Anaerobic

K14599	dbfA1	dibenzofuran dioxygenase subunit Alpha	fluorene	Aerobic
K14601	fnlB	1,1a-dihydroxy-1-hydro-9-fluorenone dehydrogenase	fluorene	Aerobic
K14600	dbfA2	dibenzofuran dioxygenase subunit beta	fluorene	Aerobic
K14602	flnD1	2'-carboxy-2,3-dihydroxybiphenyl 1,2-dioxygenase large subunit	fluorene	Aerobic
K14603	flnD2	2'-carboxy-2,3-dihydroxybiphenyl 1,2-dioxygenase small subunit and ferredoxin fusion protein	fluorene	Aerobic
K14604	flnE	2-hydroxy-6-oxo-6-(2'-carboxyphenyl)-hexa-2,4-dienoate hydrolase	fluorene	Aerobic
K14582	nahB	cis-1,2-dihydro-1,2-dihydroxyNaphthalene/dibenzothiophene dihydrodiol dehydrogenase	Naphthalene	Aerobic
K11943	nidA	PAH dioxygenase large subunit	phenanthrene	Aerobic
K11944	nidB	PAH dioxygenase small subunit	phenanthrene	Aerobic
K18257	phdE	cis-3,4-dihydrophenanthrene-3,4-diol dehydrogenase	phenanthrene	Aerobic
K11945	phdF	extradiol dioxygenase	phenanthrene	Aerobic
K11946	phdG	hydratase-aldolase	phenanthrene	Aerobic
K11948	phdI	1-hydroxy-2-naphthoate dioxygenase	phenanthrene	Aerobic
K11949	phdJ	4-(2-carboxyphenyl)-2-oxobut-3-enoate aldolase	phenanthrene	Aerobic
K18275	phdK	2-formylBenzoate dehydrogenase	phenanthrene	Aerobic
K18068	pht3	phthalate 4,5-dioxygenase	phthalate	Aerobic

K18069	pht2	phthalate 4,5-dioxygenase reductase component	phthalate	Aerobic
K18251	phtAa	phthalate 3,4-dioxygenase subunit alpha	phthalate	Aerobic
K18252	phtAb	phthalate 3,4-dioxygenase subunit beta	phthalate	Aerobic
K18253	phtAc	phthalate 3,4-dioxygenase ferredoxin component	phthalate	Aerobic
K18254	phtAd	phthalate 3,4-dioxygenase ferredoxin reductase component	phthalate	Aerobic
K18255	phtB	phthalate 3,4-cis-dihydrodiol dehydrogenase	phthalate	Aerobic
K18067	pht4	phthalate 4,5-cis-dihydrodiol dehydrogenase	phthalate	Aerobic
K18256	phtC	3,4-dihydroxyphthalate decarboxylase	phthalate	Aerobic
K04102	pht5	4,5-dihydroxyphthalate decarboxylase	Naphthalene	Aerobic
K01670	nmsA	naphthyl-2-methylsuccinate synthase alpha subunit	Naphthalene	Anaerobic
K15567	nmsB	naphthyl-2-methylsuccinate synthase beta subunit	Naphthalene	Anaerobic
K15568	nmsC	naphthyl-2-methylsuccinate synthase gamma subunit	Naphthalene	Anaerobic
K15569	bnsE	naphthyl-2-methylsuccinate CoA transferase subunit	Naphthalene	Anaerobic
K15570	bnsF	naphthyl-2-methylsuccinate CoA transferase subunit	Naphthalene	Anaerobic
K15571	bnsG	naphthyl-2-methylsuccinyl-CoA dehydrogenase	Naphthalene	Anaerobic
K15572	bnsH	naphthyl-2-hydroxymethylsuccinyl-CoA hydratase	Naphthalene	Anaerobic

K15573	bnsC	naphthyl-2-hydroxymethylsuccinyl-CoA dehydrogenase BnsC subunit	Naphthalene	Anaerobic
K19958	bnsD	naphthyl-2-hydroxymethylsuccinyl-CoA dehydrogenase BnsD subunit	Naphthalene	Anaerobic
K15574	bnsA	naphthyl-2-oxomethyl-succinyl-CoA thiolase subunit	Naphthalene	Anaerobic
K15575	bnsB	naphthyl-2-oxomethyl-succinyl-CoA thiolase subunit	Naphthalene	Anaerobic
K14586	nmoAB	2-naphthoate monooxygenase	Naphthalene	Anaerobic
K14583	nahC	1,2-dihydroxyNaphthalene dioxygenase	Naphthalene	Anaerobic
A001	ppsB	Phenylphosphate_synthase_subunit_B_Thauera_aromatica_K172_CAC12686	Phenol	Anaerobic
A002	ppcB	PpcB_Aromatoleum_aromaticum_EbN1_CAI07885	Phenol	Anaerobic
A003	ped	Phenylethanol_dehydrogenase_Aromatoleum_aromaticum_EbN1_CAI07428	Ethylbenzene	Anaerobic
A004	bssD	BssD_Thauera_aromatica_K172_CAA05050	Toluene/ Benzoate	Anaerobic
A005	bssD	BssD_Aromatoleum_aromaticum_EbN1_CAI07157	Toluene/ Benzoate	Anaerobic
A006	assA	AssA_Smithella_sp_D17_KFZ44314	Alkane	Anaerobic
A007	ibsD	IbsD_Thauera_sp_pCyN2_AIS23706	p-Cymene	Anaerobic
A008	ppcA	PpcA_Aromatoleum_aromaticum_EbN1_CAI07883	Phenol	Anaerobic
A009	ppcB	PpcB_Aromatoleum_aromaticum_EbN1_CAI07885	Phenol	Anaerobic
A010	assD	AssD2_Desulfatibacillum_alkenivorans_AK_01_ACL03895	Alkane	Anaerobic

A011	HbsA	HbsA_Desulfobacula_toluolica_Tol2T_CCK78655	p-Cresol	Anaerobic
A012	ppsB	Phenylphosphate_synthase_subunit_B_Thauera_aromatica_K172_CAC12686	Ethylbenzene	Anaerobic
A013	ppcC	PpcC_Aromatoleum_aromaticum_EbN1_CAI07884	Phenol	Anaerobic
A014	apcC	ApcC_Aromatoleum_aromaticum_EbN1_Q5P5G4	Acetophenone	Anaerobic
A015	bssD	BssD_Azoarcus_sp_T_AAK50370	Toluene/ Benzoate	Anaerobic
A016	bssD	BssD_Geobacter_metallireducens_GS_15_ABB31775	Toluene/ Benzoate	Anaerobic
A017	assA	AssA2_Desulfatibacillum_alkenivorans_AK_01_ACL03892	Alkane	Anaerobic
A018	bbsA	Putative_BssA_Clostridia_bacterium_enrichment_culture_clone_BF_ADJ93876	Toluene/ Benzoate	Anaerobic
A019	assA	AssA_Peptococcaceae_bacterium_SCADC1_2_3_KFI38250	Alkane	Anaerobic
A020	pcmJ	PcmJ_functional_alpha_subunit_Geobacter_metallireducens_GS_15_ABB32355_Locustag_Gmet_2126	p-Cresol	Anaerobic
A021	bssC	BssC_Thauera_sp_DNT_1_BAC05500	Toluene/ Benzoate	Anaerobic
A022	nmsA	NmsA_Delta_proteobacterium_NaphS6_CAO72222	Naphthalene	Anaerobic
A023	assD	AssD1_Desulfatibacillum_alkenivorans_AK_01_ACL03425	Alkane	Anaerobic
A024	nmsA	NmsA_Delta_proteobacterium_NaphS2_and_NaphS3_CAO72219_and_CAO72220	Naphthalene	Anaerobic
A025	bssD	TutE_Thauera_aromatica_T1_AAC38452	Toluene/ Benzoate	Anaerobic
A026	assA	AssA1_Desulfatibacillum_alkenivorans_AK_01_ACL03428	Alkane	Anaerobic

A027	bssD	Putative_BssD_Magnetospirillum_sp_TS6_BAD42364	Toluene/ Benzoate	Anaerobic
A028	ebdB	EbdB_Aromatoleum_aromaticum_EbN1_CAI07431	Ethylbenzene	Anaerobic
A029	apcD	ApcD_Aromatoleum_aromaticum_EbN1_Q5P5G5	Acetophenone	Anaerobic
A030	apcB	ApcB_Aromatoleum_aromaticum_EbN1_Q5P5G3	Acetophenone	Anaerobic
A031	cmdB	CmdB_Aromatoleum_aromaticum_pCyN1_AIS23702	p-Cymene	Anaerobic
A032	bssD	BssD_Thauera_sp_DNT_1_BAC05499	Toluene/ Benzoate	Anaerobic
A033	apcA	ApcA_Aromatoleum_aromaticum_EbN1_Q5P5G2	Acetophenone	Anaerobic
A034	ebdA	EbdA_Azoarcus_sp_EB1_AAK76387	Ethylbenzene	Anaerobic
A035	ppsA	Phenylphosphate_synthase_subunit_A_Thauera_aromatica_K172_CAC12685	Ethylbenzene	Anaerobic
A036	pcmI	PcmI_alpha_prime_subunit_isoform_of_PcmJ_Geobacter_metallireducens_GS_15_ABB32354 _Locustag_Gmet_2125	p-Cresol	Anaerobic
A037	assA	AssA_Partial_sequence_Smithella_sp_SDB_KQC08433	Alkane	Anaerobic
A038	bssA	BssA_Desulfobacula_toluolica_Tol2_CCK78310	Toluene/ Benzoate	Anaerobic
A039	assA	AssA_Desulfosarcina_sp_BuS5_WP_027352796	Alkane	Anaerobic
A040	hbsD	HbsD2_Desulfobacula_toluolica_Tol2_CCK78652	p-Cresol	Anaerobic
A041	assA	AssA1_Smithella_sp_SCADC_KFO69021	Alkane	Anaerobic
A042	bssA	BssA_Magnetospirillum_sp_TS6_BAD42366	Toluene/ Benzoate	Anaerobic

A043	nmsD	NmsD_partial_Bacterium_enrichment_culture_clone_N47_ADB04295	Naphthalene	Anaerobic
A044	bssA	BssA_Azoarcus_sp_T_AAK50372	Toluene/ Benzoate	Anaerobic
A045	hbsD	HbsD1_Desulfobacula_toluolica_Tol2_CCK78651	p-Cresol	Anaerobic
A046	nmsA	NmsA_partial_Bacterium_enrichment_culture_clone	Naphthalene	Anaerobic
A047	pcmI	PcmI_alpha_prime_subunit_isoform_of_PcmJ_Geobacter_sp_FRC_32_ACM18493_Locustag_Geob_0119	p-Cresol	Anaerobic
A048	bssD	BssD_Desulfobacula_toluolica_Tol2_CCK78312	Toluene/ Benzoate	Anaerobic
A049	assA	AssA2_Smithella_sp_SCADC_KGL06511	Alkane	Anaerobic
A050	pcmJ	PcmJ_functional_alpha_subunit_Geobacter_sp_FRC_32_ACM18492_Locustag_Geob_0118	p-Cresol	Anaerobic
A051	assD	Putative_AssD_Smithella_sp_SCADC_KFO69022	Alkane	Anaerobic
A052	masG	MasG_Aromatoleum_sp_HxN1_CAO03077	Alkane	Anaerobic
A053	assD	AssD_Smithella_sp_SDB_KQC10643	Alkane	Anaerobic
K00125	fdhB	formate dehydrogenase (coenzyme F420) beta subunit	Methanogenesis	Anaerobic
K00200	fwdA	formylmethanofuran dehydrogenase subunit A	Methanogenesis	Anaerobic
K00201	fwdB	formylmethanofuran dehydrogenase subunit B	Methanogenesis	Anaerobic
K00202	fwdC	formylmethanofuran dehydrogenase subunit C	Methanogenesis	Anaerobic
K00203	fwdD	formylmethanofuran dehydrogenase subunit D	Methanogenesis	Anaerobic

K00204	fwdH	4Fe-4S ferredoxin	Methanogenesis	Anaerobic
K00205	fwdF	4Fe-4S ferredoxin	Methanogenesis	Anaerobic
K00319	mtd	methylenetetrahydromethanopterin dehydrogenase	Methanogenesis	Anaerobic
K00320	mer	5,10-methylenetetrahydromethanopterin reductase	Methanogenesis	Anaerobic
K00399	mcrA	methyl-coenzyme M reductase alpha subunit	Methanogenesis	Anaerobic
K00400	K00400	methyl coenzyme M reductase system, component A2	Methanogenesis	Anaerobic
K00401	mcrB	methyl-coenzyme M reductase beta subunit	Methanogenesis	Anaerobic
K00402	mcrG	methyl-coenzyme M reductase gamma subunit	Methanogenesis	Anaerobic
K03421	mcrC	methyl-coenzyme M reductase subunit C	Methanogenesis	Anaerobic
K03422	mcrD	methyl-coenzyme M reductase subunit D	Methanogenesis	Anaerobic
K00577	mtrA	tetrahydromethanopterin S-methyltransferase subunit A	Methanogenesis	Anaerobic
K00578	mtrB	tetrahydromethanopterin S-methyltransferase subunit B	Methanogenesis	Anaerobic
K00579	mtrC	tetrahydromethanopterin S-methyltransferase subunit C	Methanogenesis	Anaerobic
K00580	mtrD	tetrahydromethanopterin S-methyltransferase subunit D	Methanogenesis	Anaerobic
K00581	mtrE	tetrahydromethanopterin S-methyltransferase subunit E	Methanogenesis	Anaerobic
K00582	mtrF	tetrahydromethanopterin S-methyltransferase subunit F	Methanogenesis	Anaerobic

K00583	mtrG	tetrahydromethanopterin S-methyltransferase subunit G	Methanogenesis	Anaerobic
K00584	mtrH	tetrahydromethanopterin S-methyltransferase subunit H	Methanogenesis	Anaerobic
K00672	ftr	formylmethanofuran--tetrahydromethanopterin N-formyltransferase	Methanogenesis	Anaerobic
K01499	mch	methenyltetrahydromethanopterin cyclohydrolase	Methanogenesis	Anaerobic
K03388	hdrA2	heterodisulfide reductase subunit A2	Methanogenesis	Anaerobic
K03389	hdrB2	heterodisulfide reductase subunit B2	Methanogenesis	Anaerobic
K03390	hdrC2	heterodisulfide reductase subunit C2	Methanogenesis	Anaerobic
K04480	mtaB	methanol--5-hydroxybenzimidazolylcobamide Co-methyltransferase	Methanogenesis	Anaerobic
K08264	hdrD	heterodisulfide reductase subunit D	Methanogenesis	Anaerobic
K08265	hdrE	heterodisulfide reductase subunit E	Methanogenesis	Anaerobic
K11260	fwdG	4Fe-4S ferredoxin	Methanogenesis	Anaerobic
K11261	fwdE	formylmethanofuran dehydrogenase subunit E	Methanogenesis	Anaerobic
K13942	hmd	5,10-methenyltetrahydromethanopterin hydrogenase	Methanogenesis	Anaerobic
K14080	mtaA	[methyl-Co(III) methanol/glycine betaine-specific corrinoid protein]:coenzyme M methyltransferase	Methanogenesis	Anaerobic
K14081	mtaC	methanol corrinoid protein	Methanogenesis	Anaerobic
K14082	mtbA	[methyl-Co(III) methylamine-specific corrinoid protein]:coenzyme M methyltransferase	Methanogenesis	Anaerobic

K14083	mttB	trimethylamine---corrinoid protein Co-methyltransferase	Methanogenesis	Anaerobic
K14084	mttC	trimethylamine corrinoid protein	Methanogenesis	Anaerobic
K14126	mvhA	F420-non-reducing hydrogenase large subunit	Methanogenesis	Anaerobic
K14127	mvhD	F420-non-reducing hydrogenase iron-sulfur subunit	Methanogenesis	Anaerobic
K14128	mvhG	F420-non-reducing hydrogenase small subunit	Methanogenesis	Anaerobic
K16176	mtmB	methylamine---corrinoid protein Co-methyltransferase	Methanogenesis	Anaerobic
K16177	mtmC	monomethylamine corrinoid protein	Methanogenesis	Anaerobic
K16178	mtbB	dimethylamine---corrinoid protein Co-methyltransferase	Methanogenesis	Anaerobic
K16179	mtbC	dimethylamine corrinoid protein	Methanogenesis	Anaerobic
K22480	hdrA1	heterodisulfide reductase subunit A1	Methanogenesis	Anaerobic
K22481	hdrB1	heterodisulfide reductase subunit B1	Methanogenesis	Anaerobic
K22482	hdrC1	heterodisulfide reductase subunit C1	Methanogenesis	Anaerobic
K22516	fdhA	formate dehydrogenase (coenzyme F420) alpha subunit	Methanogenesis	Anaerobic
K00362	nirB	Disissimilatory nitrate reduction	Nitrogen Metabolism	Anaerobic
K00363	nirD	Disissimilatory nitrate reduction	Nitrogen Metabolism	Anaerobic
K03385	nrfA	Disissimilatory nitrate reduction	Nitrogen Metabolism	Anaerobic

K00394	aprA	Dissimilatory sulfate reduction	Sulfur Metabolism	Anaerobic
K00395	aprB	Dissimilatory sulfate reduction	Sulfur Metabolism	Anaerobic
K11180	dsrA	Dissimilatory sulfate reduction	Sulfur Metabolism	Anaerobic
K11181	dsrB	Dissimilatory sulfate reduction	Sulfur Metabolism	Anaerobic

Table S2. Metagenomic sequences and Nonpareil estimations

Co-Assembly	Time	Raw reads (Million)	Trimmed reads (Million)	Coverage	Diversity Index
4.E25-BAN	0	16.5	16	0.997	11.4
4.E25-BAN	12	16.1	15.6	0.914	14.7
4.E25-BAN	24	17.1	16.6	0.75	17.9
7.E10-BAS	0	17.8	17.3	0.997	11.4
7.E10-BAS	12	14.7	14.2	0.82	16.8
7.E10-BAS	24	18.3	17.7	0.798	16.6
5.B20-ANM	0	16.8	16.3	0.994	11.5
5.B20-ANM	12	16.9	16.5	0.958	15
5.B20-ANM	24	23.9	23.1	0.75	18.1
8.B20-BAA	0	15.5	15	0.985	11.5
8.B20-BAA	12	14	13.6	0.899	15.2
8.B20-BAA	24	17.3	16.8	0.867	15.3
Reactor 5	24	20.2	19.8	0.94	17.5
Reactor 8	24	23.8	23.3	0.845	18.9

Table S4. Soil source zone BTEX concentrations (mgL^{-1}) before and twelve months after the bioremediation application

BTEX (mg/L)	4.E25-BAN			5.B20-ANM			7.E10-BAS			8.B20-BAN		
	T0	T12	T24	T0	T12	T24	T0	T12	T24	T0	T12	T24
Benzene	< 0.008	< 0.008	< 0.008	0.716	0.97	< 0.008	0.744	0.032	< 0.008	0.114	< 0.008	< 0.008
Toluene	0.125	1.6	< 0.008	9.457	9.647	< 0.008	31.394	0.863	< 0.008	3.602	2.251	< 0.008
Ethylbenzene	0.219	0.644	< 0.008	14.261	16.099	< 0.008	2.796	0.177	< 0.008	7.725	0.566	< 0.008
Xylenes	0.803	1.035	< 0.008	44.398	49.656	< 0.008	30.255	30.25	< 0.008	20.858	1.366	< 0.008
Total BTEX	1.148	3.287	< 0.024	60.828	76.373	< 0.024	65.19	2.562	< 0.024	32.301	2.124	< 0.024

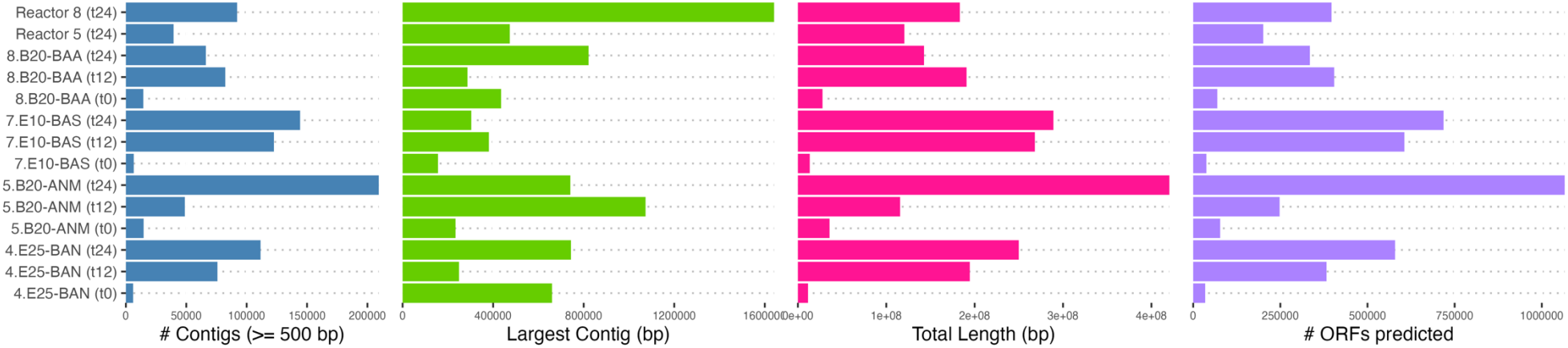


Figure S1. Coassembly and ORFs prediction statistics. Colors scale represent the lowest (red) and highest (green) from each coassembly statistics

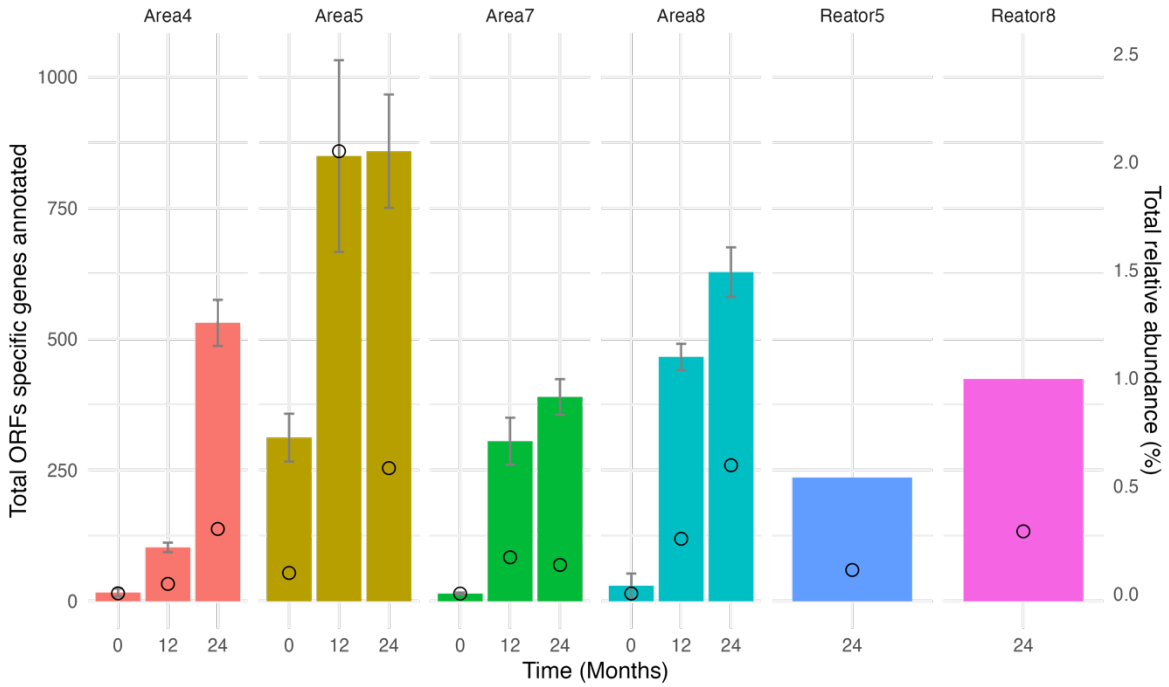


Figure S2. Impacted areas and reactors metagenomes. Detailed information about the samples and areas are presented in Table 2. Bars represent the total ORFs annotated to specific genes (Table S3) and circles represent the total relative abundance of the specific genes in each dataset.

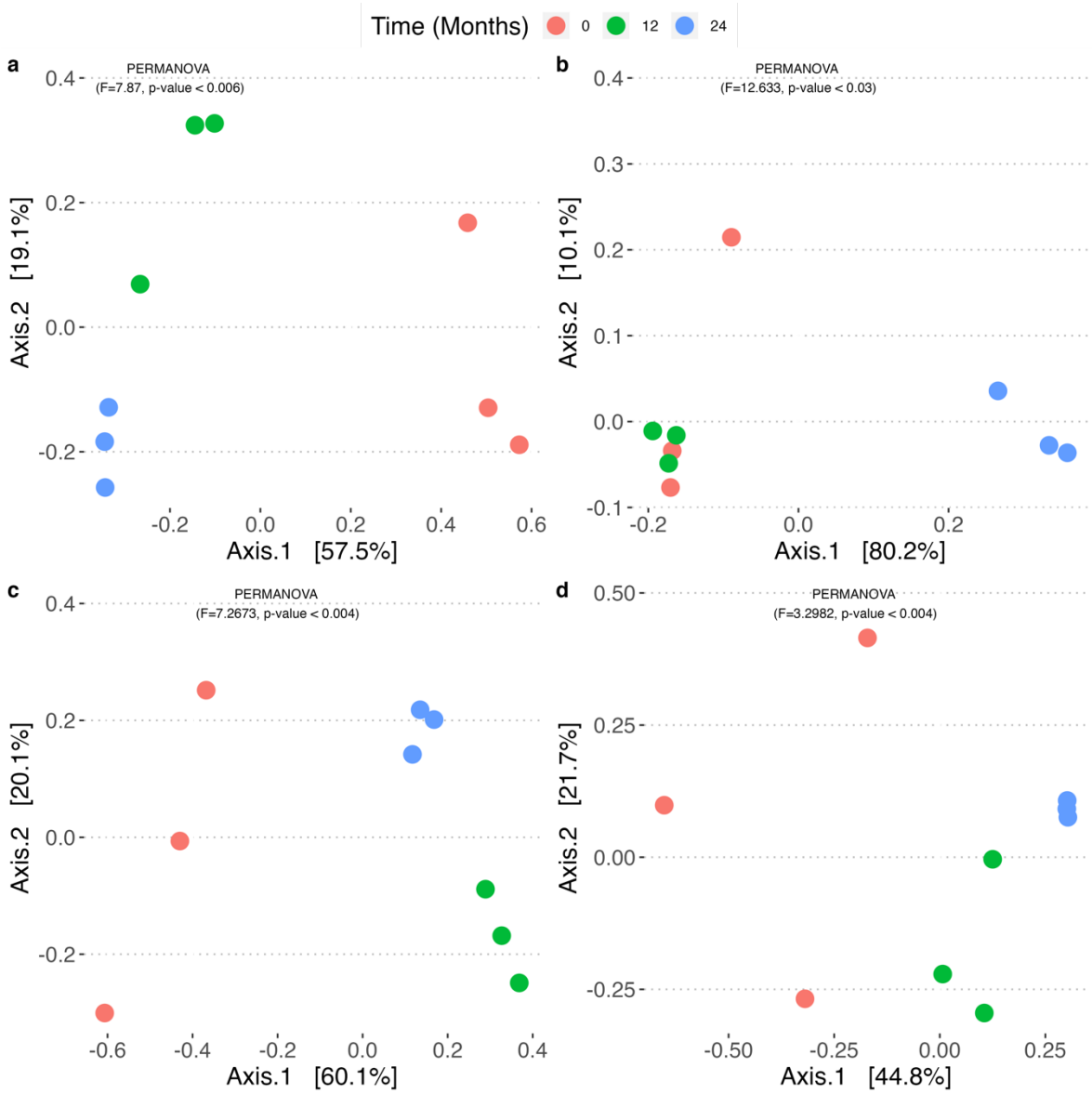


Figure S3. PCoA using Bray-Curtis distance by area and sampling year. a) Area 4.E25-BAN; b) area 5.B20-ANM; c) area 7.E10-BAS; d) area 8.B20-BAA.

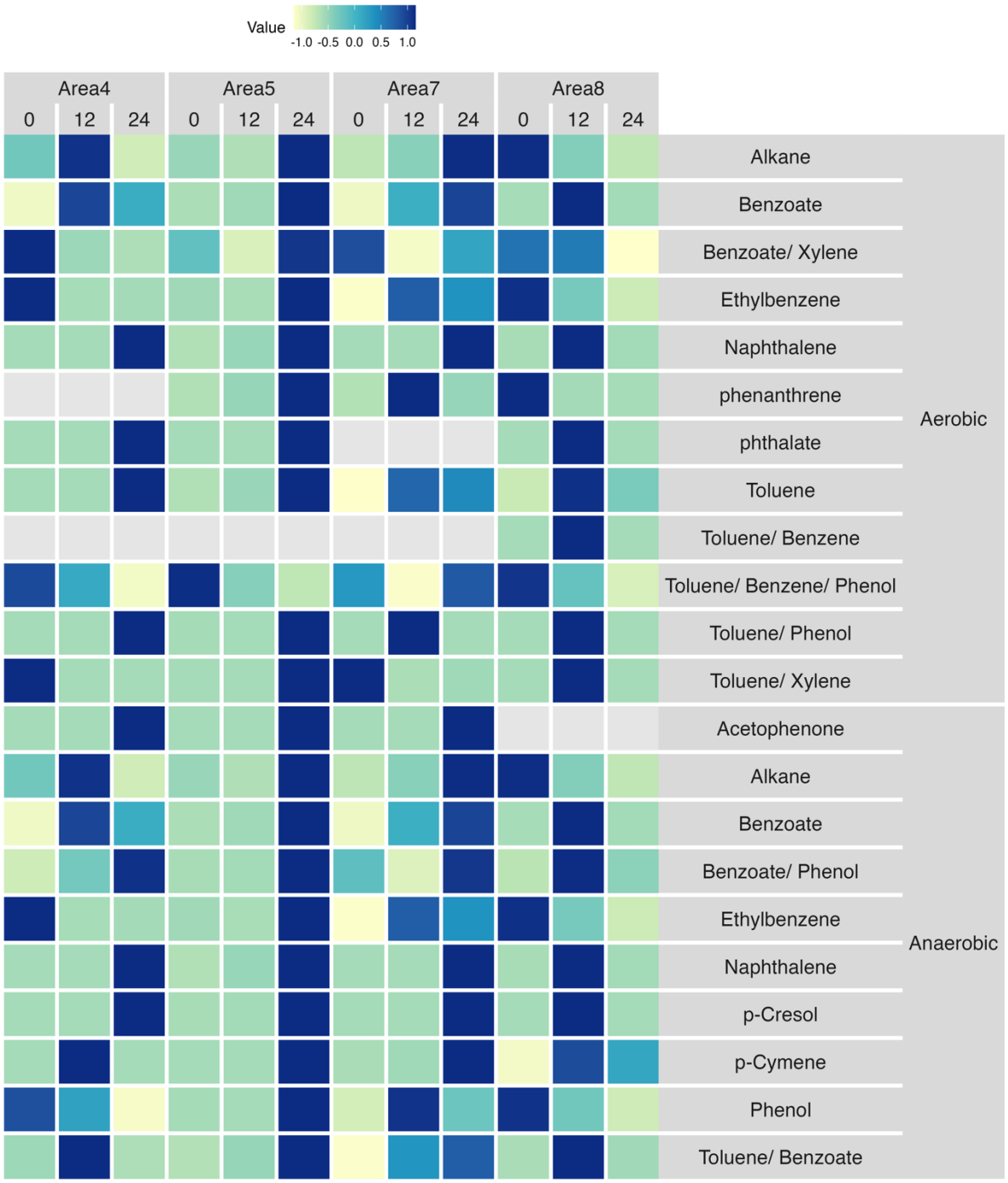


Figure S4. Aerobic and anaerobic pathways enrichment in the areas before the bioremediation application. Heatmap showing scaled relative abundance of the metabolisms according to the KEGG metabolic pathways and AnHyDeg databases (See Table S2).

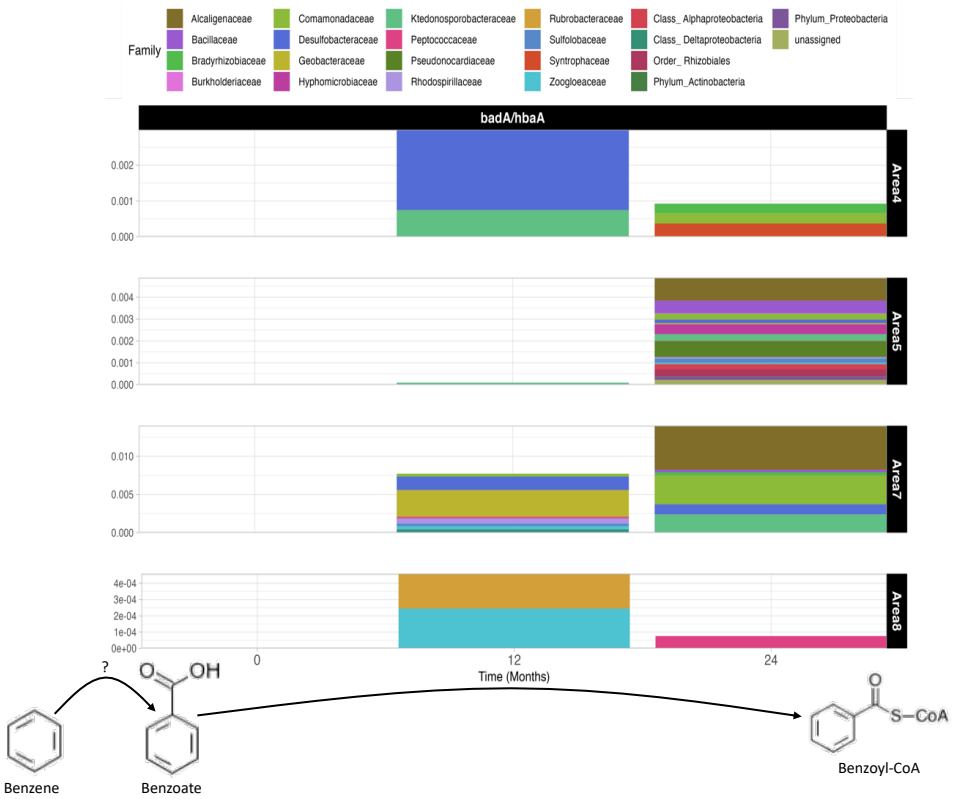


Figure S5. Relative abundance of genes encoding enzymes involved in benzene carboxylation peripheral metabolism identified in each area before and after the bioremediation application and classified at family level.

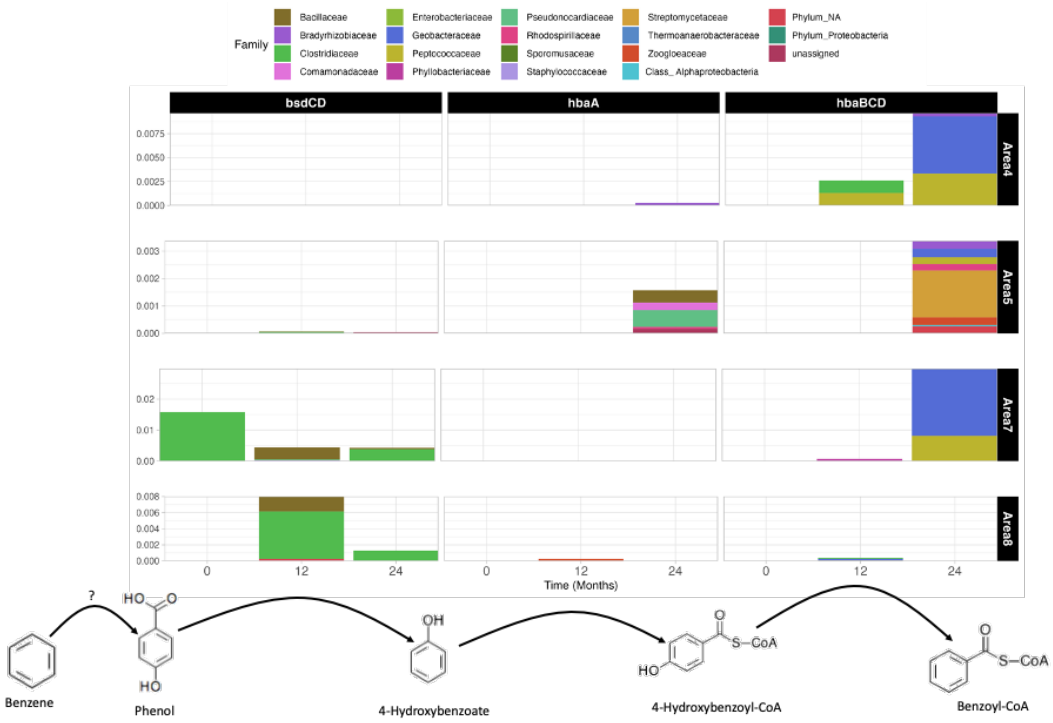


Figure S6. Relative abundance of genes encoding enzymes involved in benzene hydroxylation peripheral metabolism identified in each area before and after the bioremediation application and classified at family level.

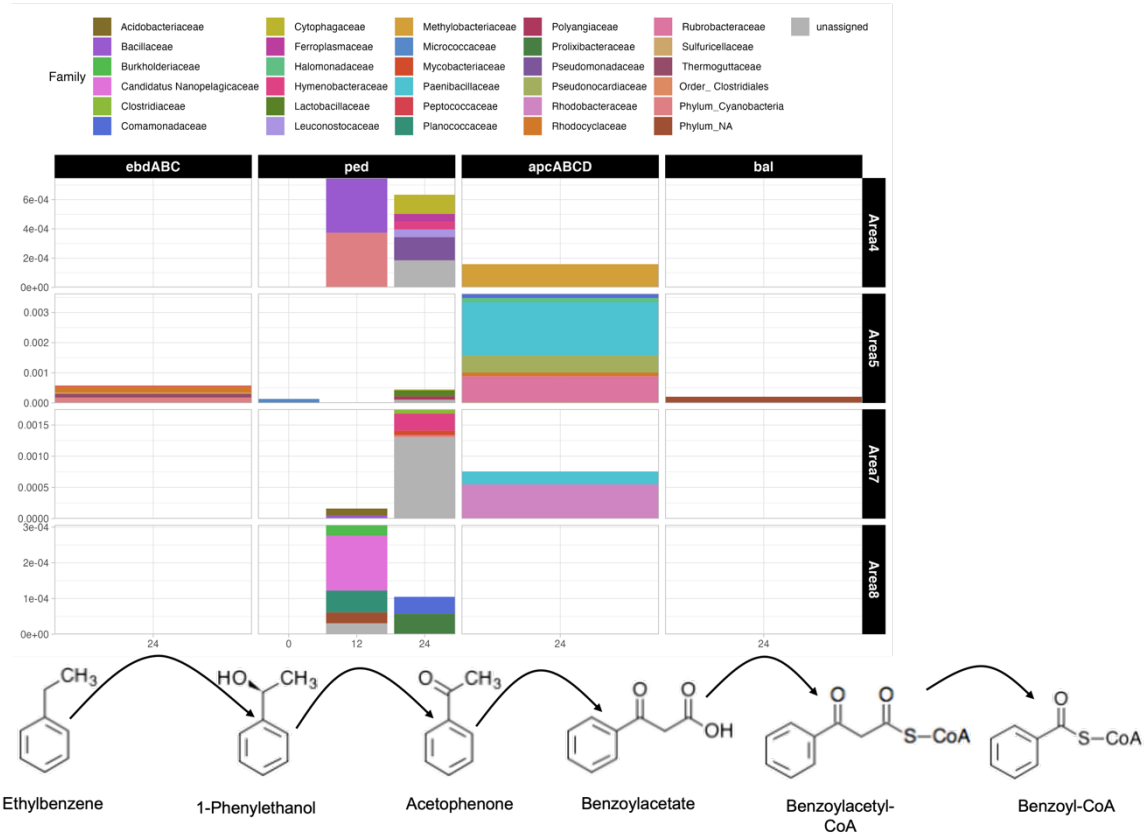


Figure S7. Relative abundance of genes encoding enzymes involved in ethylbenzene peripheral metabolism identified in each area before and after the bioremediation application and classified at family level.

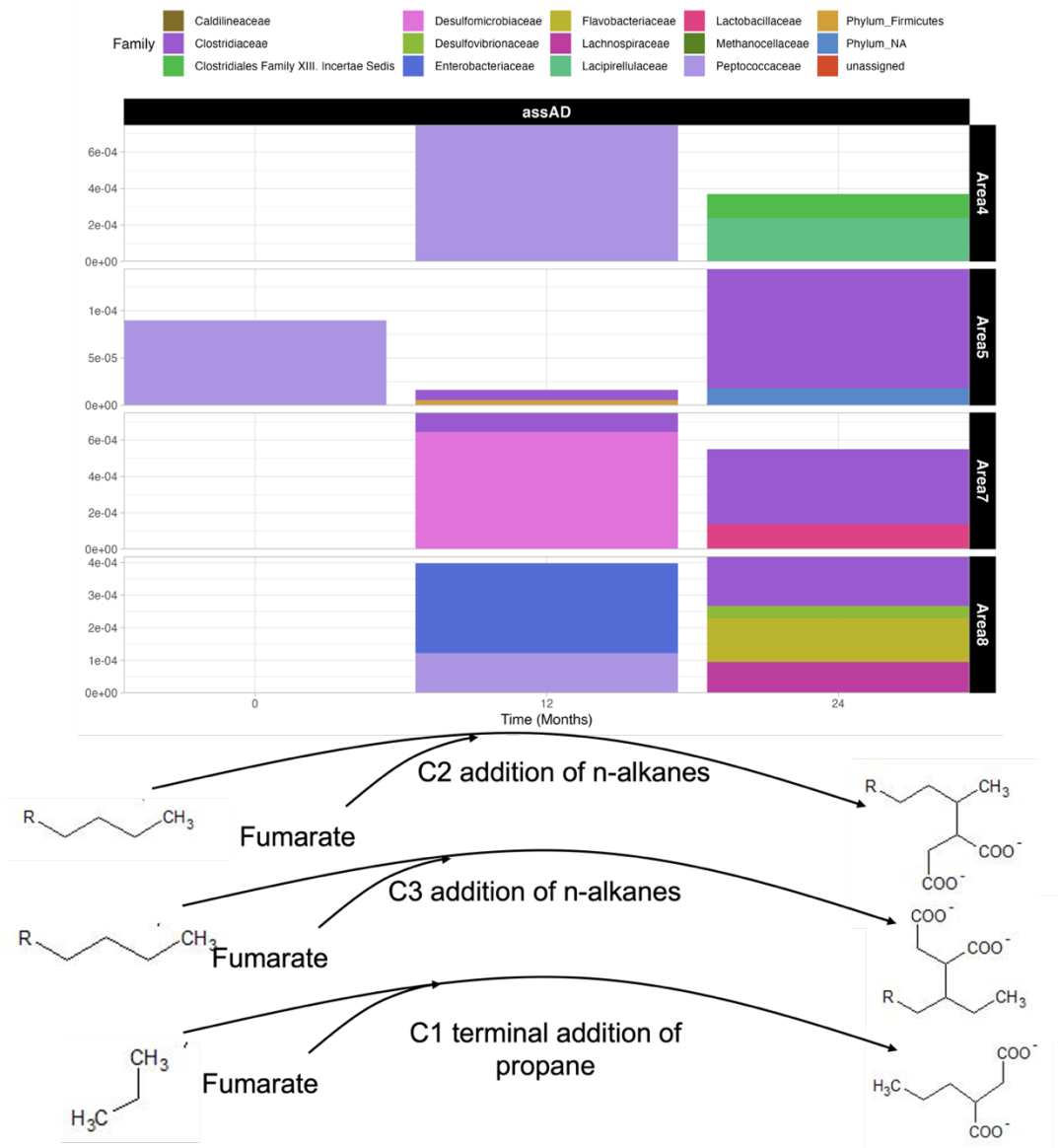


Figure S8. Relative abundance of genes encoding enzymes involved in alkanes anaerobic metabolism identified in each area before and after the bioremediation application and classified at family level.

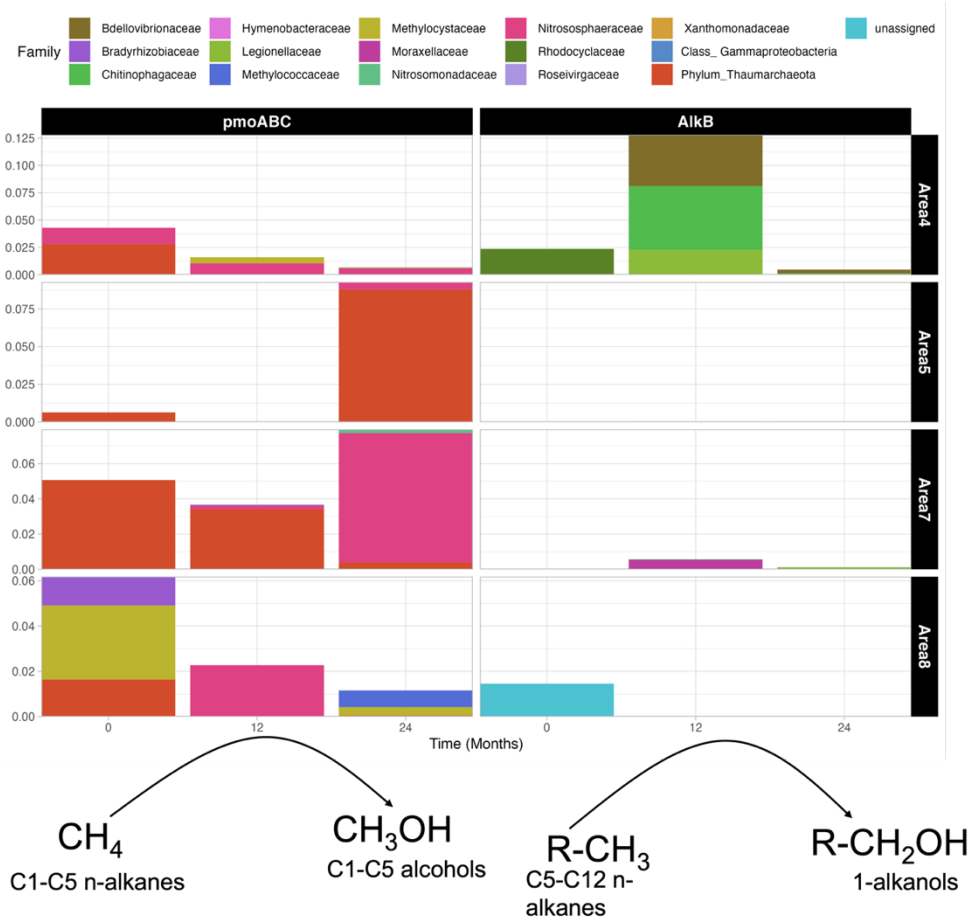


Figure S9. Relative abundance of genes encoding enzymes involved in alkanes aerobic metabolism identified in each area before and after the bioremediation application and classified at family level.

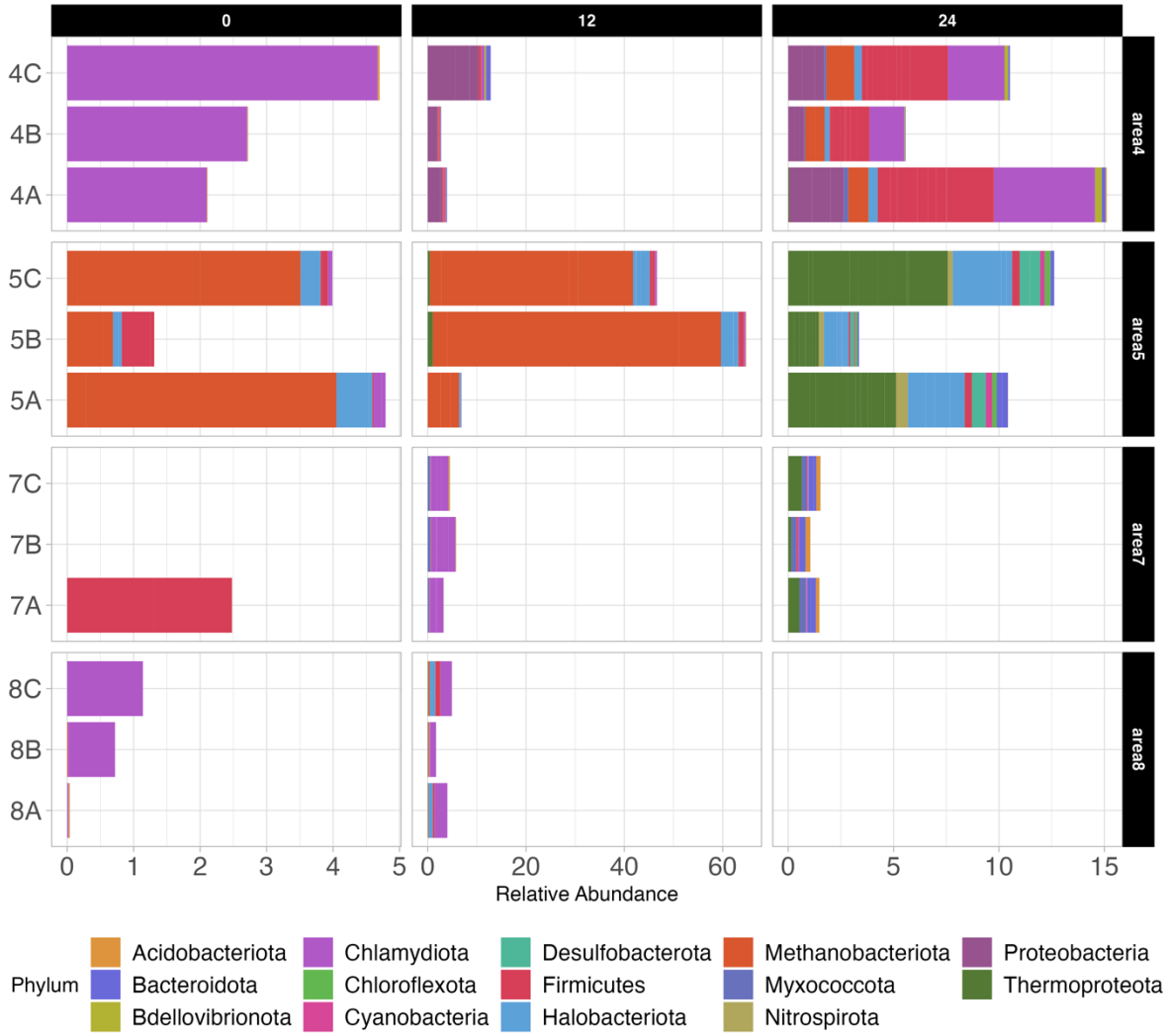


Figure S10. Relative abundance of the Metagenome-assembled genomes at phylum level in the areas before (time 0), after 12 months (time 12) the application of the bioremediation treatment at source zone and three months (24 time) after the reapplication of the bioremediation.

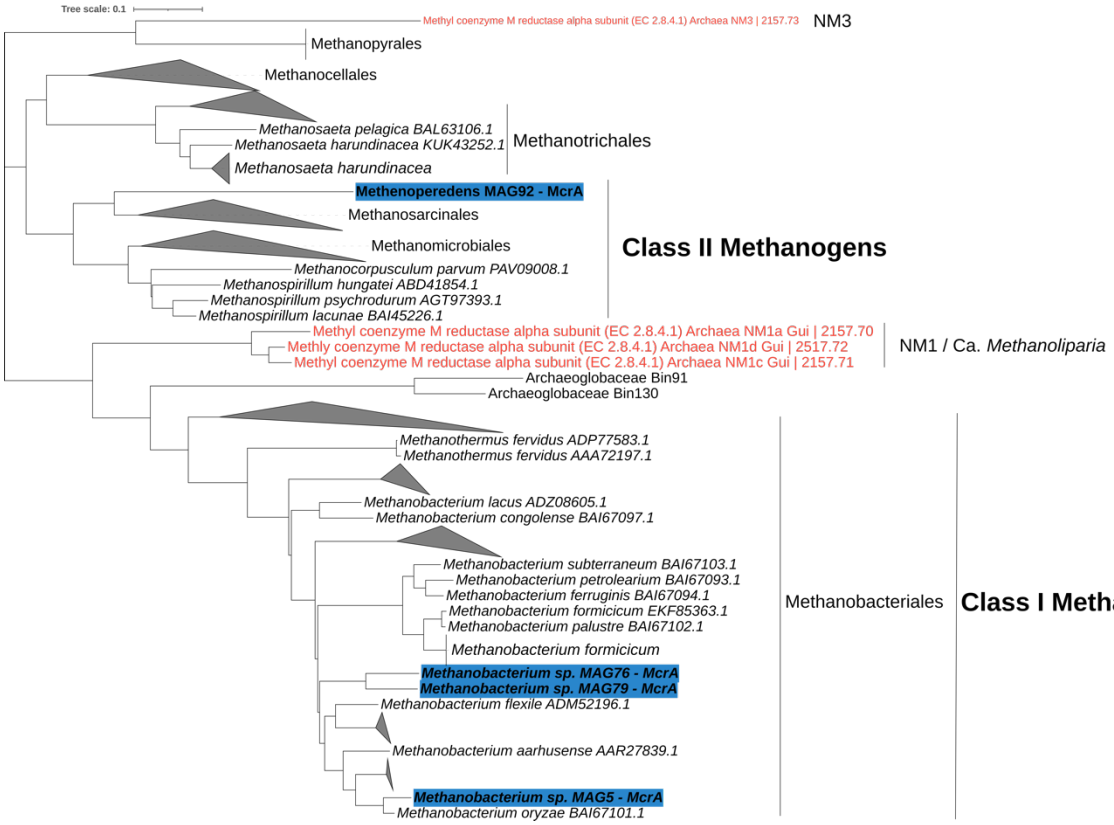


Figure S11. Phylogenetic tree of *mcr*A gene sequences recovered from the archaeal MAGs. Sequences of *mcr*A extracted from MAGs are shown in blue and Ca. *Methanoliparia* (NM1) and NM3 lineages are shown in red. The final tags for *mcrA* sequences refer to accession numbers.

References

Aburto-Medina A, Ball AS. Microorganisms involved in anaerobic benzene degradation. Annals of microbiology 2015; 65: 1201-1213.

Alneberg J, Bjarnason BS, de Bruijn I, Schirmer M, Quick J, Ijaz UZ, et al. Binning metagenomic contigs by coverage and composition. Nature Methods 2014; 11: 1144-1146.

Alvarez PJ, Hunt CS. The effect of fuel alcohol on monoaromatic hydrocarbon biodegradation and natural attenuation. Revista Latinoamericana de Microbiologia 2002; 44: 83.

Anderson MJ. Permutational multivariate analysis of variance (PERMANOVA). Wiley statsref: statistics reference online 2014: 1-15.

Andrews S. FastQC: a quality control tool for high throughput sequence data. 2010.

Bacosa HP, Mabuhay-Omar JA, Balisco RAT, Omar DM, Inoue C. Biodegradation of binary mixtures of octane with benzene, toluene, ethylbenzene or xylene (BTEX): insights on the potential of Burkholderia, Pseudomonas and Cupriavidus isolates. World Journal of Microbiology and Biotechnology 2021; 37: 122.

Baniasadi M, Mousavi SM. A Comprehensive Review on the Bioremediation of Oil Spills. In: Kumar V, Kumar M, Prasad R, editors. Microbial Action on Hydrocarbons. Springer Singapore, Singapore, 2018, pp. 223-254.

Bao Y-J, Xu Z, Li Y, Yao Z, Sun J, Song H. High-throughput metagenomic analysis of petroleum-contaminated soil microbiome reveals the versatility in xenobiotic aromatics metabolism. Journal of Environmental Sciences 2017; 56: 25-35.

Beals EW. Bray-Curtis ordination: an effective strategy for analysis of multivariate ecological data. Advances in ecological research. 14. Elsevier, 1984, pp. 1-55.

Bell TH, Stefani FOP, Abram K, Champagne J, Yergeau E, Hijri M, et al. A Diverse Soil Microbiome Degrades More Crude Oil than Specialized Bacterial Assemblages Obtained in Culture. Applied and Environmental Microbiology 2016; 82: 5530-5541.

Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 2014; 30.

Borrel G, Adam PS, McKay LJ, Chen L-X, Sierra-García IN, Sieber CMK, et al. Wide diversity of methane and short-chain alkane metabolisms in uncultured archaea. Nature Microbiology 2019; 4: 603-613.

Bowers RM, Kyrpides NC, Stepanauskas R, Harmon-Smith M, Doud D, Reddy TBK, et al. Minimum information about a single amplified genome (MISAG) and a metagenome-assembled genome (MIMAG) of bacteria and archaea. Nature Biotechnology 2017; 35: 725-731.

BRASIL. Resolução nº 420, de 28 de dezembro de 2009. In: Ambiente. MdMACNdM, editor. 420, Brasilia, 2009.

Bücker F, de Moura TM, da Cunha ME, de Quadros PD, Beker SA, Cazarolli JC, et al. Evaluation of the deteriogenic microbial community using qPCR, n-alkanes and FAMEs biodegradation in diesel, biodiesel and blends (B5, B10, and B50) during storage. Fuel 2018; 233: 911-917.

Burdukiewicz M, Gagat P, Jabłoński S, Chilimoniuk J, Gaworski M, Mackiewicz P, et al. PhyMet2: a database and toolkit for phylogenetic and metabolic analyses of methanogens. Environmental Microbiology Reports 2018; 10: 378-382.

Callaghan AV, Wawrik B. AnHyDeg: A Curated Database of Anaerobic Hydrocarbon Degradation Genes. . GitHub, Oklahoma, 2016.

Canabarro NI, Silva-Ortiz P, Nogueira L, Cantarella H, Maciel-Filho R, Souza GM. Sustainability assessment of ethanol and biodiesel production in Argentina, Brazil, Colombia, and Guatemala. Renewable and Sustainable Energy Reviews 2023; 171: 113019.

Chaudhry V, Chauhan PS, Mishra A, Goel R, Asif MH, Mantri SS, et al. Insights from the draft genome of Paenibacillus lentimorbus NRRL B-30488, a promising plant growth promoting bacterium. Journal of Biotechnology 2013; 168: 737-738.

Chaumeil P-A, Mussig AJ, Hugenholtz P, Parks DH. GTDB-Tk: a toolkit to classify genomes with the Genome Taxonomy Database. Bioinformatics 2019; 36: 1925-1927.

Chen M, Xu P, Zeng G, Yang C, Huang D, Zhang J. Bioremediation of soils contaminated with polycyclic aromatic hydrocarbons, petroleum, pesticides, chlorophenols and heavy metals by composting: Applications, microbes and future research needs. Biotechnology Advances 2015; 33: 745-755.

Chen YD, Barker JF, Gui L. A strategy for aromatic hydrocarbon bioremediation under anaerobic conditions and the impacts of ethanol: A microcosm study. Journal of Contaminant Hydrology 2008; 96: 17-31.

Corseuil HX, Monier AL, Gomes AP, Chiaranda HS, do Rosario M, Alvarez PJ. Biodegradation of soybean and castor oil biodiesel: implications on the natural attenuation of monoaromatic hydrocarbons in groundwater. Groundwater Monitoring & Remediation 2011; 31: 111-118.

Costa AHR, Nunes CC, Corseuil HX. Biorremediação de águas subterrâneas impactadas por gasolina e etanol com o uso de nitrato. Engenharia Sanitária e Ambiental 2009; 14: 265-274.

Cyplik P, Schmidt M, Szulc A, Marecik R, Lisiecki P, Heipieper HJ, et al. Relative quantitative PCR to assess bacterial community dynamics during biodegradation of diesel and biodiesel fuels under various aeration conditions. Bioresource Technology 2011; 102: 4347-4352.

da Silva MLB, Corseuil HX. Groundwater microbial analysis to assess enhanced BTEX biodegradation by nitrate injection at a gasohol-contaminated site. International Biodeterioration & Biodegradation 2012; 67: 21-27.

Dong Y, Lang Z, Kong X, Lu D, Liu Z. Kinetic and multidimensional profiling of accelerated degradation of oil sludge by biostimulation. Environmental Science: Processes & Impacts 2015; 17: 763-774.

Dueholm MS, Albertsen M, D'Imperio S, Tale VP, Lewis D, Nielsen PH, et al. Complete Genome Sequences of Pseudomonas monteilii SB3078 and SB3101, Two Benzene-, Toluene-, and Ethylbenzene-Degrading Bacteria Used for Bioaugmentation. Genome Announcements 2014; 2: 10.1128/genomea.00524-14.

Durán RE, Méndez V, Rodríguez-Castro L, Barra-Sanhueza B, Salvà-Serra F, Moore ER, et al. Genomic and physiological traits of the marine bacterium Alcaligenes aquatilis QD168 isolated from Quintero Bay, Central Chile, reveal a robust adaptive response to environmental stressors. Frontiers in microbiology 2019; 10: 528.

Durante-Rodríguez G, Gómez-Álvarez H, Blázquez B, Fernández-Llamosas H, Martín-Moldes Z, Sanz D, et al. Anaerobic pathways for the catabolism of aromatic compounds. 2018.

Edgar RC. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC bioinformatics 2004; 5: 1-19.

El-Naas MH, Acio JA, El Telib AE. Aerobic biodegradation of BTEX: Progresses and Prospects. Journal of Environmental Chemical Engineering 2014; 2: 1104-1122.

Espinoza-Tofalos A, Daghio M, Palma E, Aulenta F, Franzetti A. Structure and functions of hydrocarbon-degrading microbial communities in bioelectrochemical systems. Water 2020; 12: 343.

Gao Y, Du J, Bahar MM, Wang H, Subashchandrabose S, Duan L, et al. Metagenomics analysis identifies nitrogen metabolic pathway in bioremediation of diesel contaminated soil. Chemosphere 2021; 271: 129566.

Gieg LM, Duncan KE, Suflita JM. Bioenergy production via microbial conversion of residual oil to natural gas. Applied and environmental microbiology 2008; 74: 3022-3029.

Gieg LM, Fowler SJ, Berdugo-Clavijo C. Syntrophic biodegradation of hydrocarbon contaminants. Current Opinion in Biotechnology 2014; 27: 21-29.

Gielnik A, Pechaud Y, Huguenot D, Cébron A, Esposito G, van Hullebusch ED. Functional potential of sewage sludge digestate microbes to degrade aliphatic hydrocarbons during bioremediation of a petroleum hydrocarbons contaminated soil. Journal of Environmental Management 2021; 280: 111648.

Graham ED, Heidelberg JF, Tully BJ. BinSanity: unsupervised clustering of environmental microbial assemblies using coverage and affinity propagation. PeerJ 2017; 5: e3035.

Griffiths B, Ritz K, Bardgett RD, Cook R, Christensen S, Ekelund F, et al. Ecosystem response of pasture soil communities to fumigation-induced microbial diversity reductions: an examination of the biodiversity–ecosystem function relationship. Oikos 2000; 90: 279-294.

Gubry-Rangin C, Hai B, Quince C, Engel M, Thomson BC, James P, et al. Niche specialization of terrestrial archaeal ammonia oxidizers. Proceedings of the National Academy of Sciences 2011; 108: 21206-21211.

Hermuth K, Leuthner B, Heider J. Operon structure and expression of the genes for benzylsuccinate synthase in Thauera aromatica strain K172. Archives of Microbiology 2002; 177: 132-138.

Hidalgo KJ, Sierra-Garcia IN, Dellagnezze BM, de Oliveira VM. Metagenomic Insights Into the Mechanisms for Biodegradation of Polycyclic Aromatic Hydrocarbons in the Oil Supply Chain. Frontiers in Microbiology 2020; 11.

Hidalgo KJ, Teramoto EH, Soriano AU, Valoni E, Baessa MP, Richnow HH, et al. Taxonomic and functional diversity of the microbiome in a jet fuel contaminated site as revealed by combined application of in situ microcosms with metagenomic analysis. Science of The Total Environment 2019: 135152.

Humans IWGotEoCRt, Cancer IAfRo, Organization WH. Diesel and gasoline engine exhausts and some nitroarenes. Vol 46: World Health Organization, 1989.

Hyatt D, Chen G-L, LoCascio PF, Land ML, Larimer FW, Hauser LJ. Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics 2010; 11: 119.

Islam TH, Ghosh B, Magnet MMH, Fatema K, Akter S, Khan MAR, et al. Isolation and identification of petroleum degrading bacteria from oil contaminated soil & water and assessment of their potentiality in bioremediation. Journal of Environmental Science, toxocology and Food Technology 2013; 5: 55 - 58.

Ite AE, Ibok UJ. Role of plants and microbes in bioremediation of petroleum hydrocarbons contaminated soils. International Journal of Environmental Bioremediation & Biodegradation 2019; 7: 1-19.

Jiménez N, Richnow HH, Vogt C, Treude T, Krüger M. Methanogenic Hydrocarbon Degradation: Evidence from Field and Laboratory Studies. Journal of Molecular Microbiology and Biotechnology 2016; 26: 227-242.

Jung J, Philippot L, Park W. Metagenomic and functional analyses of the consequences of reduction of bacterial diversity on soil functions and bioremediation in diesel-contaminated microcosms. Scientific Reports 2016; 6: 1-10.

Kaida N, Habib S, Yasid NA, Shukor MY. Biodegradation of Petroleum Hydrocarbons by Bacillus spp.: A Review. Bioremediation Science and Technology Research 2018; 6: 14-21.

Kanehisa M, Furumichi M, Tanabe M, Sato Y, Morishima K. KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res 2017; 45: D353-d361.

Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic acids research 2000; 28: 27-30.

Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res 2016; 44: D457-62.

Kang D, Li F, Kirton ES, Thomas A, Egan RS, An H, et al. MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. PeerJ Preprints 2019; 7: e27522v1.

Ke X, Lu Y, Conrad R. Different behaviour of methanogenic archaea and Thaumarchaeota in rice field microcosms. FEMS Microbiology Ecology 2014; 87: 18-29.

Khodaei K, Nassery HR, Asadi MM, Mohammadzadeh H, Mahmoodlu MG. BTEX biodegradation in contaminated groundwater using a novel strain (Pseudomonas sp. BTEX-30). International Biodeterioration & Biodegradation 2017; 116: 234-242.

Khomarbaghi Z, Shavandi M, Amoozegar MA, Dastgheib SMM. Bacterial community dynamics during bioremediation of alkane- and PAHs-contaminated soil of Siri island, Persian Gulf: a microcosm study. International Journal of Environmental Science and Technology 2019; 16: 7849-7860.

Kleinsteuber S, Schleinitz KM, Breitfeld J, Harms H, Richnow HH, Vogt C. Molecular characterization of bacterial communities mineralizing benzene under sulfate-reducing conditions. FEMS Microbiology Ecology 2008; 66: 143-157.

Koshlaf E, Ball AS. Soil bioremediation approaches for petroleum hydrocarbon polluted environments. AIMS microbiology 2017; 3: 25.

Kozlowski JA, Stieglmeier M, Schleper C, Klotz MG, Stein LY. Pathways and key intermediates required for obligate aerobic ammonia-dependent chemolithotrophy in bacteria and Thaumarchaeota. The ISME Journal 2016; 10: 1836-1845.

Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular biology and evolution 2016; 33: 1870-1874.

Kunapuli U, Griebler C, Beller HR, Meckenstock RU. Identification of intermediates formed during anaerobic benzene degradation by an iron-reducing enrichment culture. Environmental Microbiology 2008; 10: 1703-1712.

Kunapuli U, Lueders T, Meckenstock RU. The use of stable isotope probing to identify key iron-reducing microorganisms involved in anaerobic benzene degradation. The Isme Journal 2007; 1: 643.

Kuntze K, Vogt C, Richnow H-H, Boll M. Combined application of PCR-based functional assays for the detection of aromatic-compound-degrading anaerobes. Applied and environmental microbiology 2011; 77: 5056-5061.

Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nature methods 2012; 9: 357.
Laso-Pérez R, Hahn C, Vliet DMv, Tegetmeyer HE, Schubotz F, Smit NT, et al. Anaerobic Degradation of Non-Methane Alkanes by “Candidatus Methanoliparia” in Hydrocarbon Seeps of the Gulf of Mexico. mBio 2019; 10: e01814-19.
Lehtovirta-Morley LE, Stoecker K, Vilcinskas A, Prosser JI, Nicol GW. Cultivation of an obligate acidophilic ammonia oxidizer from a nitrifying acid soil. Proceedings of the National Academy of Sciences 2011; 108: 15892-15897.
Letunic I, Bork P. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic acids research 2019; 47: W256-W259.
Li B, Wei Z, Huang Z, Xiao X, Ming S, Jiao H, et al. Removal of toluene from synthetic waste gas through aerobic denitrification in biotrickling reactor. Environmental Engineering Science 2020; 37: 769-781.
Liu Z. Benzene and toluene biodegradation with different dissolved oxygen concentrations: Arizona State University, 2015.
Luo F, Gitiafroz R, Devine CE, Gong Y, Hug LA, Raskin L, et al. Metatranscriptome of an Anaerobic Benzene-Degrading, Nitrate-Reducing Enrichment Culture Reveals Involvement of Carboxylation in Benzene Ring Activation. Applied and Environmental Microbiology 2014; 80: 4095-4107.
Margesin R, Schinner F. Biodegradation and bioremediation of hydrocarbons in extreme environments. Applied microbiology and biotechnology 2001; 56: 650-663.
Masset J, Calusinska M, Hamilton C, Hiligsmann S, Joris B, Wilmotte A, et al. Fermentative hydrogen production from glucose and starch using pure strains and artificial co-cultures of Clostridium spp. Biotechnology for biofuels 2012; 5: 1-15.
Mesbaiah FZ, Eddouaouda K, Badis A, Chebbi A, Hentati D, Sayadi S, et al. Preliminary characterization of biosurfactant produced by a PAH-degrading Paenibacillus sp. under thermophilic conditions. Environmental Science and Pollution Research 2016; 23: 14221-14230.
Mikheenko A, Saveliev V, Gurevich A. MetaQUAST: evaluation of metagenome assemblies. Bioinformatics 2016; 32.
Mohanty S, Jasmine J, Mukherji S. Practical Considerations and Challenges Involved in Surfactant Enhanced Bioremediation of Oil. BioMed Research International 2013; 2013: 328608.
Morris L, O'Brien A, Natera SHA, Lutz A, Roessner U, Long SM. Structural and functional measures of marine microbial communities: An experiment to assess implications for oil spill management. Marine Pollution Bulletin 2018; 131: 525-529.
Mugge RL, Salerno JL, Hamdan LJ. Microbial Functional Responses in Marine Biofilms Exposed to Deepwater Horizon Spill Contaminants. Frontiers in Microbiology 2021; 12.
Müller JB, Ramos DT, Larose C, Fernandes M, Lazzarin HSC, Vogel TM, et al. Combined iron and sulfate reduction biostimulation as a novel approach to enhance BTEX and PAH source-zone biodegradation in biodiesel blend-contaminated groundwater. Journal of hazardous materials 2017; 326: 229-236.
Ng YF, Ge L, Chan WK, Tan SN, Yong JWH, Tan TTY. An environmentally friendly approach to treat oil spill: Investigating the biodegradation of petrodiesel in the presence of different biodiesels. Fuel 2015; 139: 523-528.
Norris RD. In-situ bioremediation of soils and ground water contaminated with petroleum hydrocarbons. Handbook of bioremediation. CRC Press, 2017, pp. 17-38.
Nurk S, Meleshko D, Korobeynikov A, Pevzner PA. metaSPAdes: a new versatile metagenomic assembler. Genome research 2017; 27: 824-834.
Okoh E, Yelebe ZR, Oruabena B, Nelson ES, Indiamaowei OP. Clean-up of crude oil-contaminated soils: bioremediation option. International Journal of Environmental Science and Technology 2020; 17: 1185-1198.
Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'hara R, et al. Package 'vegan'. Community ecology package, version 2013; 2.
Pacwa-Płociniczak M, Płaza GA, Piotrowska-Seget Z. Monitoring the changes in a bacterial community in petroleum-polluted soil bioaugmented with hydrocarbon-degrading strains. Applied Soil Ecology 2016; 105: 76-85.
Papudeshi B, Haggerty JM, Doane M, Morris MM, Walsh K, Beattie DT, et al. Optimizing and evaluating the reconstruction of Metagenome-assembled microbial genomes. BMC genomics 2017; 18: 1-13.

Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome research 2015: gr. 186072.114.

Perfumo A, Banat IM, Marchant R, Vezzulli L. Thermally enhanced approaches for bioremediation of hydrocarbon-contaminated soils. Chemosphere 2007; 66: 179-184.

Pester M, Schleper C, Wagner M. The Thaumarchaeota: an emerging view of their phylogeny and ecophysiology. Curr Opin Microbiol 2011; 14: 300-6.

Peters KE, Walters CC, Moldowan JM. The biomarker guide: Volume 2, Biomarkers and isotopes in petroleum systems and earth history: Cambridge University Press, 2007.

Rabus R, Boll M, Heider J, Meckenstock RU, Buckel W, Einsle O, et al. Anaerobic Microbial Degradation of Hydrocarbons: From Enzymatic Reactions to the Environment. Journal of Molecular Microbiology and Biotechnology 2016; 26: 5-28.

Rama F, Ramos DT, Müller JB, Corseuil HX, Miotliński K. Flow field dynamics and high ethanol content in gasohol blends enhance BTEX migration and biodegradation in groundwater. Journal of contaminant hydrology 2019; 222: 17-30.

Ramos DT. Bioestimulação de processos metanogênicos com acetato de amônio para degradação acelerada de hidrocarbonetos de petróleo em águas subterrâneas contaminadas com diesel B20. Engenharia Ambiental. Doutor. Universidade Federal de Santa Catarina, Florianóplis, 2013.

Ramos DT, da Silva MLB, Chiaranda HS, Alvarez PJ, Corseuil HX. Biostimulation of anaerobic BTEX biodegradation under fermentative methanogenic conditions at source-zone groundwater contaminated with a biodiesel blend (B20). Biodegradation 2013; 24: 333-341.

Ramos DT, da Silva MLB, Nossa CW, Alvarez PJJ, Corseuil HX. Assessment of microbial communities associated with fermentative–methanogenic biodegradation of aromatic hydrocarbons in groundwater contaminated with a biodiesel blend (B20). Biodegradation 2014; 25: 681-691.

Ramos SM, Nunes CC, Corseuil HX. Biorremediação de águas subterrâneas impactadas por gasolina E10 com o uso de sulfato, 2010.

Richardson EL, King CK, Powell SM. The use of microbial gene abundance in the development of fuel remediation guidelines in polar soils. Integrated Environmental Assessment and Management 2015; 11: 235-241.

Rodriguez-R LM, Gunturu S, Tiedje JM, Cole JR, Konstantinidis KT. Nonpareil 3: fast estimation of metagenomic coverage and sequence diversity. MSystems 2018; 3: e00039-18.

Roy A, Dutta A, Pal S, Gupta A, Sarkar J, Chatterjee A, et al. Biostimulation and bioaugmentation of native microbial community accelerated bioremediation of oil refinery sludge. Bioresource Technology 2018; 253: 22-32.

Satapanajaru T, Chokejaroenrat C, Sakulthaew C, Yoo-Iam M. Remediation and restoration of petroleum hydrocarbon containing alcohol-contaminated soil by persulfate oxidation activated with soil minerals. Water, Air, & Soil Pollution 2017; 228: 1-15.

Segata N, Börnigen D, Morgan XC, Huttenhower C. PhyloPhlAn is a new method for improved phylogenetic and taxonomic placement of microbes. Nature communications 2013; 4: 1-11.

Shade A, Peter H, Allison SD, Baho DL, Berga M, Bürgmann H, et al. Fundamentals of microbial community resistance and resilience. Frontiers in microbiology 2012; 3: 417.

Shahi A, Aydin S, Ince B, Ince O. Evaluation of microbial population and functional genes during the bioremediation of petroleum-contaminated soil as an effective monitoring approach. Ecotoxicology and Environmental Safety 2016; 125: 153-160.

Shao Z, Wang W. Enzymes and genes involved in aerobic alkane degradation. Frontiers in Microbiology 2013; 4.

Sherry A, Gray ND, Ditchfield AK, Aitken CM, Jones DM, Röling WFM, et al. Anaerobic biodegradation of crude oil under sulphate-reducing conditions leads to only modest enrichment of recognized sulphate-reducing taxa. International Biodeterioration & Biodegradation 2013; 81: 105-113.

Starke R, Keller A, Jehmlich N, Vogt C, Richnow HH, Kleinsteuber S, et al. Pulsed 13C2-Acetate Protein-SIP Unveils Epsilonproteobacteria as Dominant Acetate Utilizers in a Sulfate-Reducing Microbial Community Mineralizing Benzene. Microbial Ecology 2016; 71: 901-911.

Steffi H, Sabine K, Antonis C, Steffen K, Tillmann L, Hans-Hermann R, et al. Functional characterization of an anaerobic benzene-degrading enrichment culture by DNA stable isotope probing. Environmental Microbiology 2010; 12: 401-411.

Steiner LV, Ramos DT, Liedke AMR, Serbent MP, Corseuil HX. Ethanol content in different gasohol blend spills influences the decision-making on remediation technologies. Journal of environmental management 2018; 212: 8-16.
Stepanova AY, Gladkov EA, Osipova ES, Gladkova OV, Tereshonok DV. Bioremediation of Soil from Petroleum Contamination. Processes 2022; 10: 1224.
Tahhan RA, Abu-Ateih RY. Biodegradation of petroleum industry oily-sludge using Jordanian oil refinery contaminated soil. International Biodeterioration & Biodegradation 2009; 63: 1054-1060.
Techtmann SM, Hazen TC. Metagenomic applications in environmental monitoring and bioremediation. Journal of Industrial Microbiology & Biotechnology 2016; 43: 1345-1354.
Terrón-González L, Martín-Cabello G, Ferrer M, Santero E. Functional Metagenomics of a Biostimulated Petroleum-Contaminated Soil Reveals an Extraordinary Diversity of Extradiol Dioxygenases. Applied and Environmental Microbiology 2016; 82: 2467-2478.
Timmusk S, Teder T, Behers L. Paenibacillus polymyxa A26 and Its Surfactant-Deficient Mutant Degradation of Polycyclic Aromatic Hydrocarbons. Stresses 2021; 1: 266-276.
Ulrich AC, Beller HR, Edwards EA. Metabolites Detected during Biodegradation of 13C6-Benzene in Nitrate-Reducing and Methanogenic Enrichment Cultures. Environmental Science & Technology 2005; 39: 6681-6691.
Uritskiy GV, DiRuggiero J, Taylor J. MetaWRAP—a flexible pipeline for genome-resolved metagenomic data analysis. Microbiome 2018; 6: 1-13.
van Beilen JB, Funhoff EG. Alkane hydroxylases involved in microbial alkane degradation. Applied Microbiology and Biotechnology 2007; 74: 13-21.
Van Beilen JB, Li Z, Duetz WA, Smits TH, Witholt B. Diversity of alkane hydroxylase systems in the environment. Oil & gas science and technology 2003; 58: 427-440.
Van Elsas JD, Chiurazzi M, Mallon CA, Elhottovā D, Krištůfek V, Salles JF. Microbial diversity determines the invasion of soil by a bacterial pathogen. Proceedings of the National Academy of Sciences 2012; 109: 1159-1164.
von Netzer F, Kuntze K, Vogt C, Richnow HH, Boll M, Lueders T. Functional Gene Markers for Fumarate-Adding and Dearomatizing Key Enzymes in Anaerobic Aromatic Hydrocarbon Degradation in Terrestrial Environments. Journal of Molecular Microbiology and Biotechnology 2016; 26: 180-194.
Wang M, Garrido-Sanz D, Sansegundo-Lobato P, Redondo-Nieto M, Conlon R, Martin M, et al. Soil Microbiome Structure and Function in Ecopiles Used to Remediate Petroleum-Contaminated Soil. Frontiers in Environmental Science 2021; 9.
Wartell B, Boufadel M, Rodriguez-Freire L. An effort to understand and improve the anaerobic biodegradation of petroleum hydrocarbons: A literature review. International Biodeterioration & Biodegradation 2021; 157: 105156.
Weidenweber S, Schühle K, Demmer U, Warkentin E, Ermler U, Heider J. Structure of the acetophenone carboxylase core complex: prototype of a new class of ATP-dependent carboxylases/hydrolases. Scientific Reports 2017; 7: 1-10.
Wood DE, Lu J, Langmead B. Improved metagenomic analysis with Kraken 2. Genome biology 2019; 20: 1-13.
Wu H-j, Du X-y, Wu W-j, Zheng J, Song J-y, Xie J-c. Metagenomic analysis reveals specific BTEX degrading microorganisms of a bacterial consortium. AMB Express 2023; 13: 1-12.
Wu M, Li W, Dick WA, Ye X, Chen K, Kost D, et al. Bioremediation of hydrocarbon degradation in a petroleum-contaminated soil and microbial population and activity determination. Chemosphere 2017; 169: 124-130.
Wu Y-W, Simmons BA, Singer SW. MaxBin 2.0: an automated binning algorithm to recover genomes from multiple metagenomic datasets. Bioinformatics 2015; 32: 605-607.
Xiong W, Mathies C, Bradshaw K, Carlson T, Tang K, Wang Y. Benzene removal by a novel modification of enhanced anaerobic biostimulation. Water Research 2012; 46: 4721-4731.
Yergeau E, Sanschagrin S, Beaumier D, Greer CW. Metagenomic analysis of the bioremediation of diesel-contaminated Canadian high arctic soils. PloS one 2012; 7.
Zaan BMvd, Saia FT, Stams AJM, Plugge CM, Vos WMd, Smidt H, et al. Anaerobic benzene degradation under denitrifying conditions: Peptococcaceae as dominant benzene degraders and evidence for a syntrophic process. Environmental Microbiology 2012; 14: 1171-1181.

Zafra G, Taylor TD, Absalón AE, Cortés-Espinosa DV. Comparative metagenomic analysis of PAH degradation in soil by a mixed microbial consortium. Journal of Hazardous Materials 2016; 318: 702-710.

Zhang T, Tremblay P-L, Chaurasia AK, Smith JA, Bain TS, Lovley DR. Anaerobic benzene oxidation via phenol in Geobacter metallireducens. Applied and environmental microbiology 2013: AEM. 03134-13.

Zhang Z, Lo IMC. Biostimulation of petroleum-hydrocarbon-contaminated marine sediment with co-substrate: involved metabolic process and microbial community. Applied Microbiology and Biotechnology 2015; 99: 5683-5696.

Zhao D, Liu C, Liu L, Zhang Y, Liu Q, Wu W-M. Selection of functional consortium for crude oil-contaminated soil remediation. International Biodeterioration & Biodegradation 2011; 65: 1244-1248.

Chapter IV - Manuscript 5: Petroleum-associated Genome Database - PaGeD: a repository of genes and genomes related to the oil supply chain

Petroleum-associated Genome Database - PaGeD: a repository of genes and genomes related to the oil supply chain

K.J. Hidalgo[a,b6], L.N. Lemos[a] , A.J. Giachini[c] , M.R. Schneider[c], A.U. Soriano[d], M.P. Baessa[d], L.F. Martins[d], and V.M. Oliveira[a]

[a] Microbial Resources Division, Research Center for Chemistry, Biology and Agriculture (CPQBA), University of Campinas – UNICAMP, Av. Alexandre Cazellato, 999, ZIP 13148-218, Paulínia, SP, Brazil.
[b] Graduate Program in Genetics and Molecular Biology, Institute of Biology, University of Campinas (UNICAMP), Rua Monteiro Lobato 255, Cidade Universitária ZIP 13083-862, Campinas, SP, Brazil.
[c] Núcleo Ressacada de Pesquisas Em Meio Ambiente (REMA) - Department of Microbiology, Federal University of Santa Catarina (UFSC), Campus Universitário Sul da Ilha - Rua José Olímpio da Silva, 1326 - Bairro Tapera, 88049-500 Florianópolis, SC, Brazil.
[d] PETROBRAS/ R&D Center (CENPES), Av. Horácio Macedo, 950. ZIP 21941-915 Cidade Universitária, Ilha do Fundão, Rio de Janeiro, Brazil.

[6] Corresponding author: K.J. Hidalgo
E-mail: khidalgo@javeriana.edu.co
Telephone: +55 19 98172 1510

Abstract

Oil & Gas industry associated microbiomes play an important role in a range of processes that take place in the corresponding stages of the oil supply chain, due to their active participation in biogeochemical cycling. Microbes can cause detrimental damages to the industry, such as in reservoir oil biodegradation, souring, microbiologically influenced corrosion (MIC), biofouling, etc. However, at the same time, the microbiota can be beneficial, for instance, for oil spill bioremediation, biodesulfurization and/or production of biosurfactants to improve the secondary recovery in oil wells. Nevertheless, knowledge about the biotechnological potential of microorganisms in petroleum-associated environments is still scarce. Genome-centric analyses can help to fill this gap. We present the Petroleum-associated Genome Database (PaGeD), comprising 3,334 genomes (3,014 MAGs, 319 isolate genomes and 1 SAG) organized into 2,522 species-level clusters from oil associated samples, such as oil polluted environments, microbiologically influenced corrosion related samples, oil reservoirs, produced, injection and formation water, among others. The analyses revealed limited geographic distribution and high isolation source specificity, i.e, specific microbial communities in each type of sample. Over 50% of the species-level clusters represent novel taxa, showing the reduced representation of cultured microorganisms from oil-associated sources. PaGeD expands the current understanding of the oil-associated microbiomes and provides a large genome catalogue for future analyses to decipher beneficial and harmful microorganisms and their impact on the oil and gas industry.

1. INTRODUCTION

Oil & Gas industry is one of the major and most important industries in the world, mainly because fossil fuels as still the primary energy sources globally (Lu et al., 2019). Due to the extreme temperatures, pressures, high concentrations of salt, heavy metals and organic solvents, as well as other physical and chemical parameters, for several decades the O&G industry believed that microbial life was not possible in the oil reservoirs, pipelines, machineries, and processes (Augustinovic et al., 2012; Wunch et al., 2021). Nonetheless, microorganisms have demonstrated the ability to adapt to these hostile conditions, proliferating and thriving in such environments (Foght, 2010; Sierra-Garcia, 2016). This is mainly by the fact that water is present in many stages of the oil production, such as secondary oil recovery and hydraulic fracturing (Wunch et al., 2021). A diverse range of sources of microorganisms are related with the O&G industry, including offshore and onshore oil reservoirs, produced, injection and formation water, pipeline and equipment biofilm, oil-polluted soil, sediments, or water, among others.

In this context, many questions arise: i) Which microorganisms can grow under these conditions? ii) What are the adaptations that allow them to colonize these environments? iii) What damages do they cause to the industry? and iv) Are there microorganisms with beneficial potential? The only way to answer all these questions is scrutinizing and understanding the microbiome present in all these habitats at all levels of information. Until recently, the knowledge of the oil-associated microbiomes was very limited by the culture-based methods, making it difficult to study the microbial community ecology and functioning. Besides, because most of the microbes in oil-associated environments are extremophilic, environmental conditions were very difficult to be reproduced at the laboratory, except for a few research groups in the world. In the last decade, the massification of the large-scale DNA sequencing and the bioinformatics development allowed us to learn more about oil-associated microbiomes and their metabolic potential, expanding our understanding of the microbial community structure, composition and functions in oil-associated habitats, and providing a deeper knowledge of the key problems and opportunities in the O&G industry (Wunch et al., 2021).

Studies with cultured microorganisms continue to provide valuable information that are essential for application in research biotechnology. On the other hand, the main limitation for obtaining genetic information from non-cultured microorganisms was solved with development of metagenome shotgun sequencing (Parks et al., 2017). This approach allows one to recover nearly all the genetic material from any type of sample. Later, the development of powerful algorithms made it possible to reconstruct near complete genomes from metagenome datasets (Metagenome-assembled genomes – MAGs) (Parks et al., 2017). Thus, now it is possible to obtain several genomes from uncultured microorganisms.

The genome repository of oil systems (GROS) (Karthikeyan et al., 2020) was a pioneering initiative to enrich knowledge of oil-associated microbial diversity. Hundreds of metagenome-assembled genomes were obtained as part of this work, being available in a web-based database at the Microbial Genome Atlas (Rodriguez-R et al., 2018). This catalog is focused on oil spills, mainly on the Deep-Water Horizon spill at the Gulf of Mexico, since this was the first major oil spill extensively characterized by sequencing (Karthikeyan et al., 2020). However, currently there is not any organized source of genomes associated with the overall oil supply chain. In order to expand this repository, we retrieved genomes from different sources related with the O&G industry, such as produced and injection water, MIC related samples, oil polluted water and soil, among others, to create the Petroleum-associated Genome Database (PaGeD). Currently, PaGeD is under construction and the final aim is to make it available on a platform that allows the user to interactively search and filter the genomes according to some specific characteristic or property, such as isolation source or metabolic potential.

2. MATERIAL AND METHODS

2.1. Genome collection

To explore the diversity of bacteria and archaea in the Oil & Gas industry, all the prokaryotic genomes publicly available in July 2023, obtained from any type of sample/environment related with the Oil & Gas industry (e.g., oil reservoir, produced or formation water, fracturing fluids, deep seeps, soil or water contaminated, etc.), were

downloaded. To retrieve the genomes, surveys were carried out with related keywords in the main genome databases available, such as IMG (Chen et al., 2019) and NCBI (O'Leary) as well as the genomes available in the Genome Repository of Oil Systems - GROS from the Microbial Genome Atlas - MiGA webserver (Karthikeyan et al., 2020; Rodriguez-R et al., 2018). To expand this data set, the key words were used in scientific article databases (e.g., Google Scholar, Scopus, etc.) to find genomes not deposited anywhere. To avoid including duplicate genomes due to redundancy between the databases, a manual curation was performed. These procedures yielded 3,885 genomes (Figure 1). Details on the genome accession numbers are provided in Table S1. Metadata of each genome were retrieved from each database used, focusing on the geographic origin, isolation source, geographic coordinates, and type of genome (e.g., Metagenome-assembled genome or isolate genome). Information about the number and genome type by isolation source in the habitat categories are provide in Table S2.

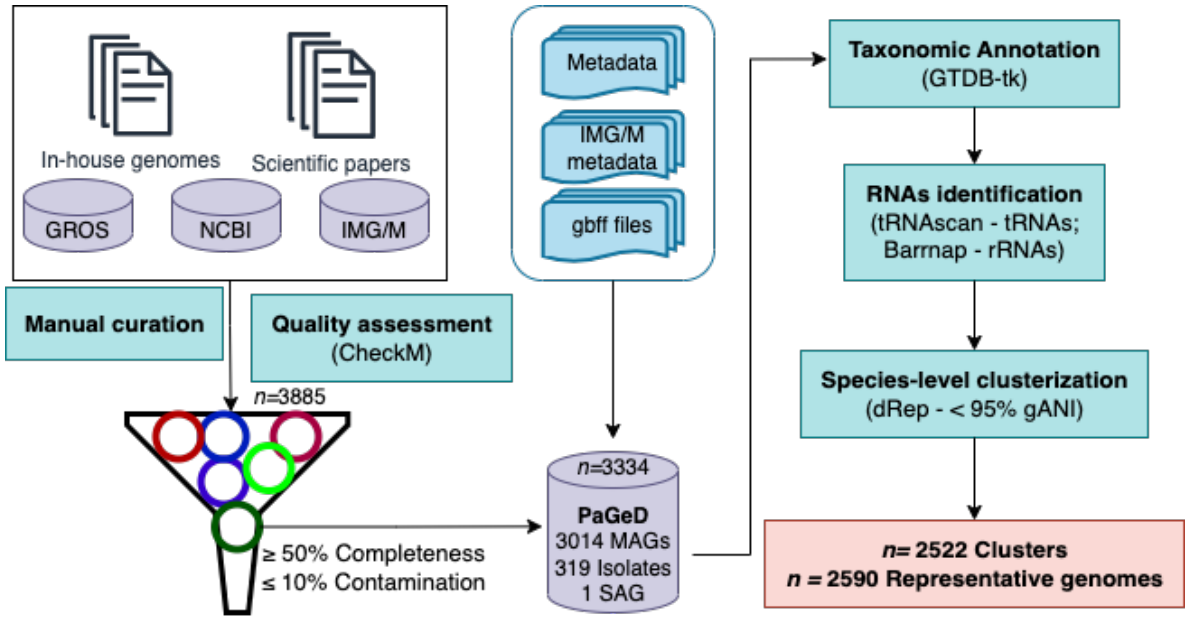


Figure 1. Workflow for genome retrieval, filtering, annotation and dereplication to construct the PaGeD.

2.2. Assessing genome quality and taxonomic classification

Quality (completeness and contamination) of the nonredundant 3,885 genomes was estimated with CheckM v 1.1.3 (Parks et al., 2015) and only genomes with at least 50% completeness and less than 10% contamination, according with the MiMAG standards (Bowers et al., 2017), were selected. As additional information, tRNAs and rRNAs were

identified using tRNAscan-SE v2.0.12 (Chan et al., 2021) and Barrnap (Seemann, unpublished, https://github.com/tseemann/barrnap, accessed on 6 July 2023), respectively. Based on these results, 3,334 genomes of the 3,885 were classified as medium and high-quality genomes and used to form the PaGeD dataset (Figure 1).

All retained genomes were taxonomically annotated using GTDB-tk v2.3.2 (Release 214.1 April 2023) (Chaumeil et al., 2019). The total set of 3,334 genomes were clustered into 2,515 species-level groups (ANI>95% (Jain et al., 2018), >30% alignment coverage) and representative genomes of each species were established using dRep v3.4.3 (Olm et al., 2017). For phylogenetic reconstruction, the PaGeD dataset was submitted to GToTree software v1.7 (Lee, 2019). The tree was constructed with genomes harboring at least 30% of the marker genes set and plotted with iTOL v6 (Letunic and Bork, 2019).

3. RESULTS AND DISCUSSION

Herein, the “Petroleum-associated Genomes Database” (PaGeD), a wide collection of genomes obtained from culturable microorganisms and from metagenome-assembled genomes (MAG), was created and curated. The genomes of PaGeD were reported as related to the Oil & Gas industry, spanning environments as offshore and onshore oil reservoirs, natural oil seeps, processing fluids (e.g., produced, formation and/or injection water, fracking fluids), MIC samples, oil spills or even laboratory microcosms or enrichment cultures simulating oil contamination. These isolation sources were categorized in habitats (Table S3). Engineered (i.e., activate sludges, bioreactors, etc) and marine habitats were the ones with more derived genomes (Figure 2A). The present collection has genomes from a total of 74 countries across six continents (South America, North America, Europe, Africa, Asia and Oceania), as well as the Pacific, Atlantic, Indian and North Oceans (Figure 2B). Due to the extensive use of next-generation sequencing in different research projects at the Deepwater horizon spill DWH at the Gulf of Mexico (USA and Mexico) (Kimes et al., 2014; Mason et al., 2012; Mason et al., 2014; Rodriguez-r et al., 2015), most of the genomes were obtained from this zone (Karthikeyan et al., 2020) (Figure 2B, Figure S1).

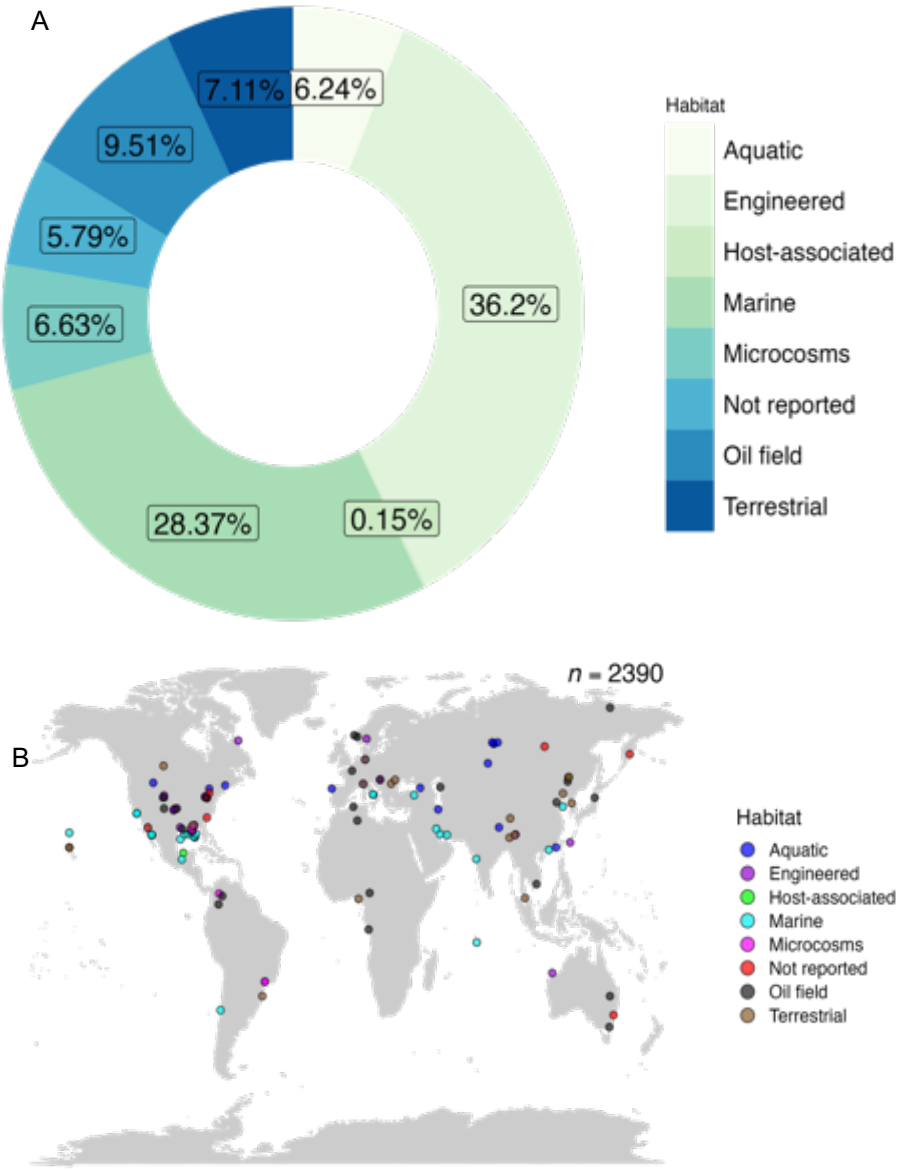


Figure 2. (a) PaGeD genome distribution across habitats based on the associated metadata available in the IMG/M and NCBI databases. **(b)** Geographic distribution of PaGeD genomes within each habitat.

From the 3,334 genomes currently available in the PaGeD database, 3,014 correspond to MAGs and 319 to bacterial or archaeal isolate genomes (n=319). All of them meet or exceed the medium quality (completeness average = 83.86% ± 14.80; contamination average = 2.35% ± 2.43), according with MiMAG standards (Bowers et al., 2017) (Figure 3). In addition, ~36% of the genomes have at least partial sequence of the 16S rRNA gene. The genome assemblies varied from one to 2,899 contigs, with a mean of 238 contigs per genome (Q3 = 311) (Figure 3). The genome sizes of the PaGeD ranged from 0.27 Mb to 12.25 Mb (Figure 3). Most of the small-length genomes were affiliated to

the Nanoarchaeota and Patescibacteria phyla, and large-length genomes belonged mainly to Myxococcota and Planctomycetota.

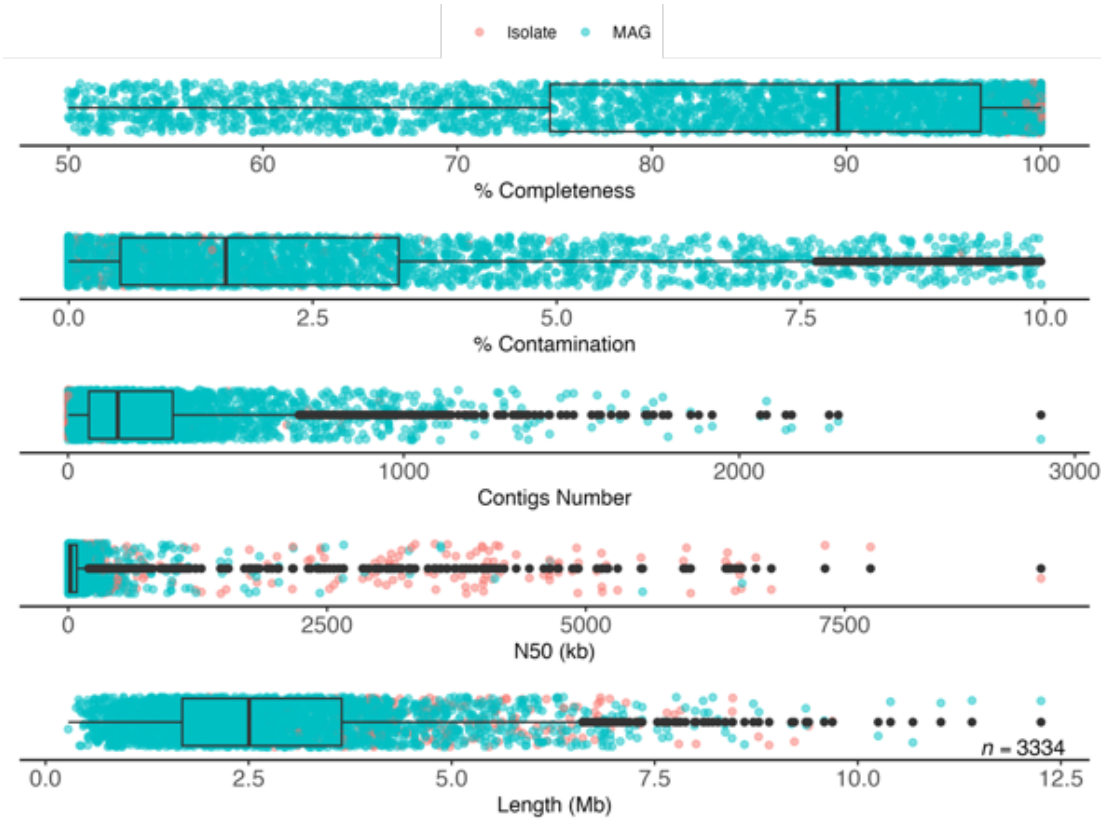


Figure3. Distribution of quality metrics across the PaGeD, showing the minimum, first quartile, median, third quartile and maximum values for completeness, contamination, contig number, N50 e length. The genome type (i.e. MAG or isolate) is represented by the dot color. See Table S3 for quality statistics for all genomes.

These genomes were grouped into species-level clusters based on the 95% gANI cut-off, yielding 2,522 unique clusters, of which 47 clusters contain at least five members, and 2,089 were singletons, disclosing high species diversity in petroleum-associated environments. These results showed an increase in the diversity known in Oil & Gas industry associated environments compared to the GROS (Karthikeyan et al., 2020), a first initiative towards the assessment and compilation of Oil & Gas related genomes. Cultured species genomes comprised only ~10% of the unique clades (Figure 4A). Species-level clusters were represented by 90.4% and 8.15% of MAGs and isolate genomes, respectively, while only 1.45% were represented by both cultured species genomes and MAGs (Figure 4B, Figure S2). PaGeD contains substantial novelty, since almost 50% of the representative species (centroid genomes at each species-level

clusters) were not taxonomically classified at genus level, showing high potential for the discovery of new species associated to oil & gas related environments.

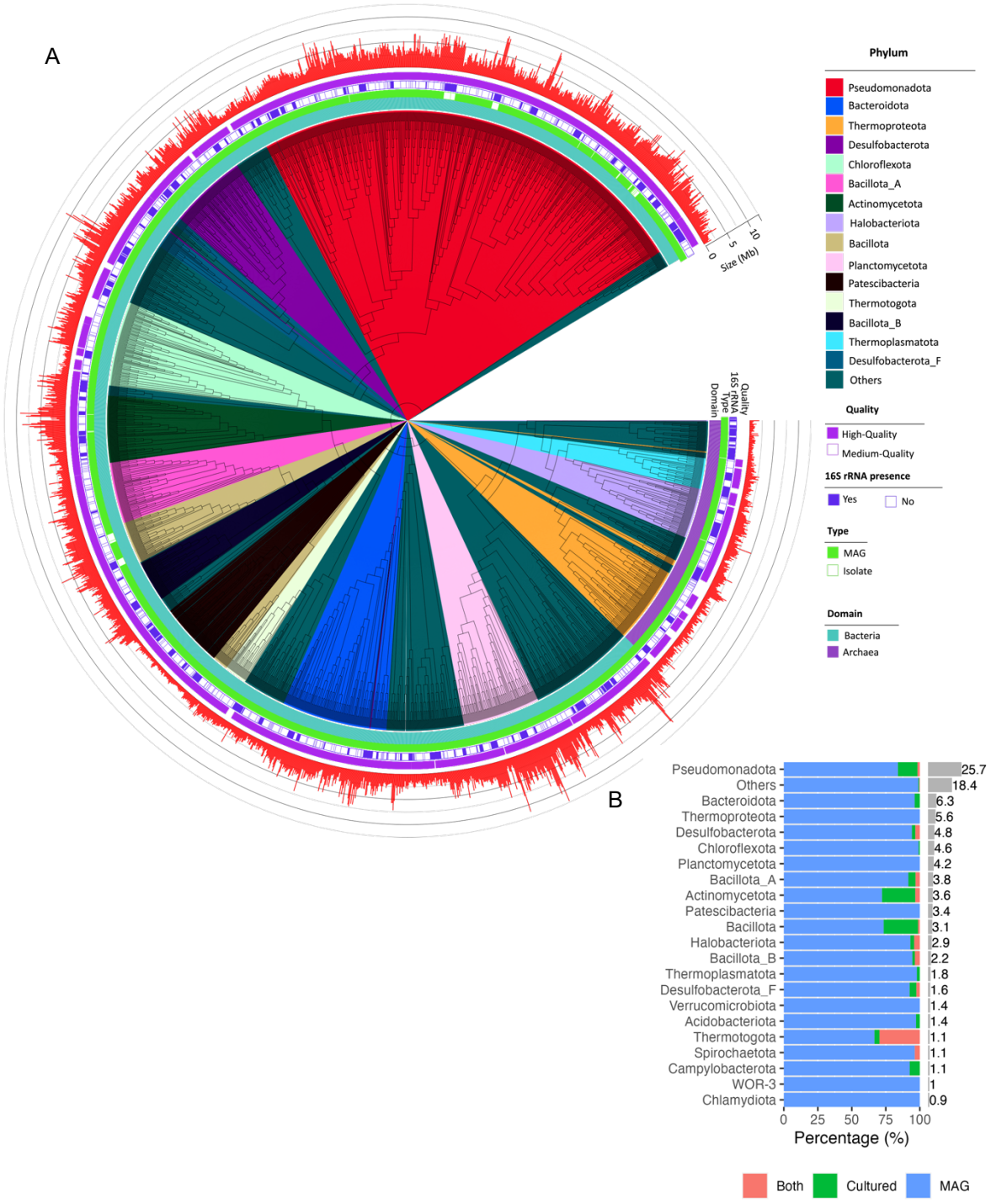


Figure4. (a) Phylogenetic tree based on a concatenated alignment of 25 bacteria-archaea distributed single-copy genes from 2,381 (at least 30% of the markers genes present) genomes of PaGeD. **(b)** Proportion of the cultivated and uncultivated genomes identified for each taxonomy group. Gray bars indicate the percentage of genomes of each phylum.

PaGeD revealed high site-specificity of the genomes, since ~73% of the species-level clusters with more than one member (n=433) were found only in a unique isolation source and ~21% were obtained from two isolation sources (Figure 5A). The number of species-level clusters decreased dramatically with increasing prevalence in the different types of isolation sources, suggesting that petroleum-associated genomes are dominated by endemic over cosmopolitan species (Wang et al., 2023; Yang et al., 2016). Based on the representative species (centroid genomes in the species-level clusters) found in only one isolation source, hydrothermal vent sediments from the Guaymas Basin were the most differentiated site (Figure 5B), showing a unique microbiome, as it has been reported by other studies (Cruaud et al., 2017; Zhou et al., 2022).

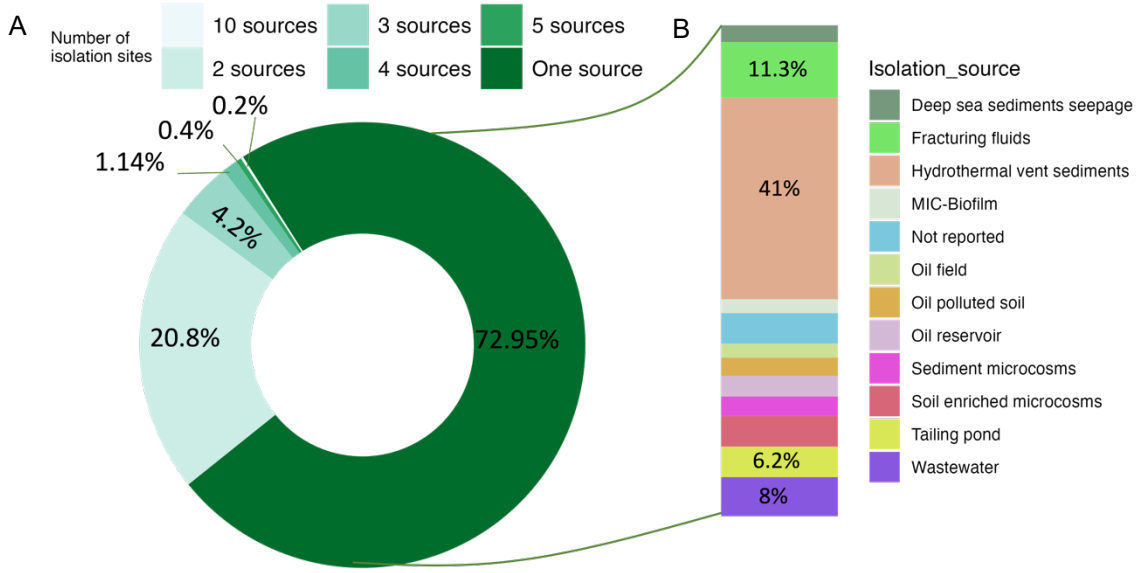


Figure5. (a) Percentage of species-level clusters according to the number of isolation sources. (b) Distribution of species-level clusters found in only one isolation source according to the type of environment.

Taxonomic classification and phylogenetic analysis revealed that PaGeD genomes are distributed in 109 different phyla (94 bacterial and 15 archaeal phyla), 248 classes, 553 orders, 999 families and 1,799 genera (Figure 4B). PaGeD is dominated by Pseudomonadota (before Proteobacteria), with almost 26.15% (872 / 3,334) of the genomes, followed by Bacteroidota 5.8% (193 / 3,334), Thermoproteota 5.6% (187 / 3,334), Desulfobacterota 5.2% (175 / 3,334) and Chloroflexota 4.0% (135 / 3,334) (Figure 4B). Moreover, one class, 8 orders, 36 families, 197 genera and 437 species do not currently have representatives in the GTDB, consequently representing potential novel lineages. Pseudomonadota was the most widely distributed phylum, with presence in all

continents (Figure S3A), habitats (e.g., marine, aquatic, terrestrial, microcosms, engineered and oil field), and isolation sources such as hydrothermal vent sediments, microcosms, oil polluted soils, among others (Figure S3B). *Acinetobacter radioresistens* was the species with more genomes (n=24) in PaGeD, followed by *Pseudomonas aeruginosa* (n=18) *Stutzerimonas stutzeri* (n=13), *Cellulosimicrobium funkei* (n=12), *Rhodococcus erythropolis* (n=11) and *Thermotoga petrophila* (n=1) (Figure 6). However, Acinetobacter was poorly distributed accross the isolation sources because it was only found in MIC samples. Various species from the *Acinetobacter* genus have been reported as well-known biofilm formers at corrosion (Lerm et al., 2013; Zhu et al., 2003). In contrast, Pseudomonas aeruginosa (n=18) was found in diverse types of samples, such as soil, sea water, produced water, oil reservoir and crude oil, among others (Figure 6A). Pseudomonas aeruginosa is an extremely adaptable and metabolically versatile bacterium, that has been detected in an extensive diversity of habitats, including, water, soil, oil polluted environments (Crone et al., 2020). P. aeruginosa is a prominent bacterium in the production of biosurfactants, which are surface active compounds useful in the bioremediation of polluted environments (Liu et al., 2018), as well as in the Microbial-Enhanced Oil recovery (MEOR) (Câmara et al., 2019). Interestingly, almost 100 (~3%) genomes were affiliated to the recently proposed phylum Patescibacteria (Brown et al., 2015). Members of the phylum were obtained from widely diverse isolation sources, being more prevalent in activated sludge/bioreactors (n=21), wastewater (n=15) and tailing ponds (n=12) (Figure S4). These bacteria are characterized by the small cell and genome size (Tian et al., 2020). In-depth analysis of the soil Patescibacteria metabolic potential, by comparing gene/pathway content of several genomes of this phylum with that of other soil bacteria, showed that Patescibacteria genomes lack essential biosynthetic capacities. A symbiotic lifestyle has been proposed as a tangible explanation for such genome conformation (Nascimento Lemos et al., 2020).

However, besides these exceptions of widely distributed bacteria across the petroleum-associated samples, the species-level clusters showed to be site-specific and with a limited geographical distribution (Figure 6B and Figure S5). Some studies focusing on only one type of isolation source (e.g., oil reservoirs, oil-polluted soils) showed similar results. For instance, a genome-centric meta-analysis performed by our research group

allowed us to assess microbial communities of oil reservoirs with different temperatures and levels of anthropogenic intervention (i.e., water injection), located at China, Alaska and Brazil. Results showed that the microbial community varied according to the site, suggesting high niche specialization (Hidalgo et al., 2021). Gittings and collaborators (2023) used 343 16S rRNA samples to study microbial communities from oil reservoirs around the world. This study showed that the oil reservoir amplicon libraries did not share any core taxa at the species, genus, family, or order levels, except for the Gammaproteobacteria class that was detected in all samples (Gittins et al., 2023). In concordance with Jiao et al. (2016), who analyzed the biogeographic pattern of the microbial communities in oil polluted soils. They observed a spatial structure of microbial communities (Jiao et al., 2016).

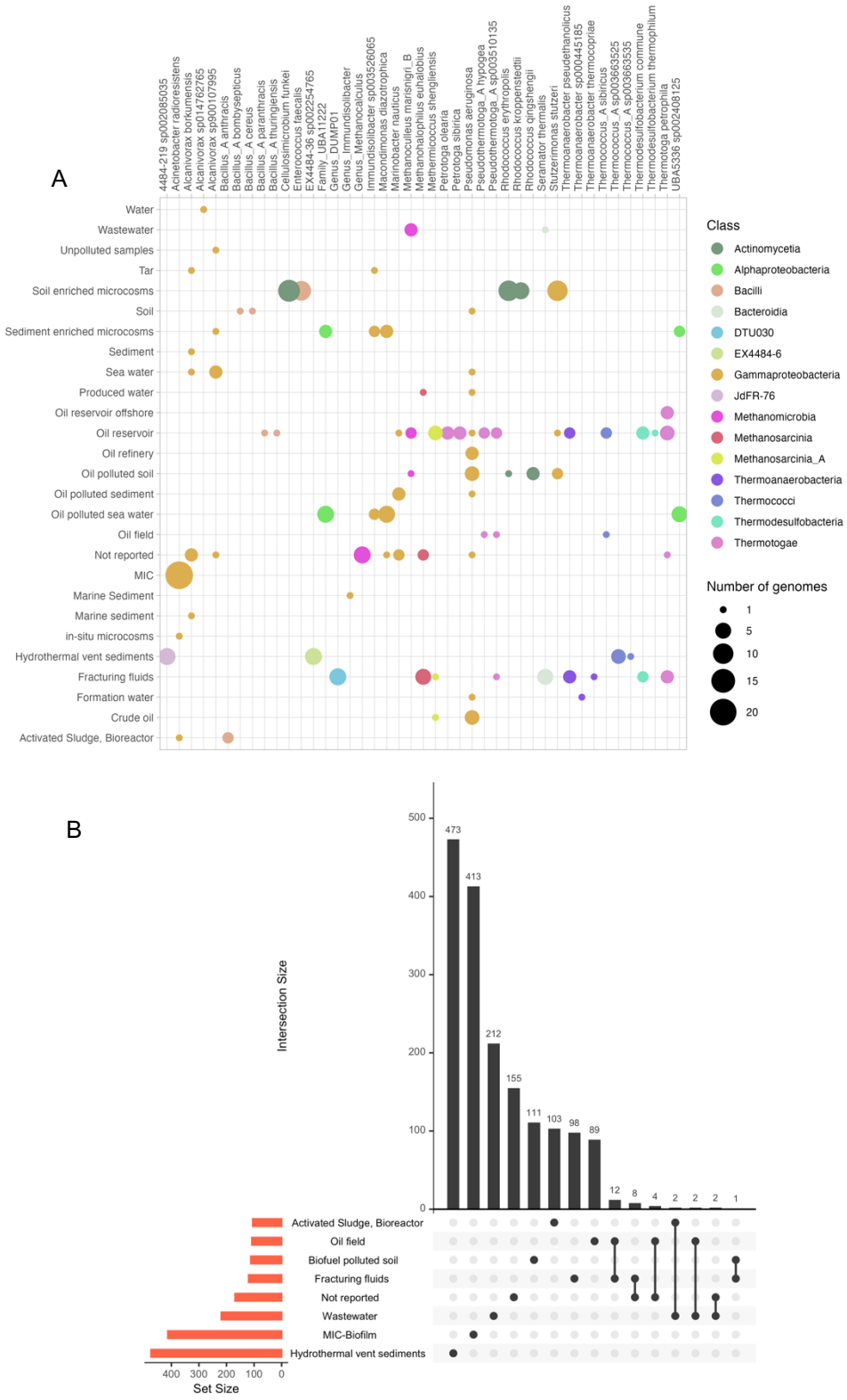


Figure6. (a) Distribution of species-level clusters with at least five members across the isolation sources. Class taxonomic level is represented by the bubble colors, and the size represents the number of genomes in each cluster. (b) Number of species-level clusters found across the isolation sources, ordered by their level of overlap. Vertical bars represent the number of species-level clusters shared between the specific isolation source highlighted with black lines and dots. Horizontal bars in the lower panel indicate the total number of species-level cluster in each isolation source.

In conclusion, the PaGeD offers a rich and comprehensive resource for performing in-depth analyses of the microbial diversity in several habitats associated to oil systems. A catalog of genomes of such environments is timely from different perspectives in the Oil & Gas industry. The knowledge of oil system associated microbial communities is quite useful in order to explore the metabolic potential and interactions of beneficial microorganisms such as hydrocarbon degraders, biosurfactant producers, among others. In addition, understanding the metabolisms of detrimental microorganisms such as sulfate reducing, acid producing and MIC-related microbes, allowing one to develop customized control strategies. Last but not least, PaGeD offers the possibility to expand the current microbial diversity knowledge, ending up with the deposit of new genomes in the databases.

Future perspectives for a robust genome collection

PaGeD is currently under construction. New genomes will be added through the direct communication with authors. In addition, a compilation of oil & gas metagenomes will be used to generate new MAGs. After that, a first version of PaGeD will be generated and functional annotation will be performed focused on carbon, nitrogen, and sulfate cycling genes, aiming to determine the functional potential of these genomes related with degradation of hydrocarbons, metal corrosion, souring, among other process of interest to the O&G industry. The resistome will also be annotated, searching for biocide resistance genes used in industry to deal with contamination by microorganisms in different industrial processes. Likewise, genes for tolerance to chemical substances will be targeted in order to understand the adaptations of these microorganism to the extreme conditions imposed by these environments. Combined results will allow us to establish a gene catalog of microorganisms associated with the O&G industry and likely elect genome and/or gene biomarkers for different environments and/or processes. Finally, in the future PaGeD will be available interactively through a shiny app, where the user will be able to search for different parameters.

Supplementary material

Table S2. Number and genome type by habitat and isolation source classification

Habitat	Isolation_source	Isolate	MAG
	Groundwater	2	101
	Hot springs	0	3
	Oil polluted mangrove	1	0
Aquatic	Oil polluted sediment	17	0
	Oil polluted water	4	0
	Sediment	2	0
	Water	0	77
	Activated Sludge, Bioreactor	10	96
	Fracturing fluids	3	187
	Lab experiment	1	0
	MIC	1	23
	MIC-Biofilm	0	416
Engineered	MIC-biofilm	1	8
	Machinery Facilities	1	12
	Sea water microcosms	0	29
	Sediment microcosms	0	37
	Tailing pond	0	118
	Wastewater	0	264
Host-associated	Host-associated	2	3
	Deep sea sediments seepage	0	82
	Deep sea water	2	0
	Hydrothermal vent sediments	0	662
	Marine Sediment	2	26
	Marine sediment	5	2
Marine	Oil polluted sea water	4	69
	Oil polluted sediment	7	1
	Oil spill offshore	0	6
	Sea water	12	47
	Sediment	1	0
	Unpolluted samples	0	18
	Crude oil microcosms	0	2
	Deep sea sediment microcosms	0	8
	Microcosms	2	0
Microcosms	Sediment enriched microcosms	0	29
	Sediment microcosms	0	21
	Soil enriched microcosms	2	138
	in-situ microcosms	0	19

Not reported	Not reported	66	127
Oil field	Crude oil	12	11
	Formation water	5	9
	Injection water	4	0
	Oil field	10	107
	Oil polluted water	0	11
	Oil refinery	6	0
	Oil reservoir	48	62
	Oil reservoir offshore	5	0
	Produced water	16	8
	Water-oil	2	1
Terrestrial	Biofuel polluted soil	0	112
	Bioremediation	1	0
	Oil polluted sediment	1	0
	Oil polluted soil	50	45
	Soil	9	2
	Tar	1	15
	Vulcano	1	0

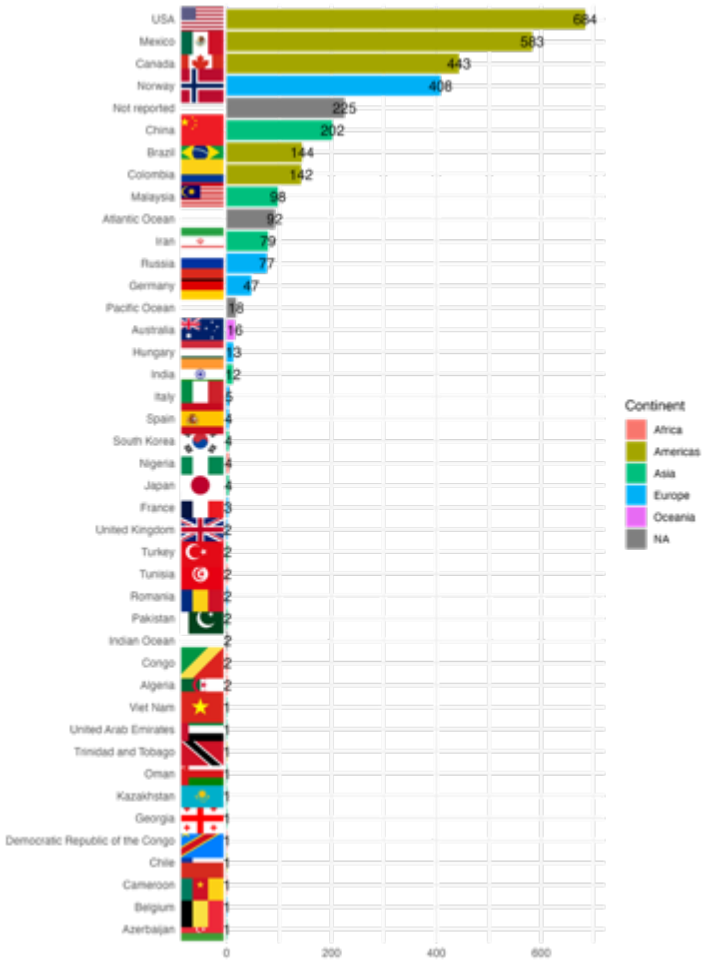


Figure S1. PaGeD distribution by countries.

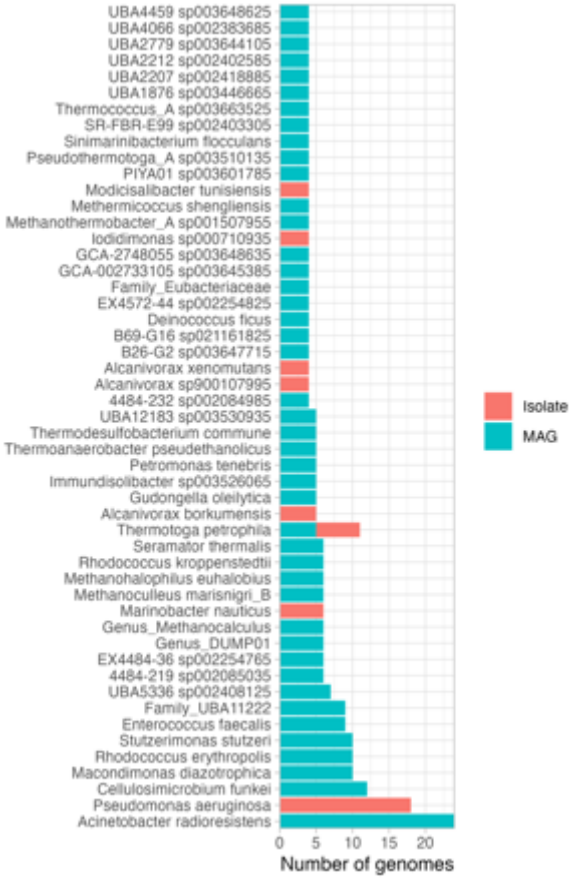
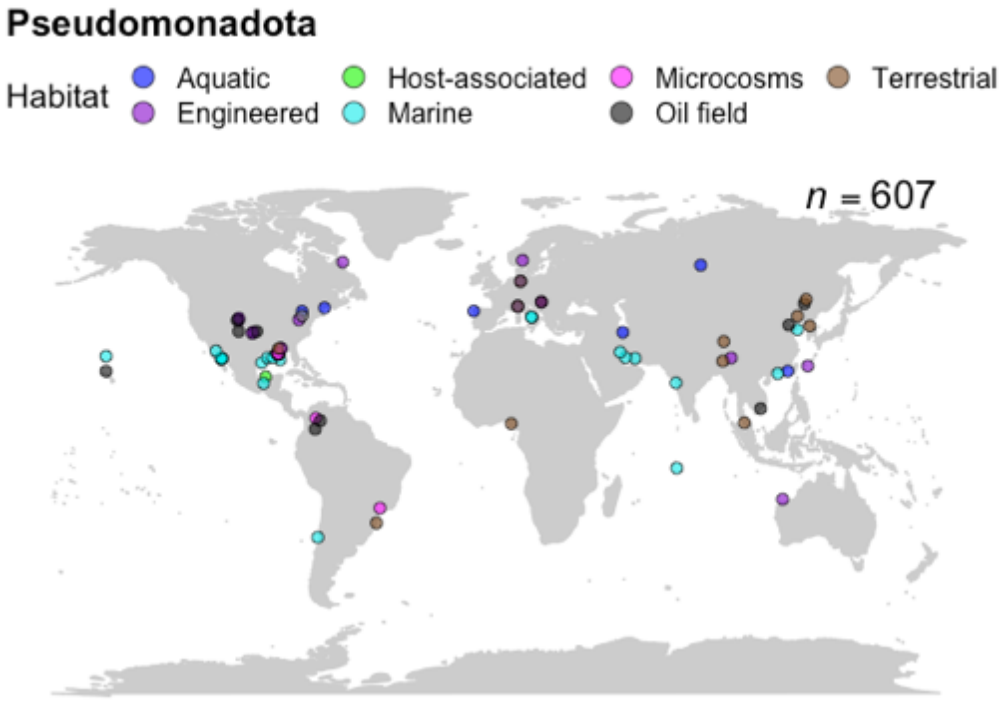


Figure S2. Type of genomes (MAG or isolate) members of the species-level clusters. Only species-level cluster with more than three genomes were plotted.

A



B

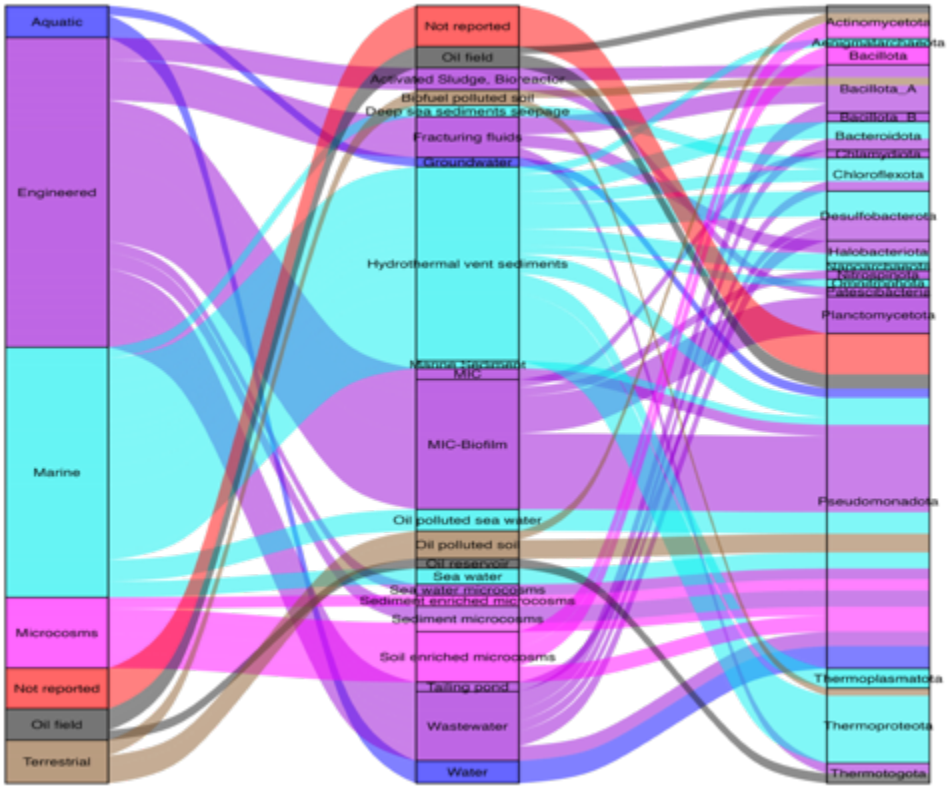


Figure S3. (a) Geographic and habitat distribution of the Pseudomonadota phylum. **(b)** Phyla distribution across the habitats and the isolation sources. For improve clarity only a subset of phyla with the most connections are shown.

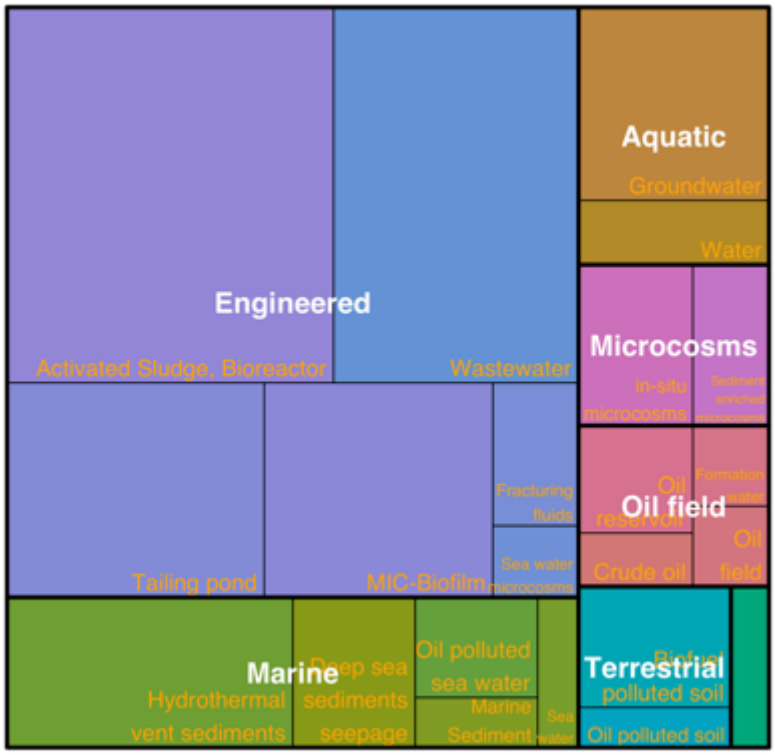


Figure S4. Patescibacteria phylum distribution across the habitats and isolations sources.

Figure S5. Species-level clusters with more than 3 genomes geographic distribution. The color represents individual cluster.

References

Augustinovic Z, Birketveit O, Clements K, Freeman M, Gopi S, Ishoey T, et al. Microbes–oilfield enemies or allies. Oilfield review 2012; 24: 1-14.

Bowers RM, Kyrpides NC, Stepanauskas R, Harmon-Smith M, Doud D, Reddy TBK, et al. Minimum information about a single amplified genome (MISAG) and a metagenome-assembled genome (MIMAG) of bacteria and archaea. Nature Biotechnology 2017; 35: 725-731.

Brown CT, Hug LA, Thomas BC, Sharon I, Castelle CJ, Singh A, et al. Unusual biology across a group comprising more than 15% of domain Bacteria. Nature 2015; 523: 208-211.

Câmara JMDA, Sousa MASB, Barros Neto EL, Oliveira MCA. Application of rhamnolipid biosurfactant produced by Pseudomonas aeruginosa in microbial-enhanced oil recovery (MEOR). Journal of Petroleum Exploration and Production Technology 2019; 9: 2333-2341.

Chan PP, Lin BY, Mak AJ, Lowe TM. tRNAscan-SE 2.0: improved detection and functional classification of transfer RNA genes. Nucleic acids research 2021; 49: 9077-9096.

Chaumeil P-A, Mussig AJ, Hugenholtz P, Parks DH. GTDB-Tk: a toolkit to classify genomes with the Genome Taxonomy Database. Bioinformatics 2019; 36: 1925-1927.

Chen I-MA, Chu K, Palaniappan K, Pillay M, Ratner A, Huang J, et al. IMG/M v. 5.0: an integrated data management and comparative analysis system for microbial genomes and microbiomes. Nucleic acids research 2019; 47: D666-D677.

Crone S, Vives-Flórez M, Kvich L, Saunders AM, Malone M, Nicolaisen MH, et al. The environmental occurrence of Pseudomonas aeruginosa. APMIS 2020; 128: 220-231.

Cruaud P, Vigneron A, Pignet P, Caprais J-C, Lesongeur F, Toffin L, et al. Comparative Study of Guaymas Basin Microbiomes: Cold Seeps vs. Hydrothermal Vents Sediments. Frontiers in Marine Science 2017; 4.

Foght J. Microbial communities in oil shales, biodegraded and heavy oil reservoirs, and bitumen deposits. Handbook of hydrocarbon and lipid microbiology 2010.

Gittins DA, Bhatnagar S, Hubert CRJ. Environmental Selection and Biogeography Shape the Microbiome of Subsurface Petroleum Reservoirs. mSystems 2023; 8: e00884-22.

Hidalgo KJ, Sierra-Garcia IN, Zafra G, de Oliveira VM. Genome-Resolved Meta-Analysis of the Microbiome in Oil Reservoirs Worldwide. Microorganisms 2021; 9: 1812.

Jain C, Rodriguez-R LM, Phillippy AM, Konstantinidis KT, Aluru S. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. Nature communications 2018; 9: 5114.

Jiao S, Liu Z, Lin Y, Yang J, Chen W, Wei G. Bacterial communities in oil contaminated soils: Biogeography and co-occurrence patterns. Soil Biology and Biochemistry 2016; 98: 64-73.

Karthikeyan S, Rodriguez-R LM, Heritier-Robbins P, Hatt JK, Huettel M, Kostka JE, et al. Genome repository of oil systems: An interactive and searchable database that expands the catalogued diversity of crude oil-associated microbes. Environmental Microbiology 2020; 22: 2094-2106.

Kimes NE, Callaghan AV, Suflita JM, Morris PJ. Microbial transformation of the Deepwater Horizon oil spill—past, present, and future perspectives. Frontiers in microbiology 2014; 5: 603.

Lee MD. GToTree: a user-friendly workflow for phylogenomics. Bioinformatics 2019; 35: 4162-4164.

Lerm S, Westphal A, Miethling-Graff R, Alawi M, Seibt A, Wolfgramm M, et al. Thermal effects on microbial composition and microbiologically induced corrosion and mineral precipitation affecting operation of a geothermal plant in a deep saline aquifer. Extremophiles 2013; 17: 311-327.

Letunic I, Bork P. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic acids research 2019; 47: W256-W259.

Liu WJ, Duan XD, Wu LP, Masakorala K. Biosurfactant Production by Pseudomonas aeruginosa SNP0614 and its Effect on Biodegradation of Petroleum. Applied Biochemistry and Microbiology 2018; 54: 155-162.

Lu H, Guo L, Azimi M, Huang K. Oil and Gas 4.0 era: A systematic review and outlook. Computers in Industry 2019; 111: 68-90.

Mason OU, Hazen TC, Borglin S, Chain PS, Dubinsky EA, Fortney JL, et al. Metagenome, metatranscriptome and single-cell sequencing reveal microbial response to Deepwater Horizon oil spill. The ISME journal 2012; 6: 1715-1727.

Mason OU, Scott NM, Gonzalez A, Robbins-Pianka A, Bælum J, Kimbrel J, et al. Metagenomics reveals sediment microbial community response to Deepwater Horizon oil spill. The ISME Journal 2014; 8: 1464-1475.

Nascimento Lemos L, Manoharan L, William Mendes L, Monteiro Venturini A, Satler Pylro V, Tsai SM. Metagenome assembled-genomes reveal similar functional profiles of CPR/Patescibacteria phyla in soils. Environmental Microbiology Reports 2020; 12: 651-655.

O'Leary N. W right MW, Brister JR, C iufo S, Haddad D, McVeigh R, et al. 2016. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucleic Acids Res; 44: D733-D745.

Olm MR, Brown CT, Brooks B, Banfield JF. dRep: a tool for fast and accurate genomic comparisons that enables improved genome recovery from metagenomes through de-replication. The ISME journal 2017; 11: 2864-2868.

Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome research 2015: gr. 186072.114.

Parks DH, Rinke C, Chuvochina M, Chaumeil P-A, Woodcroft BJ, Evans PN, et al. Recovery of nearly 8,000 metagenome-assembled genomes substantially expands the tree of life. Nature Microbiology 2017; 2: 1533-1542.

Rodriguez-R LM, Gunturu S, Harvey WT, Rosselló-Mora R, Tiedje JM, Cole JR, et al. The Microbial Genomes Atlas (MiGA) webserver: taxonomic and gene diversity analysis of Archaea and Bacteria at the whole genome level. Nucleic acids research 2018; 46: W282-W288.

Rodriguez-r LM, Overholt WA, Hagan C, Huettel M, Kostka JE, Konstantinidis KT. Microbial community successional patterns in beach sands impacted by the Deepwater Horizon oil spill. The ISME journal 2015; 9: 1928-1940.

Garcia-Sierra, I. Investigation of the microbiota and processes involved in petroleum degradation in oil reservoirs. Doctoral tesis. 2016, University of Campinas, Brazil

Tian R, Ning D, He Z, Zhang P, Spencer SJ, Gao S, et al. Small and mighty: adaptation of superphylum Patescibacteria to groundwater environment drives their genome simplicity. Microbiome 2020; 8: 1-15.

Wang J, Zhang Y, Ding Y, Song H, Liu T, Zhang Y, et al. Comparing the indigenous microorganism system in typical petroleum-contaminated groundwater. Chemosphere 2023; 311: 137173.

Wunch K, Stipaničev M, Frenzel M. Microbial Bioinformatics in the Oil and Gas Industry: Applications to Reservoirs and Processes: CRC Press, 2021.

Yang S, Wen X, Shi Y, Liebner S, Jin H, Perfumo A. Hydrocarbon degraders establish at the costs of microbial richness, abundance and keystone taxa after crude oil contamination in permafrost environments. Scientific Reports 2016; 6: 37473.

Zhou Z, St. John E, Anantharaman K, Reysenbach A-L. Global patterns of diversity and metabolism of microbial communities in deep-sea hydrothermal vent deposits. Microbiome 2022; 10: 241.

Zhu XY, Lubeck J, Kilbane JJ. Characterization of Microbial Communities in Gas Industry Pipelines. Applied and Environmental Microbiology 2003; 69: 5354-5363.

DISCUSSION

In Chapter I (Review 2: *Recent advances in bioremediation of biofuel blends*), we compiled comprehensive information about the state-of-the-art of biofuel blend bioremediation. This review allowed us to notice the gap in the literature about the ecology of microbiomes in environments polluted with biofuel / fossil fuel blends , mainly the hydrocarbon degrading microbial communities. Thus, we firstly addressed our efforts to investigate if there were differences among microbial communities inhabiting soils polluted with different biofuel blends (i.e., B20, E10, E25) (*Chapter II: Shifts in structure and dynamics of the soil microbiome in fuel/biofuel blends-affected areas triggered by different bioremediation treatments*). Microbial composition and structure were shown to be different depending on the biofuel blend (i.e., biodiesel or gasohol). Similar results were obtained in a previous study that compared the microbiome in soils polluted with blends E10 and B20 (Elazhari-Ali et al. 2013). Additionally, differences between the microbiomes in soils impacted with the same biofuel but in different proportions (i.e., E10 and E25) were observed, suggesting that the proportion of the blend also contributed to shape the communities. This result was also observed in other studies comparing microbial communities in areas contaminated with E10, E24 and E85 (Rama et al., 2019; Steiner et al., 2018). Regarding the characteristics of each type of area (i.e., biodiesel- or gasohol-polluted areas), we observed that the biodiesel-polluted areas showed more diverse and complex microbial communities. Biodiesel molecular composition is more complex than the ethanol, with the presence of different chemical functional groups (i.e. ester, fatty acids), being necessary different microorganisms to decomposed these compounds (Bücker, et al., 2011).

In chapter II, further analyses were performed to investigate the effect of different bioremediation treatments on the microbial community composition, structure, and co-occurrence patterns. It was observed that the anaerobic biostimulation with injection of electron acceptors triggered significant shifts in the communities. Meanwhile, in the biodiesel areas, mainly in area 5.B20-ANM treated with biosparging and bioaugmentation, the communities before and 12 months after the bioremediation treatment were not significantly different. Here, we hypothesized that these results could be due to the long-term contamination without any treatment intervention for 12 years, resulting in a microbial

community well adapted, resilient, or even resistant to perturbations. Throughout the analyses, area 5.B20-ANM proved to be very different from the other areas. For instance, unlike the other areas, in area 5.B20-ANM the stochastic events were more important for the assembly of the community. In addition, the co-occurrence network analyses carried out were more sensitive to unravel the perturbations in the community. For instance, area 5.B20-ANM did not show differences before and after the bioremediation treatment in the community composition analysis, while the co-occurrence network analysis showed that after the treatment the community interaction network was less complex, with a big decrease in the number of interactions. It has been proposed by some authors that, in case of perturbation, microbial interactions can be affected first, changing the functions of the ecosystem even before the species disappear (Valiente-Banuet et al., 2014). The resilience/ resistance of the microbial communities could be affected in the long term by the alteration of the network structure (Tylianakis et al, 2010, Vacher et al 2016). Moreover, we observed that almost all networks presented higher number of negative interactions after the treatment when compared with time zero (before the treatment), suggesting more competition or less cooperation. Some studies have demonstrated that substrates more complex promote more cooperative interactions and reduce the competitiveness (Deng and Wang, 2016; Lindemann, 2020). So, we think that the increase of the negative interactions might be a consequence of the bioremediation treatments that stimulated the degradation of the pollutants and the increase of less complex molecules. In addition, the network analyses also allowed to identify the keystone species using the node metrics. We observed that most of the keystone species had low relative abundances, showing that rare taxa also have relevant roles in maintaining the community homeostasis, as it was also observed by other authors (Jiao et al., 2017; Pan et al., 2021, Qian et al., 2020). It was also identified that more keystone species appeared after the treatment, and that these taxa are well-known hydrocarbon degraders, such as *Clostridium*, *Mycobacterium*, *Extensimonas* (Hennesse and Li, 2016; Morris et al., 2013; Wang et al., 2021), suggesting that these microorganisms can be used as target for future bioremediation intervention in the areas.

In terms of functional potential (Chapter III: *Functional redundancy as a key microbial strategy to cope with pollution in biofuel impacted soils*), we observed that the specific

functional profiles (i.e., hydrocarbon degradation and related genes) in the affected soils were influenced by previous bioremediation treatments. Thus, areas that had been submitted to any active bioremediation treatment in the past showed similar functional profiles, while area 5.B20-ANM that underwent only natural attenuation showed significant functional differences. In total, only 16% of hydrocarbon degradation genes searched were found in all areas, showing a relatively weak metabolic potential for hydrocarbon degradation, mainly observed in areas 5 and 7. This result was congruent with the higher concentrations of BTEX compounds in those areas when comparing with areas 4 and 8.

We observed that the biofuel type shaped the microbial community composition and structure. However, the functional potential was more related with previous bioremediation interventions in the affected areas, suggesting that microbial functional redundancy plays an important role in the recovery of polluted environments. This has been already observed in other studies (Bell et al., 2016; Morris et al., 2018; Mugge et al., 2021).

Additionally, chapter III also showed that 12 months after the bioremediation treatment, the microbial functional profile of area 5.B20-ANM was different, being the area with lower relative abundance of hydrocarbon degradation genes. All these results were reflected in the concentrations of BTEX in the soil, which were still high at time 12. However, after the second bioremediation treatment applied in the source zone, a decrease of all BTEX compounds in all areas was observed, and the increase of the number and relative abundance of the genes related with hydrocarbon degradation. These results suggest the power of the bioremediation treatments to modulate, shape and improve the microbial communities and functional potential profiles. In chapter III, it was also demonstrated that the same genes and metabolic pathways were enriched in the areas, independently of the bioremediation treatments, although they were assigned to different microbial taxa. These results corroborated the microbial community composition analyses carried out in chapter II, where it was showed that the enriched communities were different among the areas. Altogether, these results corroborate the fundamental role of the functional redundancy in the recovery of contaminated areas.

All the results presented above highlight the importance of the holistic knowledge of microbial communities in polluted areas, with the aim of understanding all microbial

processes that lead to the recovery of contaminated areas through active and/ or passive bioremediation approaches.

Finally, this work proposed the creation of a genome and gene database from petroleum associated environments/ samples (chapter IV: *Petroleum-associated Genome Database - PaGeD*) in order to have a rich and comprehensive resource for performing in-depth analysis of the microbial diversity in habitats associated to oil systems. Preliminary analyses using this genome collection showed high niche specialization among the microbial communities associated to oil systems. Furthermore, PaGeD showed a huge potential for the discovery of new species, since most of the species found do not yet exist in the available databases nor have been cultivated yet. Thus, PaGeD can contribute to expand the current microbial diversity knowledge.

In summary, the work presented in this thesis has contributed to increase our understanding of the biofuel/ fossil fuel blend contamination microbiology. Notably, the work conducted has showed that: i) the microbial community composition, structure, and co-occurrence patterns varied according to the biofuel type; ii) the microbiome of long-term no intervened soil is more resilient to perturbation, delaying the degradation; iii) the keystone species are well-known hydrocarbon degraders; iv) all bioremediation treatments enriched the same hydrocarbon degrading genes, but these were associated to different taxa, demonstrating the importance of the functional redundancy as a mechanism for hydrocarbon biodegradation; and iv) the PaGeD repository offers a tool with huge potential to explore the microbial diversity in different habitats associated with oil systems (i.e. oil reservoirs, contaminated environments, produced fluids, etc) around the world, characterized by a high niche specialization of the microbial communities.

FUTURE PERSPECTIVES

The knowledge of microbial communities in polluted environments brings important insights about how these communities can help in the degradation of contaminants, contributing to decision-making about the most appropriate approach to bioremediation. In recent years, more and more companies are using molecular biology tools and bioinformatics for the decommissioning of contaminated sites. However, there is still a lack of standard protocols for monitoring and evaluating contaminated environments that, in addition to geochemical and physical analyses, include microbiological analyses, such as composition, structure, co-occurrence patterns and functional potential of the microbial communities, as well as quantification of microbial groups of interest (i.e., degraders), and / or of key genes/enzymes in the degradation processes. These protocols should also include an important theoretical foundation from the different areas of knowledge to allow robust correlation of the data, enabling a holistic view of the polluted sites. With the growth of studies like these, it will be possible to generate models to predict the behavior of the communities, degradation rates, treatment types, duration and efficiency, paving the way for the worldwide use of bioremediation as an unequivocally efficient, cost-effective and sustainable approach for the recovery of contaminated sites.

References

Bell TH, Stefani FOP, Abram K, Champagne J, Yergeau E, Hijri M, et al. A Diverse Soil Microbiome Degrades More Crude Oil than Specialized Bacterial Assemblages Obtained in Culture. Applied and Environmental Microbiology 2016; 82: 5530-5541.

Bücker F, Santestevan NA, Roesch LF, Jacques RJS, Peralba MdCR, de Oliveira Camargo FA, et al. Impact of biodiesel on biodeterioration of stored Brazilian diesel oil. International Biodeterioration & Biodegradation 2011; 65: 172-178.

Deng Y-J, Wang SY. Synergistic growth in bacteria depends on substrate complexity. Journal of Microbiology 2016; 54: 23-30

Elazhari-Ali A, Singh AK, Davenport RJ, Head IM, Werner D. Biofuel components change the ecology of bacterial volatile petroleum hydrocarbon degradation in aerobic sandy soil. Environmental Pollution 2013; 173: 125-132.

Hennessee CT, Li QX. Effects of Polycyclic Aromatic Hydrocarbon Mixtures on Degradation, Gene Expression, and Metabolite Production in Four Mycobacterium Species. Applied and Environmental Microbiology 2016; 82: 3357-3369.

Jiao S, Chen W, Wei G. Biogeography and ecological diversity patterns of rare and abundant bacteria in oil-contaminated soils. Molecular ecology 2017; 26: 5305-5317.

Lindemann SR. A piece of the pie: engineering microbiomes by exploiting division of labor in complex polysaccharide consumption. Current Opinion in Chemical Engineering 2020; 30: 96-102.

Morris L, O'Brien A, Natera SHA, Lutz A, Roessner U, Long SM. Structural and functional measures of marine microbial communities: An experiment to assess implications for oil spill management. Marine Pollution Bulletin 2018; 131: 525-529.

Morris BE, Henneberger R, Huber H, Moissl-Eichinger C. Microbial syntrophy: interaction for the common good. FEMS microbiology reviews 2013; 37: 384-406.

Mugge RL, Salerno JL, Hamdan LJ. Microbial Functional Responses in Marine Biofilms Exposed to Deepwater Horizon Spill Contaminants. Frontiers in Microbiology 2021; 12.

Pan Z, Chen Y, Zhou M, McAllister TA, Guan LL. Microbial interaction-driven community differences as revealed by network analysis. Computational and Structural Biotechnology Journal 2021; 19: 6000-6008.

Qian X, Li S, Wu B, Wang Y, Ji N, Yao H, et al. Mainland and island populations of Mussaenda kwangtungensis differ in their phyllosphere fungal community composition and network structure. Scientific Reports 2020; 10: 952.

Tylianakis JM, Laliberté E, Nielsen A, Bascompte J. Conservation of species interaction networks. Biological conservation 2010; 143: 2270-2279.

Vacher C, Tamaddoni-Nezhad A, Kamenova S, Peyrard N, Moalic Y, Sabbadin R, et al. Learning ecological networks from next-generation sequencing data. Advances in ecological research. 54. Elsevier, 2016, pp. 1-39.

Valiente-Banuet A, Aizen M, Alcántara J, Arroyo J, Cocucci A, Galetti M, et al. Beyond species loss: extinction of interactions in a changing world. Functional Ecology, 2015, vol. 29, p. 299-307 2014.

Wang M, Garrido-Sanz D, Sansegundo-Lobato P, Redondo-Nieto M, Conlon R, Martin M, et al. Soil Microbiome Structure and Function in Ecopiles Used to Remediate Petroleum-Contaminated Soil. Frontiers in Environmental Science 2021; 9.

ANNEX

Anexo 1: Declaração de Bioética e/ou Biossegurança

COORDENADORIA DE PÓS-GRADUAÇÃO
INSTITUTO DE BIOLOGIA
Universidade Estadual de Campinas
Caixa Postal 6109. 13083-970, Campinas, SP, Brasil
Fone (19) 3521-6378. email: cpgib@unicamp.br

DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Qualificação, intitulada " Assessment of the effect of different bioremediation approaches on the soil microbiome of fuel-affected areas", desenvolvida no Programa de Pós-Graduação em Genética e Biologia Molecular do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos e animais.

Assinatura: _____
Nome do(a) aluno(a): Kelly Johanna Hidalgo Martinez

Assinatura: _____
Nome do(a) orientador(a): Valéria Maia Merzel

Data: 10/10/2022

Anexo 2: Termos de autorização para reprodução de conteúdo publicado

Declaração

As copias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas cientificas ou anais de congressos sujeitos a arbitragem, que constam na minha Dissertação/Tese de Mestrado/Doutorado, intitulada **Assessment of the effect of different bioremediation approaches on the soil microbiome of fuel-affected áreas**, não infringem os dispositivos da Lei n,º 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 18 de Julho de 2023

Assinatura: Kelly Hidalgo M.
Nome do(a) autor(a): **Kelly Johanna Hidalgo Martinez**
RG n.º G370387-I

Assinatura:_____
Nome do(a) orientador(a): **Valéria Maia Merzel**
RG n.º 12.573.419-0