UNIVERSIDAD DE BURGOS

Advances in bioremediation techniques for the degradation of hydrocarbons in soils

Sandra Curiel Alegre

Supervisors:

Juan Carlos Rad Moradillo

Rocío Barros García

Thesis for the degree of:

Doctor of Advances in Food Science and Biotechnologies

Burgos, January 2024

Dr. JUAN CARLOS RAD MORADILLO, Profesor Titular del Área de Edafología y Química Agrícola del Departamento de Química de la Universidad de Burgos y Dra. ROCÍO BARROS GARCÍA, Investigadora del International Research Centre in Critical Raw Materials for Advanced Industrial Technologies (ICCRAM) de la Universidad de Burgos.

INFORMAN FAVORABLEMENTE:

Que la presente memoria titulada "Advances in bioremediation techniques for the degradation of hydrocarbons in soils" ha sido realizada bajo su dirección en el Área de Edafología y Química Agrícola y en el International Research Centre in Critical Raw Materials for Advanced Industrial Technologies (ICCRAM) de la Universidad de Burgos, por la graduada Dña. Sandra Curiel Alegre y autorizan su presentación para que sea calificada como Tesis Doctoral. El trabajo experimental se encuentra bien planificado, posee una base científica rigurosa, ha empleado una bibliografía actualizada, y ha dado lugar a unos resultados de gran aplicabilidad en el sector de la recuperación de suelos contaminados con hidrocarburos mediante técnicas de bioestimulación y bioaumentación, así como tecnologías híbridas que combinan procesos de inmovilización en soportes orgánicos e introducción de sistemas bioelectroquímicos. La tesis culmina con el escalado en planta piloto del sistema optimizado que ha logrado importantes reducciones en la carga contaminante.

Burgos, 10 enero 2024

RAD MORADILLO JUAN CARLOS - 13106650P

Firmado digitalmente por RAD MORADILLO JUAN CARLOS - 13106650P
Fecha: 2024.01.10 10:00:26 +01'00'

Fdo.: Dr. Juan Carlos Rad Moradillo

BARROS GARCIA ROCIO - 53546784R

Firmado digitalmente por BARROS GARCIA ROCIO - 53546784R
Fecha: 2024.01.10 12:01:14 +01'00'

Fdo.: Dra. Rocío Barros García

“El más terrible de todos los sentimientos es el sentimiento de tener la esperanza muerta.”

Federico García Lorca

AGRADECIMIENTOS/ACKNOWLEDGEMENTS

En primer lugar, me gustaría agradecer al Dr. Carlos Rad por estos años de aprendizaje, apoyo y conocimiento transmitido, gracias por la confianza depositada en mí y la gran oportunidad que me dio desde mis comienzos como técnico de laboratorio. A la Dra. Rocío Barros por seguir los pasos de Carlos y darme el apoyo necesario para continuar en un proyecto tan bonito como GREENER, su apoyo tanto profesional como personal han hecho que este sueño se haga realidad poco a poco.

Agradecer a la Comisión Europea la financiación del proyecto GREENER (G.A. 826312) enmarcado en el programa Horizonte 2020, gracias al cual se ha podido desarrollar la presente investigación. También agradecer a la Consejería de Educación de la Junta de Castilla y León, por la ayuda predoctoral concedida a través de la Orden EDU/1508/2020, cofinanciada por el Fondo Social Europeo.

También me gustaría dar las gracias a todos los que de una manera u otra han pasado tantísimas horas conmigo en el laboratorio. A Dani por estar siempre, y ayudarnos con una sonrisa, gracias porque no sé qué sería de todos nosotros sin tenerte en cualquier momento al pie del cañón. A Sandra Guti, Paulis y Rajaa, porque fueron las primeras compañeras con las que tuve la suerte de coincidir e hicieron que las horas de trabajo siempre fueran más amenas, gracias por vuestra enorme amistad. A Blanca, Alberto, Elena, las dos Andreas, Aqib y Silvia por tanto apoyo, ayuda y tiempo compartido, gracias. A todo el equipo de laboratorio de ICCRAM con quien he tenido la suerte de aprender muchísimo, dando mis pinitos por la microbiología, sin los cuales hubiese sido imposible sacar nada adelante, en especial gracias a Dalia por estos últimos meses de ayuda personalizada. Al resto de compañeros de ICCRAM, porque son un equipo

increíble en todos los sentidos y esto también es un poco suyo. Gracias de manera especial a Bea, por su apoyo incondicional, y por convertirse en una verdadera amiga durante esta etapa.

Gracias a todo el Consorcio de GREENER, en especial a la Universidad Autónoma de Madrid por el gran trabajo desarrollado en colaboración. Thanks to the Jožef Stefan Institute for give me the opportunity to learn from them, for the time share together and for start a friendship since that time. Thanks Aleš, Tomaž, Maja, Dmitrii and Farzaneh.

Gracias a toda mi familia, soy muy afortunada de haber nacido en una tan buena como la mía. En especial, a mis padres, por una vida dedicada a mi hermano y a mí, por su sacrificio, por sus valores, por estar siempre en todo lo que nos hemos propuesto, por su intensa lucha, y por alegrarse tanto o más que nosotros cada vez que lo hemos conseguido. A mi tato, Iván, porque eres la definición de lo que es un buen hermano mayor porque, aunque llevemos muchos años viviendo lejos, siempre te siento cerca, gracias por hacer mi vida más bonita. No quiero olvidarme de destacar a mi abuelo Seve, una vida más justa le hubiese dejado estudiar, espero que una nieta doctora sea un orgullo para ti, tanto como lo eres tú para mí.

A mis amigos, esa familia que elegimos, los que llegaron por casualidad y los que sabían que llegaban para quedarse, por todas las risas y momentos compartidos. Sabéis de primera mano lo que os quiero y lo afortunada que soy de teneros.

Por último, gracias Migue. Por todo, no puedo estar más agradecida de tenerte en mi vida. Por aguantar todo el proceso que ha sido llegar hasta aquí, mis días malos cuando algo no salía en el laboratorio, mis horas pasadas en el ordenador sin tener tiempo para nada, gracias por estar siempre y ser un apoyo fundamental para conseguir esto.

RESUMEN

En la actualidad, uno de los problemas más importantes al cual nos tenemos que enfrentar como sociedad es la contaminación del medio ambiente. Se ha puesto especial interés en la descontaminación del agua y del aire, pero cada vez son más los emplazamientos contaminados en los que el suelo se encuentra en condiciones pésimas pudiendo causar grandes daños en muchos casos ya irreversibles.

Aunque en el suelo cada vez aparecen contaminantes más diversos, y en muchos casos mezclas de ellos, uno de los contaminantes principales al que tenemos que hacer frente son los hidrocarburos, su complejidad y naturaleza recalcitrante hacen que sean muy difíciles de degradar y supongan un coste económico muy alto. Por lo tanto, el objetivo principal de la presente tesis doctoral fue mejorar los métodos de biorremediación ya existentes, en un suelo altamente contaminado con hidrocarburos de cadena larga y otros contaminantes como los metales pesados, de manera que se realice una restauración eficaz, sostenible y económicamente viable de las áreas contaminadas con este tipo de contaminantes. A lo largo de esta investigación se ofrece un análisis exhaustivo de la aplicación de las técnicas de bioestimulación, bioaumentación, y bioelectroquímica, evaluando la optimización de estas tecnologías, la eficacia en la degradación de contaminantes, la mejora con la incorporación de aditivos o enmiendas orgánicas, y la sostenibilidad ambiental. Además, este trabajo aborda las limitaciones y los retos asociados a la aplicación de las técnicas de biorremediación, haciendo hincapié en la necesidad de seguir investigando y desarrollando estas técnicas para optimizar su eficacia.

En primer lugar, se hace un análisis de la importancia del suelo y la contaminación presente en él, así como de todas las tecnologías existentes que se usan habitualmente para la mitigación de los hidrocarburos, para concluir con los objetivos. Siguiendo con los antecedentes que nos llevan al desarrollo de esta investigación y el alcance de las técnicas de aplicación, poniendo en contexto el desarrollo de la presente tesis doctoral a través del Proyecto Europeo GREENER H2020. En segundo lugar, y como parte central, se desarrollan tres capítulos que corresponden con tres experimentos, dos a escala laboratorio y uno a escala piloto, en los que se desarrollan distintas soluciones para

optimizar la degradación principalmente de los hidrocarburos del petróleo, mostrándose la importancia de la selección de las condiciones del suelo para mejorar la eficacia de las técnicas. Además, para la mejora de las técnicas de biostimulación y bioaumentación ensayadas durante el desarrollo de estos experimentos, se probaron distintas enmiendas y aditivos orgánicos, soluciones nutritivas y un sistema de bioelectroquímica pasivo. Por último, se hace un repaso no sólo de lo anteriormente citado, si no una visión general de todos los experimentos desarrollados a lo largo del periodo en el cual se ha desarrollado la presente tesis doctoral, haciendo hincapié en el desarrollo de las tecnologías desde la escala laboratorio, a la escala piloto, y como último paso a escala real, pudiéndose entender de manera más precisa el funcionamiento de las técnicas y la mejora en la optimización y eficacia de ellas.

Los resultados obtenidos contribuyen al avance de la biorremediación de los suelos contaminados con hidrocarburos del petróleo y otros contaminantes xenobióticos como los metales pesados y al desarrollo de enfoques integrados para la gestión sostenible del suelo. Este documento aporta valiosas ideas sobre el potencial de las técnicas de biorremediación para hacer frente a la contaminación del suelo y la necesidad de seguir investigando en esta línea para desarrollar técnicas más eficaces.

ABSTRACT

Nowadays, one of the most important problems to be solved as a society is environmental pollution. Special attention has been received to the decontamination of water and air, but there are more contaminated sites where the soil is in a very poor condition and can cause significant damage, which in many cases is already irreversible.

Although different pollutants are appearing in the soil, and in many cases mixtures of them, one of the main pollutants to be treated are petroleum hydrocarbons, their complexity and recalcitrant nature make them very difficult to degrade and involve a very high economic cost. Therefore, the main objective of this doctoral thesis was to improve existing bioremediation methods, in a soil highly contaminated with long chain hydrocarbons and other pollutants such as heavy metals, to carry out an effective, sustainable, and economically viable restoration of the areas contaminated with this type of pollutants. Throughout this research, an exhaustive analysis of the application of biostimulation, bioaugmentation and bioelectrochemical technologies is showed, evaluating the optimisation of these technologies, the effectiveness in the degradation of pollutants, the improvement with the incorporation of additives or organic amendments, and environmental sustainability. In addition, this work addresses the limitations and challenges associated with the application of bioremediation techniques, with particular emphasis on the need for further research and development of these techniques to optimise their effectiveness.

Firstly, an analysis is made of the importance of the soil and the contamination present in it, as well as all the existing technologies that are commonly used for the mitigation of hydrocarbons, to conclude with the objectives. Following with the background that led us to the development of this research and the scope of the application techniques, putting in context the development of this doctoral thesis through the European Project GREENER H2020. Secondly, and as a central part, three chapters are developed corresponding to three experiments, two at laboratory scale and one at pilot scale, in which different solutions are developed to optimise the degradation mainly of petroleum hydrocarbons, showing the importance of the selection of soil conditions to improve the effectiveness of the techniques. In addition, for the improvement of the

biostimulation and bioaugmentation techniques tested during the development of these experiments, different organic amendments and additives, nutrient solutions and a passive bioelectrochemical system were tested. Finally, a review is made not only of the above mentioned, but also an overview of all the experiments developed throughout the period in which this doctoral thesis has been developed, emphasising the development of technologies from laboratory scale, to pilot scale, and as a last step to real scale, being able to understand more precisely the operation of the techniques and the improvement in their optimisation and effectiveness.

The results obtained contribute to the advancement of bioremediation of soils contaminated with petroleum hydrocarbons and other xenobiotic pollutants and to the development of integrated approaches for sustainable soil management. This thesis provides valuable insights into the potential of bioremediation techniques to address soil contamination and the need for further research to develop more effective techniques.

CONTENT

FIGURES

TABLES

ACRONYMS

AC: Activated Carbon

AIT: Austrian Institute of Technology

AMC: Amino-4-MethylCoumarin

ANOVA: ANalysis Of VAriance

BA: Bioaugmentation

BAME: Bacterial Acid Methyl Ester

BES: Bioelectrochemical Systems

BET: Specific Surface Area according Brunauer-Emmett-Teller isotherm

BH: Biochar

BHB: Bushnell Haas Broth

BS: Biostimulation

BSR: Basal Soil Respiration

CFU: Colony Forming Unit

CT: Control

DCM: DiChloroMethane

DM: Dry Matter

DNA: DeoxyriboNucleic Acid

DOI: Digital Object Identifier

DPBS: Phosphate-Buffered Saline

DW: Dry Weight

E: Epoxioctane

EC: Electrical Conductivity

EGD: European Green Deal

EI: Electron Ionization

ENAR: Enhanced Natural Attenuation Remediation

EOC: Extractable Organic Carbon

EPA: Environmental Protection Agency

EPHs: Extractable Petroleum Hydrocarbons

ETN: Extractable Total Nitrogen

EU: European Union

FAME: Fatty Acid Methyl Ester

FAO: Food and Agriculture Organization of the United Nations

FID: Flame Ionization Detector

FS: Fatty acid (Saturated) esters

FTIR-ATR: Fourier Transform InfraRed Spectroscopy - Attenuated Total Reflectance

GC-MS: Gas Chromatography-Mass Spectrometry

GREENER: InteGRated systems for Effective ENvironmEntal Remediation

HC: Hydrocarbon

HD: Hydrocarbon Degradation

HOCs: Hydrophobic Organic Pollutants

HSD: Honestly Significant Difference

HWRC: Highest Water Retention Capacity

H_x: Hopanes, x is the carbon number

HX: Hexane

ICCRAM: International research Center in Critical RAw Materials for Advanced Industrial Technologies

ISEBE: International Symposium on Environmental Biotechnology and Engineering

ITPS: Intergovernmental Technical Panel on Soils

IUSS: International Union of Soil Science

JASP: Jeffrey's Amazing Statistics Program

JRC: Joint Research Center

KPIs: Key Performance Indicators

LC: Liquid Chromatography

LOI: Loss Of Ignition

MES: Microbial Electrochemical System (Microbial Electrochemical Snorkel)

MUB: Modified Universal Buffer

MUF: 4-MethylUmbelliFerone

OAE: Oleic Acid Ester

OD: Optical Density

OM: Organic Matter

OTUs: Operational Taxonomic Units

P: Plasticizer / Phosphorus

PAS: Phosphate-buffered mineral medium Salts

PBS: Phosphate-Buffered Saline

PCA: Plate Count Agar

PCBs: PolyChlorinated Biphenyls

PCoA: Principal Coordinate Analysis

PCR: Polymerase Chain Reaction

PFASs: PolyFluoroAlkylated Substances

PLFAs: PhosphoLipid Fatty Acids

POPs – Persistent Organic Pollutants

PostHoc: “Post hoc ergo propter hoc,” meaning “after this, therefore because of this.”

PUFAs: PolyUnsaturated Fatty Acids

RML: Rhamnolipid

RNA: RiboNucleic Acid

RPMs: Revolutions Per Minute

SARA: Saturates, Aromatics, Resins, and Asphaltenes

SDGs: Sustainable Development Goals

SEM: Scanning Electron Microscopy

SIM: Single Ion Chromatograms

SVE: Soil Vapor Extraction

SVOCs: Semi-Volatile Organic Compounds

SWHC: Soil Water Holding Capacity

TEN: Total Extractable Nitrogen

TN: Total Nitrogen

TOC: Total Organic Carbon

TPHs: Total Petroleum Hydrocarbons

UAM: Universidad Autónoma de Madrid

UBU: Universidad de Burgos

UBUCOMP: Universidad de Burgos - Grupo de Compostaje

UCM: Unresolved Complex Mixture

VC: Vermicompost

VOCs: Volatile Organic Compounds

WRC: Water Retention Capacity (same as SWHC)

CHAPTER 1

General Introduction, Objectives and Thesis Outline

1.1. Soil as part of life.

Soil is an environmental matrix that carries life for all living things (Daâssi & Qabil Almaghribi, 2022). Soil is an evolved natural body formed by the disintegration and decomposition of rocks and minerals in the form of a profile, which serves as mechanical support and for the growth of plants because it contains air, water, nutrients, and organisms (Hartemink, 2016). The natural soil environment provides a complex habitat in which mineral and organic matter are three-dimensionally organized and combined (de Souza Machado et al., 2020). Over time, the interaction of climate, living things, and topography changes rocks, resulting in many types of soils. These types of soils differ in terms of the composition of minerals and particle sizes of sand, silt, and clay, and in terms of the structure that they produce (Guarino et al., 2017). Biological and physicochemical properties of soil, environmental factors and anthropogenic influences make soils to play a critical role, affecting ecosystem services, environmental quality, agricultural sustainability, climate change and human health (Hou et al., 2020). Most of the terrestrial life, as well as terrestrial ecosystems that consequently benefit humans, are due to soils (de Souza Machado et al., 2020). It is a fundamental part of the natural environment, most plants need the soil as a source of water and nutrients, therefore, humans need it, as they feed on plants or consume animals that feed on plants (de Souza Machado et al., 2020). To maintain the fertility of agricultural soils, plants use nutrients from the soil and store them in their tissues. Some of these nutrients are transferred to animals or humans directly or indirectly, and others are returned to the soil in the form of organic or inorganic fertilisers. However, these nutrient sources often do not completely replace the nutrients used through harvesting, resulting in long-term loss of nutrients and degradation (Silver et al., 2021). Maintaining soils with a equilibrate amount of nutrients leads to healthy soils, suitable for agriculture and the life of micro and macro fauna.

1.2. Soil contamination.

The presence of a substance where it should not be present, or in concentrations higher than background concentrations, implies contamination, which may have produced adverse biological effects on indigenous communities, or may do so over time (Chapman, 2007). When contamination occurs in soil it is defined as soil pollution, which occurs if a chemical or substance out of place and/or at a higher than normal concentration is present in the soil and has adverse effects on non-target organisms present on it (FAO & ITPS, 2015). Soil contamination can be caused by different types of chemicals or pollutants present in the Earth, which are harmful to living organisms. A pollutant is a substance or energy that has undesirable or negative effects when introduced into the environment and affects the usefulness of a resource (Havugimana et al., 2017). Apart from natural disasters such as earthquakes and erosion, the main sources of soil pollution are industrial and household waste (Ukaogo et al., 2020). Deforestation, the burning of bushes, the dumping of agricultural and domestic wastewater, the use of chemicals in the collection of aquatics and the improper disposal of electronic waste are activities in which human beings pollute, in many cases due to lack of knowledge that they become dangerous to the environment (Ukaogo et al., 2020). However, there are other sources of anthropogenic pollution caused by industrial activities that generate highly toxic pollutants, such as mining activities, foundries, the chemical, electronics, and oil industries, as well as the burning of fossil fuels (Khan et al., 2021).

The presence of chemicals or substances in soils that are out of place or in exceeding background concentrations of natural environments are becoming more and more common, and these substances result in adverse effects on any non-target organism (Rodríguez-Eugenio et al., 2018). Although it should be noted that one of the growing problems is the increase of soils that are contaminated with more than one different pollutant, which makes remediation more difficult to achieve, posing a high risk to natural ecosystems and the organisms living in them, plants and animals (Panwar & Mathur, 2023), causing adverse effects on human health through the food chain (Ye et al., 2017).

Ossai et al., (2022) explained that the most common chemicals causing contamination in the world are pesticides, and solvents. However, Van Liedekerke et al. (2014), showed in the "Progress in the management of Contaminated Sites in Europe" that the most frequent contaminants in water and soils in Europe were heavy metals, petroleum, petroleum-derived compounds, and chlorinated organic solvents, as given in Figure 1.

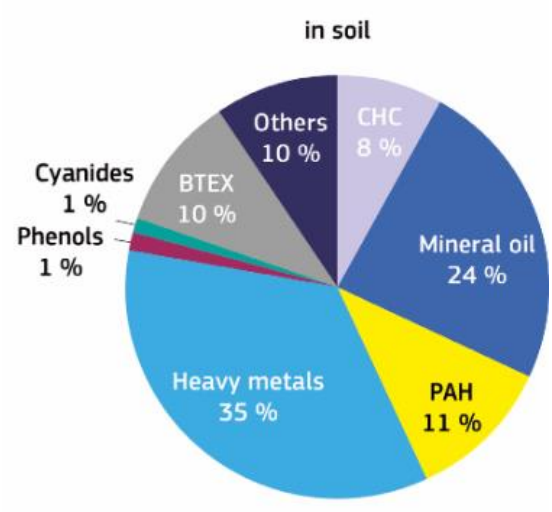


Figure 1. Most frequent contaminants in European water and soils. (Van Liedekerke et al., 2014)

Therefore, with the Stockholm Convention, which deals with persistent organic pollutants (POPs), the treatment of these toxic substances began to be regulated through an international agreement, where over the years the list has been increasing and classified as POPs that must be eliminated, banned, or those that must be controlled due to their unintentional production. The first list specified by the Stockholm Convention in Regulation (EC) 850/2004 only recognized 12 POPs causing adverse effects on the ecosystem and humans, and classified them into 3 specific categories:

- Pesticides: aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, hexachlorobenzene, mirex, and toxaphene.
- Industrial chemicals: hexachlorobenzene, and polychlorinated biphenyls (PCBs).
- By-products: hexachlorobenzene; polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (PCDD/PCDF), and PCBs.

This first agreement, Regulation (EC) 850/2004, signed on 23 May 2001 in Stockholm, which did not enter into force until 17 May 2004, was signed by 151 countries, but after

years of negotiations more countries have joined, with 186 countries now signing new agreements. The latest agreement signed is Regulation (EU) 2019/1021, where new POPs were added, which entered into force on 15 July 2019 and was revised on 13 December 2022.

Special attention should be paid to hydrocarbons, there are different types, such as saturated, unsaturated, and aromatic. Saturated hydrocarbons are alkanes, which are the simplest, because they contain only single bonds between carbon atoms. Unsaturated hydrocarbons are alkenes and alkynes, which are more complex and contain at least one double bond and one triple bond respectively. Finally, aromatic hydrocarbons, such as benzene, have a ring structure with delocalized electrons. Their different structural and chemical properties make them important for various industrial or chemical needs (Ossai et al., 2020). Petroleum-based products continue to be an essential supply of energy for both industrial and domestic use. In addition, leaks are common when petroleum and its derivative products are being discovered, extracted, refined, transported, and stored (Guarino et al., 2017). However, although hydrocarbons are classified as saturated, unsaturated, or aromatic, a more specific classification is made for those derived from petroleum (Varjani, 2017). These can be classified as:

- Saturated or aliphatic: Hydrocarbons without double bonds that make up the largest percentage of crude oil.
- Aromatic: With one or more aromatic rings that are usually replaced by different alkyl groups.
- Resins and asphaltenes: Contain non-hydrocarbon polar compounds, which do not appear in the previous ones. They have very complex carbon structures, with nitrogen, sulphur and oxygen atoms appearing in them.

The nature of these compounds gives them a specific chemical behaviour that affects their biodegradability in the environment (Varjani, 2017). High molecular weight hydrocarbons including long-chain aliphatic and polycyclic aromatic hydrocarbons (PAH), are common persistent organic contaminants that are toxic to many organisms and deleterious to the environment. The persistence of hydrocarbons in the soil is influenced by several variables, including molecule structure, soil characteristics, soil microbial

diversity, and environmental conditions. Aliphatic hydrocarbons can survive in the soil for months to years, while PAHs can persist for years or decades due to their complex structure and low volatility (Yap et al., 2021). PAHs are one of the most dangerous environmental contaminants, that are produced by the pyrolysis or incomplete combustion of organic compounds. It can also be produced by the maturation of fossilised organic matter in the form of coal or crude oil, and therefore also present in the heavy fractions of petroleum hydrocarbons (Gao et al., 2018). PAHs cause a serious problem due to its mutagenic and carcinogenic toxicity (Panwar & Mathur, 2023). PAHs are one of the pollutants included in Regulation (EU) 2019/1021 through the Stockholm Convention, in Annex III (Part B), concerning substances subject to emission reduction provisions, where the indicators defined were benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene and indeno(1,2,3-cd)pyrene, therefore, a special interest currently resides in them. Petroleum hydrocarbon pollution has a negative effect on physical, chemical, and biological properties, so environmental sustainability is directly linked to its presence in the soil.

Effective remediation techniques are crucial to mitigate long term effects of hydrocarbons on soil quality and environmental safety. Remediating persistent hydrocarbons from soils is generally a slow and expensive process, particularly for the high molecular weight fractions derived from oil refinery sludge.

In addition, to knowing the type of contaminant present in the soil and against which we are going to act, it is important to know how these compounds have reached the soil, when and where they are found. Contaminants can appear in a diffuse form, that is, spread over large areas in which there is no identified source of contamination, and we cannot be sure that there is a single source, nor can we be sure when this contamination occurred (Hartemink, 2016). This type of pollution involves the passage of pollutants between different media such as soil, air, and water. On the other hand, acute pollution refers to that which is due to point pollution by a fully identified source and in the short term (Picariello et al., 2020), which allows us to act more directly on both the source and its effect.

1.3. European Soil Protection Policies

In the 21st century globalized world, political decisions on laws, marketing, and subsidies, together with management decisions made by farmers, foresters, and planners, play an important role in ensuring soil sustainability, but increasingly public opinion may be the most crucial factor (Keesstra et al., 2016). In a context of demographic expansion and climate change, there is an urgent need to preserve soils globally to meet social demands (Cimpoiasu et al., 2021). To address the main risks of soil organic matter decline, soil deterioration and soil contamination, the European Commission developed the Soil Protection Strategy in 2006, even though several EU policies were already contributing to soil governance but were insufficient to ensure an adequate level of protection (Heuser, 2022). Further, to mitigate this problem of soil degradation, the International Decade of Soils (2015-2024) was declared by the International Union of Soil Science (IUSS), in which seeks to increase public awareness of soil protection, and thus guide us towards more sustainable policies. The fundamental stakeholders in the use of sustainable soil management practices are governments and scientists, their action seeks scientific knowledge and expand its applicability by including it in policy instruments (Hou et al., 2020). The Food and Agriculture Organization of the United Nations (FAO) try to connect this growing soil knowledge with the Sustainable Development Goals (SDGs). To achieve the SDGs proposed by the United Nations, soil health is very important, although it has not been directly mentioned in any of them, so interdisciplinary research must be encouraged to find real solutions. This interdisciplinary research will be carried out between soil scientists and researchers, in fields related to social sciences, climate sciences, ecology and environmental sciences (Hou et al., 2020).

The European Green Deal (EGD), which seeks to improve soil techniques with the Zero Pollution Action Plans, the Farm to Fork and Biodiversity 2030 initiatives, and the Climate Law framework, represent some of the many policies that focus on addressing environmental and climate concerns (Heuser, 2022). Soil degradation is expected to receive significant political attention and legal importance, as food security, poverty reduction and climate change adaptation and mitigation cannot be achieved without

fertile soils, therefore EU legislation must focus on the protection and responsible use of soil (Heuser, 2022).

Intergovernmental Technical Panel of Soil identified soil pollution as the third threat to European soil functions in 2015 (FAO & ITPS, 2015). The “Zero Pollution Action Plan” and the "EU Soil Strategy for 2030" aim to ensure that, by 2050, (i) all EU soil ecosystems are healthy and more resilient and can continue to provide their crucial services, (ii) there is no land take and soil pollution is reduced to levels that are no longer harmful to people’s health or ecosystems and, (iii) protecting soils, managing them sustainably and restoring soils is a common standard.

Remediation of the contaminated soils and sediments requires active cleaning operations, which is very costly, especially for large areas with persistent contamination (European Commission, 2006). Contamination significantly reduces soil functions, like the ability to act as a carbon sink, hindering efforts to limit the global temperature increase to 1.5-2 °C, as targeted in Paris Agreement, and threatening food safety, security, and achievement of the SDGs. Despite the complexity of the identification, investigation, and remediating the contaminated soils, increased technical and legal efforts are necessary to improve ecosystems conservation and human-health protection. A common framework for supporting environmental protection, specifically in soil-contamination prevention and remediation, would contribute to these goals.

On 17th Novembre 2021 the European Union adopted the EU Soil Strategy for 2030 (European Commission, 2021) that sets out a framework and concrete measures to protect and restore soils and ensure that they are used sustainably with the global objective of achieving healthy soils by 2050. As part of this objective, on July 5th 2023, the European Commission approved the draft of the Directive on Soil Monitoring and Resilience that provides a harmonised definition of soil health, try to put in place a comprehensive and coherent monitoring framework and lays down rules on sustainable soil management and remediation of contaminated sites (EU Commission, 2023).

1.4. Remediation technologies for contaminated sites.

Remediation technologies are used to decontaminate polluted areas. Depending on the pollutant, or types of pollutants, it may be necessary to use a combination of techniques to improve the effectiveness of the techniques and achieve the desired results. Further details on the different remediation methods most commonly used to remediate hydrocarbon contaminated soil are given below.

To classify the different techniques, a distinction is made between those that are carried out in the place where the pollutant appears, *in situ* techniques, and those that are carried out by moving the soil to another place to be treated, *ex situ* techniques. In *in situ* bioremediation, the soil is treated without the need for excavation, which involves minimal technology, and is a simple process that also avoids the risk of contaminants spreading during transport (Paul et al., 2021). In contrast, *ex situ* bioremediation involves the movement of soil from the original site to another site for disposal in landfill or treatment, which is more complex and costlier (Paul et al., 2021) as it requires a lot of manpower (Akpasi et al., 2023).

1.4.1. Physical remediation.

The removal or separation of contaminants from the soil physically has given rise to these techniques. The use of physical methods provides high efficiency in the removal of pollutants, but they are quite expensive (Raffa et al., 2021). The type and concentration of the contaminants, the properties of the soil, the design and operation of the remediation system, are the factors that will define the effectiveness of the technique. Depending on the physical technique, it can be applied *in situ* or *ex situ*, knowing the site conditions and the specific contaminants to be removed.

1.4.1.1. Soil washing.

As soil washing is quick and efficient, it is frequently used to remediate severely contaminated soils (Kim et al., 2022). By doing this, the amount of contaminated soil decreases and also the concentration of contaminants. The method involves chemical and physical processes and, depending on the nature of the

contamination, the selection of the washing agent is essential for the success of the operation. Being an easy and cost-effective process, it can be used for *in situ* decontamination (Li et al., 2019b). Sometimes, depending on the form of contamination and the type of soil, the soil is taken out of the contaminated location and combined with the suitable extractant solution (Khalid et al., 2017). The use of reagents promotes the dissolution or migration of contaminants in the soil (Teng et al., 2020), causing them to leach. It can be a source of new chemical contaminants, due to the introduction in soils different types of reagents (Raffa et al., 2021).

1.4.1.2. Soil spanding.

Soil spanding is a physical remediation technique, in which contaminated soil is excavated and mixed with pure soil, with the objective of reducing the concentration of contaminants (Priya et al., 2023). The use of this technique allows the soil to be aerated, which promotes microbial activity, accelerating the spontaneously degradation of pollutants in soil (Priya et al., 2023).

1.4.1.3. Soil substitution.

Removing polluted soil and adding pure soil in its place is a technique known as soil substitution for soil restoration (Priya et al., 2023), where the replaced soil is treated as waste (Raffa et al., 2021). Soil substitution effectively reduces exposure to contaminants by successfully removing the soil under study. This technique is onerous and expensive (Raffa et al., 2021), consequently, it is carried out when pollution is discovered in tiny, specific places like close to industrial sites or underground storage tanks. (Priya et al., 2023).

1.4.2. Thermal remediation.

Thermal techniques involve the application of heat to the contaminated soil to improve the volatilization or degradation of contaminants. These methods can be used to degrade or volatilise organic substances such as PAHs, which are transformed

into gases and increase their mobility, and for *ex situ* treatment, the gases can be collected in wells (Sakshi et al., 2019).

1.4.2.1. Thermal Desorption.

Thermal desorption is the technique of heating the soil to vaporize contaminants with a low boiling point, collecting these contaminants and releasing the remediated soil (Li et al., 2022). The temperature at which thermal desorption is carried out depends on the specific contaminants being treated. However, it should be noted that specific soil conditions are required for treatment, and the soil may need to be processed before it is introduced into the thermal desorption system (Li et al., 2022). It has been shown to successfully remove persistent organic pollutants from soil, such as polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and per- and polyfluoroalkylated substances (PFASs) (Li et al., 2022; Sörengård et al., 2020).

1.4.2.2. Thermal Heating.

The soil is heated and causes the contaminants to evaporate, which are collected and treated separately. Infrared heating can be used to treat soil *in situ* or *ex situ* (Vidonish et al., 2016). If heating is applied by infrared radiation to the soil, it is called infrared heating. It is a fast process and has a key advantage in that it can treat large areas of contaminated soil. It is also effective in treating soils with considerably moisture content, which can be difficult to treat using other technologies (Yang et al., 2021). Some specific types of pollutants cannot be treated by this method; however, this method is effective for the treatment of volatile organic compounds (VOCs) and semi-volatile organic compounds (SVOCs) with low boiling points.

1.4.2.3. Soil Vapor Extraction (SVE).

It is possible to remove volatile organic compounds (VOCs), such as petroleum hydrocarbons, chlorine-based solvents, and other organic pollutants, by applying vacuum to vertical or horizontal wells screened in unsaturated soils (Stewart et al.,

2020). Through an inlet well, fresh air is introduced into the contaminated soil, the vacuum pump and induced draught fan create a negative pressure which is used to volatilise the oil and dissolve and adsorb volatile organic compounds (VOCs) from the soil (Cao et al., 2021). Before being discharged into the environment or extracted again, the resulting gases are pumped to the surface for purification (Guo et al., 2020). Because of its excellent efficiency and low-cost cost, this technology has garnered a lot of interest (Cao et al., 2021).

1.4.3. Chemical remediation.

Technologies that apply chemicals to isolate, concentrate, precipitate, contain, sequester, segregate, and eliminate pollutants from contaminated soils are examples of chemical remediation techniques (Ossai et al., 2020). By using chemical substances, pollutants in the soil can be turned less mobile, available, and hazardous through adsorption, precipitation, reduction, oxidation, complexation, or polymerization (Song et al., 2022). These techniques require the use of dangerous chemicals in many cases, which pose risks to human health and the environment.

1.4.3.1. Chemical Stabilization.

Chemical stabilization is a technique that involves adding chemicals to soil to decrease the mobility and bioavailability of contaminants (Teng et al., 2020), minimizing their potential for leaching or absorption by plants, and reducing their environmental toxicity. The presence of stabilising compounds induces numerous chemical and physical processes, such as ion exchange, desorption, adsorption, surface complexation, precipitation, densification, and agglomeration (Schlögl et al., 2023). The process does not involve the removal or destruction of contaminants, but aims to contain them within the soil, converting the contaminants into a chemically stable form, producing an immobile mass or monolithic block (Ossai et al., 2020). For example, it was shown that by using asphalt emulsions on oil-contaminated soils, these emulsions stabilised and solidified into a durable matrix, serving as a building material. Numerous important influencing factors, including soil conditions, experiment varieties, component

geochemistry, treatment duration, and stabilization rates, significantly affect stabilization efficiency (Xu et al., 2021).

1.4.3.2. Chemical Oxidation/Reduction.

The chemical oxidation technique consists of adding chemicals to the soil to oxidize or reduce contaminants and convert them into less toxic forms, through redox reactions. Remediation of hydrophobic organic pollutants (HOCs), including PAHs, is possible using chemical oxidation (Li et al., 2019a). In addition, a large degradation of hydrocarbons from petroleum is achieved where different oxidants such as Fenton or persulphate are used to deal with different organic compounds (Usman et al., 2018). However, for contaminants that are adsorbed on soil particles it is not very efficient, because the oxidation reaction usually takes place in the aqueous phase (Li et al., 2019a). Therefore, to improve the efficiency, the humidity should be greatly increased. Photocatalysts can also be used to stimulate oxidative reactions as part of photocatalytic degradation (Sakshi et al., 2019).

1.4.3.3. Encapsulation.

Encapsulation technology immobilises contaminated soil and prevents surrounding materials from becoming contaminated by combining soils with materials such as concrete, asphalt or lime (Khalid et al., 2017).

1.4.4. Biological remediation.

Biological remediation or bioremediation techniques use fungi, bacteria, or plants to biodegrade or eliminate contaminants from polluted areas, transforming them into less toxic or inert forms (Kalia et al., 2022). Depending on chromosomal genes as well as extracellular enzyme activity, bacteria and other microorganisms can degrade an extensive range of organic substances (Bala et al., 2022). They can be classified as waste management techniques, of which there are several types depending on their application site, type of pollutant and method of application (Azubuike et al., 2016). Bioremediation technologies degrades harmful substances, providing organisms with nutrients and other chemicals necessary for their survival (Bala et al., 2022).

It is a low-cost, non-invasive remediation technique and offers a persistent solution to restore natural soil conditions (Khalid et al., 2017). Like other remediation methods, bioremediation can be used *in situ* or *ex situ*, depending on the characteristics of the contaminated site and the specific types of contaminants that remain at the site.

1.4.4.1. Natural attenuation.

Natural attenuation or intrinsic bioremediation is based on the ability of micro-organisms present in soil to degrade pollutants through aerobic and anaerobic microbial processes without human intervention (Azubuike et al., 2016). As this technique does not require external intervention, it will be more economical, where environmental conditions such as pH, temperature and oxygen levels will be adjusted to improve the process. Currently, a variation of this technique is Enhanced Natural Attenuation Remediation (ENAR), in which soil is removed to promote microbial growth, aeration, nutrient availability, moisture, and enhanced degradation of contaminants (Ayilara et al., 2023).

1.4.4.2. Bioventing.

Bioventing is an *in-situ* remediation process to degrade organic pollutants using microorganisms. The technique is performed by injecting air into contaminated areas, thereby degrading the pollutants absorbed in the soil. (Ayilara et al., 2023). This technique stimulates the natural biodegradation of hydrocarbon derivatives by indigenous microorganisms with air, oxygen flow and the necessary nutrients (Yadav et al., 2021). Although it is commonly used for the removal of hydrocarbons or persistent organic pollutants from the soil, it can also be used for the removal of pesticides (Setiadi et al., 2022).

1.4.4.3. Land farming.

One of the most popular and traditional forms of bioremediation is landfarming, which can be done both *in situ* and *ex situ*. (Lukić et al., 2017). The oil industry has been using it frequently since it is a cost-effective and easy method to depollute

soils that have been unintentionally affected by oil spills (Lukić et al., 2017). Landfarming is an environmentally friendly technology, however during the remediation process some of the physicochemical properties of the soil may deteriorate, thus affecting soil health (Lee et al., 2022). To maximize the rate and effectiveness of pollutant degradation, it is crucial to monitor soil parameters such as pH, aeration, moisture, and nutrient content (Lukić et al., 2017).

1.4.4.4. Biostimulation.

Biostimulation is a technique that seeks to accelerate the natural biodegradation process under ideal physicochemical conditions: pH, temperature, oxygen, moisture content and nutrients (Kumar et al., 2018). It involves the addition of nutrients or fertilisers to stimulate the growth and metabolic activity of indigenous microbes (Udume et al., 2023). The addition of nutrients to contaminated sites limited in carbon, oxygen, nitrogen, or phosphorus, stimulates microbial activity, enhances their survival, and increases their ability to degrade contaminants (Rawat et al., 2023; Udume et al., 2023). Several studies on petroleum hydrocarbons contaminated soils showed that the addition of only nitrogen or phosphorus as nutrients was able to promote the growth of soil microbial communities, allowing for more efficient bioremediation (Wu et al., 2016; Wu et al., 2019).

The main advantage is that the native micro-organisms already present in the soil responsible for bioremediation are well adapted to the environment (Adams et al., 2020). Furthermore, through studies of soil microbial evolution, it has been shown that biostimulation ensures soil microbial uniformity and diversity, allowing for more efficient hydrocarbon degradation (Wu et al., 2019). It was also shown that in soils contaminated with petroleum hydrocarbons there was an enrichment of degrading microorganisms of the *Pseudomonadaceae* and *Burkholderiaceae* bacterial families, based on 16S rRNA sequencing of DNA analysis (Kundu et al., 2022).

The main problem is that the distribution of the additives throughout the affected area depends on the geology of the soil, where impermeable lithology caused by

compacted clays or other fine-grained materials hinders their distribution (Adams et al., 2020). Although biostimulation results sometimes give high degradation values, there may be toxicity or phytotoxicity problems due to the increased availability of the contaminants produced by the soils indigenous microorganisms (Giovanella et al., 2021).

1.4.4.5. Bioaugmentation.

Bioaugmentation adds living organisms such as fungi, bacteria, or algae to the soil to degrade or remove contaminants using them as a food resource. Bioaugmentation has become an environmentally friendly *in situ* bioremediation technology, offering the most promising solutions for successful environmental bioremediation and degradation of contaminants in different sites, including soil, water, and sediments, and allowing bioremediation of both organic and inorganic contaminants (Kuppusamy et al., 2020). The success of the technology depends mainly on the adaptation of the microbiome to the site to be decontaminated, competition with the indigenous microorganisms, pH, temperature, moisture, organic matter content, aeration, nutrient content, and other physicochemical characteristics of the soil.

When hydrocarbons are the polluting source, these techniques rely on the addition of oil-degrading microorganisms to enhance native populations (Adams et al., 2020). The identification and selection of a particular microbial strain or microbial consortium with the ability to biodegrade or biotransform the desired pollutants is the most crucial aspect for the success of the application (Kumar et al., 2018). The enzymatic capacities and preferences of different microbial species vary for the degradation of petroleum substances (Adams et al., 2020). In particular, the decomposition of PAHs has been effectively achieved by using microbial species from the genera *Pseudomonas*, *Acinetobacter*, *Rhodococcus*, and *Burkholderia* (Rawat et al., 2023). The effectiveness of the microorganisms depends on the capacity to contend with native microorganisms, predators, and diverse abiotic variables (Adams et al., 2020). It has been shown that the combination of bioaugments with other amendments allows for more effective bioremediation,

e.g. with the use of biochars a significant effect on hydrocarbon degradation was observed in the short term, and without the need to add chemical biostimulants or surfactants (Guirado et al., 2021). Furthermore, it has been shown that the addition of rhamnolipid and rhamnolipid producing bacteria together with bioaugmentation improves the microbial activity of soil endogenous or exogenous microorganisms, and consequently the degradation of the hydrocarbons contained in the soil (Joe et al., 2019). Numerous studies such as Wu et al. (2019) and Nwankwegu et al. (2022) have shown that bioaugmentation is an effective bioremediation technique for the bioremediation of petroleum hydrocarbon contaminated soils. But it is not only the type of microorganisms that affects the effectiveness of bioaugmentation (Nwankwegu et al., 2022), but also the environmental conditions, so that small-scale laboratory experiments often show better results than large-scale *in situ* experiments.

It is important to highlight how the application of omics tools has significantly improved the understanding of how bioremediation technologies, such as bioaugmentation, can be used. Omics tools can play a vital role in the bioremediation of soil organic contaminants by providing valuable information on microbial communities and their functional potentials in contaminated soils (Sharma et al., 2022). Metagenomics allows the analysis of the entire genetic content of the microbial community, shedding light on the presence of specific genes involved in the degradation pathways of organic pollutants. Metatranscriptomics focuses on gene expression patterns, allowing researchers to identify active metabolic pathways and monitor the effectiveness of bioremediation treatments (Bala et al., 2022). By combining these omics approaches, scientists can gain a comprehensive understanding of microbial ecology and functional dynamics during bioremediation, which can be translated into field applications by selecting appropriate microbial inoculants due to a deeper understanding of the functional dynamics of the microbial community during bioremediation, optimising environmental conditions for enhanced degradation of organic contaminants, facilitating real-time monitoring of bioremediation progress, developing efficient enzyme cocktails for bioremediation

purposes, preparing synthetic enzymes that can be marketed as a stand-alone solution for the degradation of organic contaminants from environmental matrices, allowing timely adjustments and ensuring successful remediation results.

1.4.4.6. Composting.

For generations, people have utilized composting, a self-heating biological process, for the disposal of organic waste (Lin et al., 2022). The composting material contains microbial populations capable of degrading organic pollutants, the process also increases the bioavailability of pollutant compounds through the elevated temperatures reached which cause volatilisation of volatile and semi-volatile substances (Lin et al., 2022). In addition, being composed of several types of microorganisms, some species may mineralise or completely metabolise a pollutant, while other species may only cometabolise or non-specifically oxidise pollutants (Lin et al., 2022). Composting can be carried out in open piles, in open or closed containers, in windrows, and in vermicomposting, depending on the required needs (Mihai et al., 2020; Sayara & Sánchez, 2020). It is an economical and ecologically friendly option for the management of urban organic waste, agricultural waste, sewage sludge, or polluted soil, and by using the by-product in agriculture or environmental restoration as a soil amendment or organic fertiliser. These technologies promote at the same time, the transition to a circular economy framework (Koolivand et al., 2020).

1.4.4.7. Mycoremediation.

Mycoremediation uses fungi to degrade, transform or immobilise in the removal of toxic compounds, achieving their elimination (Bosco & Mollea, 2019). Knowing the fungal capacity and catabolic activity of the enzymes produced is important because their performance depends on this, which implies degradation potential, toxicity of by-products, environmental adaptability, and economic viability (Kumar et al., 2021). Degradation potential can be improved by manipulating various abiotic and biotic factors (Kumar et al., 2021). Fungal growth is favoured over bacterial growth in contaminated environments, due to their hyphal network,

biomass, and extended life cycle (Akpasi et al., 2023). However, these processes are restricted to the soil surface where fungal mycelia can grow due to aerobic conditions (Akpasi et al., 2023). It is an economically efficient, environmentally sound, and effective strategy to combat soil and water pollution (Akhtar & Mannan, 2020).

1.4.4.8. Phytoremediation.

Phytoremediation is the use of plants to bioremediate contaminated areas, such as soil and water (Kafle et al., 2022). Plants with high degrading capacity often have deep roots, quick growth, large biomass, and effective absorption and transportation of heavy metals or other pollutants to their aerial parts (Priya et al., 2023). Phytoremediation is a technique with many advantages: it is efficient, environmentally friendly, can produce bioenergy from oilseed and biomass crops, is low cost, can be applied *in situ* and *ex situ*, and removes multiple pollutants (Priya et al., 2023). Although this technique is mostly used for the bioremediation of heavy metals, it can be used for more types of pollutants with a good selection of plants. There are different types of phytoremediation depending on the types, forms and means of contamination as outlined below:

I. **Phytoextraction or phytoaccumulation:** Employs accumulator plants to remove metals or organic pollutants out of soil by depositing them in different harvestable parts of the plant (Sharma et al., 2018). Contamination is taken up by the roots, passes into the shoots and is deposited in the vacuole, cell wall, cell membrane and other parts of plant tissues that are not metabolically active (Kafle et al., 2022). Harvested plants are used for various purposes when accumulated toxins are safe, such as livestock feed or biofuel manufacturing (Priya et al., 2023)

II. **Phytostabilisation or phytoimmobilisation:** Using metal-tolerant plants to immobilise heavy metals in the soil and reduce their bioavailability (Priya et al., 2023). In this way it reduces the number of contaminants that migrate by runoff, leaching, or erosion (Sharma et al., 2018).

III. **Phytovolatilisation:** Soil pollutants are taken up by plants, which then release them into the atmosphere after metabolic transformation into volatile byproducts (Sharma et al., 2018). Not widely used because of limitations, such as the likelihood of airborne toxins contaminating surrounding areas (Priya et al., 2023). It is therefore used in situations where volatilised pollutants, once released from the soil into the atmosphere, have less adverse consequences (Kafle et al., 2022).

IV. **Rhizodegradation:** This technique involves using plants and their root exudates to improve microbial activity associated with the root zone to degrade soil contaminants (Priya et al., 2023).

V. **Phytotransformation:** Plants take up pollutants that are then enzymatically broken down into simpler forms, incorporating them into plant tissues, which they use in metabolic growth processes (Sharma et al., 2018).

VI. **Phytodesalination:** Plants that are tolerant to salts can recover saline soils and extract large amounts of salts (Kafle et al., 2022).

1.5. Objectives.

To solve the problems of contamination by hydrocarbons and other xenobiotic compounds in the soil under study, and to develop the technologies envisaged, some objectives were set to be developed within this doctoral thesis. The main objective was the study of bioremediation methods to remediate a soil contaminated with recalcitrant long-chain hydrocarbons and heavy metals from site 001 of the European GREENER project, which is explained in detail in chapter 2.

The objectives of the bioremediation techniques studied for soils contaminated with hydrocarbons and potentially toxic metals and metalloids are mainly focused on achieving effective and sustainable restoration of contaminated sites. More specifically, an attempt was made to improve biostimulation and bioaugmentation techniques:

- Increase the growth and microbial activity of indigenous microorganisms in the contaminated soil under study, to improve the biodegradation capacity.

- Accelerate the bioremediation process by adding microorganisms with specific hydrocarbon degradation capabilities.

- Provide the microorganisms present in the soil with the necessary nutrients to improve their growth and increase their degradation capabilities.

- Improve working conditions and physicochemical properties of the soil to increase the efficiency of bioremediation, achieving better results more quickly.

- Increase the bioavailability of contaminants and, if possible, finally eliminate them, using different types of materials such as biosurfactants.

- Evaluate the potential changes in the microbial community dynamics and functionality in the treatments developed and improve the scale up the studied techniques.

- Study of hybrid solutions such as bioelectrochemical remediation to prove the improve of degradation capabilities.

1.6. Thesis outline.

This doctoral thesis is based on the search for the improvement of two soil bioremediation techniques such as biostimulation and bioaugmentation, selected for their low cost, their environmental friendliness and for being efficient techniques for the type of pollutants we are dealing with throughout the research work.

After this first chapter, in which we have introduced the importance of soils, their pollutants and the most widespread techniques for their decontamination, and the objectives, we will move on to second chapter, in which we will study the background of this work, the European GREENER project, under which this doctoral thesis has been carried out, and we will seek to understand the scope of this research.

In the following chapters, chapters three, four and five, the specific research work carried out at the Universidad de Burgos (UBU) in collaboration with various project partners will be developed. For soil decontamination, nutrient solutions have been used to increase the biodegradation capacity of microorganisms, organic amendments, carriers and additives, and different microbial species, and the characteristics of the experiments have been modified to improve the effectiveness of the techniques studied. The research presented in this doctoral thesis is already published in Open Access so that the whole scientific community can benefit from the results obtained or is in the process of publication and is currently under review in scientific journals.

This is a complete work in which the degradation capabilities of microorganisms susceptible of degrading hydrocarbons in soil have been investigated from the laboratory scale with the use of soil microcosms, as shown in chapters three and four, to the pilot scale, as can be seen in the fifth chapter, in which the improved techniques have been studied by performing 500 kg of pilot scale soil mesocosms in the facilities of one of the GREENER project partners in Madrid.

Finally, chapter six, the last chapter, shows the thesis overview, which put together all the experiences carried out throughout this thesis, including parts not added in the different chapters but carried out during the development of the same, in which both previous incubations and intermediate ones are done as previous experiences for the development of the chapters here present. Although the project has already finished, as a way of testing the proposed technologies for the European Commission, a full-scale experiment is being carried out on 10 tonne biopiles, also at the facilities of our project partners in Madrid. Through this last experience we hope to continue to obtain better solutions for the bioremediation of soils contaminated with hydrocarbons and other xenobiotic compounds and to give a vision of the next steps to further improve the techniques. In addition, this last chapter also includes conclusions and concluding remarks for this research work.

1.7. References.

Adams, G. O., Fufeyin, P. T., Okoro, S. E., & Ehinomen, I. (2020). Bioremediation, Biostimulation and Bioaugmention: A Review. *International Journal of Environmental Bioremediation & Biodegradation*, 3(1), 28-39. https://doi.org/10.12691/ijebb-3-1-5

Akhtar, N., & Mannan, M. A. U. (2020). Mycoremediation: Expunging environmental pollutants. *Biotechnology Reports*, 26, e00452. https://doi.org/10.1016/j.btre.2020.e00452

Akpasi, S. O., Anekwe, I. M. S., Tetteh, E. K., Amune, U. O., Shoyiga, H. O., Mahlangu, T. P., & Kiambi, S. L. (2023). Mycoremediation as a Potentially Promising Technology: Current Status and Prospects—A Review. *Applied Sciences,* 13(8), 4978. https://doi.org/10.3390/app13084978

Ayilara, M. S., Adeleke, B. S., Adebajo, M. T., Akinola, S. A., Fayose, C. A., Adeyemi, U. T., Gbadegesin, L. A., Omole, R. K., Johnson, R. M., Edhemuino, M., Ogundolie, F. A., & Babalola, O. O. (2023). Remediation by enhanced natural attenuation; an environment-friendly remediation approach. *Frontiers in Environmental Science*, 11, 1182586. https://doi.org/10.3389/fenvs.2023.1182586

Azubuike, C. C., Chikere, C. B., & Okpokwasili, G. C. (2016). Bioremediation techniques–classification based on site of application: principles, advantages, limitations and prospects. *World Journal of Microbiology and Biotechnology*, 32 (180), 1-18. https://doi.org/10.1007/s11274-016-2137-x

Bala, S., Garg, D., Thirumalesh, B. V., Sharma, M., Sridhar, K., Inbaraj, B. S., & Tripathi, M. (2022). Recent Strategies for Bioremediation of Emerging Pollutants: A Review for a Green and Sustainable Environment. *Toxics*, 10(8), 484. https://doi.org/10.3390/toxics10080484

Bosco, F., & Mollea, C. (2019). Mycoremediation in Soil. In: *Environmental Chemistry and Recent Pollution Control Approaches* (H. Saldarriaga-Noreña, M. A. Murillo-Tovar, R. Farooq, R. Dongre & S. Riaz eds.) IntechOpen. 173. https://doi.org/10.5772/intechopen.84777

Cao, W., Zhang, L., Miao, Y., & Qiu, L. (2021). Research progress in the enhancement technology of soil vapor extraction of volatile petroleum hydrocarbon pollutants. *Environmental Science: Processes and Impacts*, 23(11). https://doi.org/10.1039/d1em00170a

Chapman, P.M. (2007) Determining when contamination is pollution - weight of evidence determinations for sediments and effluents. *Environment International*, 33(4), 492-501. https://doi.org/10.1016/j.envint.2006.09.001

Cimpoiasu, M. O., Dowdeswell-Downey, E., Evans, D. L., McCloskey, C. S., Rose, L. S., & Sayer, E. J. (2021). Contributions and future priorities for soil science: Comparing perspectives from scientists and stakeholders. *European Journal of Soil Science*, 72(6), 2538-2557. https://doi.org/10.1111/ejss.13162

European Commission (2006). Proposal for a Directive of the European Parliament and of the Council establishing a framework for the protection of soil and amending Directive 2004/35/EC /*. COM/2006/0232 final - COD 2006/0086 */. https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:52006PC0232&from=EN

European Commission (2021). Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the regions EU Soil Strategy for 2030 Reaping the benefits of healthy soils for people, food, nature and climate. COM/2021/699 final. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52021DC0699

Daâssi, D., & Qabil Almaghribi, F. (2022). Petroleum-contaminated soil: environmental occurrence and remediation strategies. *3 Biotech*, 12, 139. https://doi.org/10.1007/s13205-022-03198-z

de Souza Machado, A. A., Horton, A. A., Davis, T., & Maaß, S. (2020). Microplastics and Their Effects on Soil Function as a Life-Supporting System. *Handbook of Environmental Chemistry*, 95, 199-222. https://doi.org/10.1007/698_2020_450

FAO & ITPS. 2015. *Status of the World's Soil Resources (SWSR) - Main Report*. Rome, Italy, Food and Agriculture Organization of the United Nations and Intergovernmental Technical Panel on Soils. (also available at http://www.fao.org/3/ a-i5199e.pdf)

Gao, P., Li, H., Wilson, C. P., Townsend, T. G., Xiang, P., Liu, Y., & Ma, L. Q. (2018). Source identification of PAHs in soils based on stable carbon isotopic signatures. *Critical Reviews in Environmental Science and Technology*, 48(13-15), 923-948. https://doi.org/10.1080/10643389.2018.1495983

Giovanella, P., de Azevedo Duarte, L., Kita, D. M., de Oliveira, V. M., & Sette, L. D. (2021). Effect of biostimulation and bioaugmentation on hydrocarbon degradation and detoxification of diesel-contaminated soil: A microcosm study. *Journal of Microbiology*, 59, 634-643. https://doi.org/10.1007/s12275-021-0395-2

Guarino, C., Spada, V., & Sciarrillo, R. (2017). Assessment of three approaches of bioremediation (Natural Attenuation, Landfarming and Bioaugmentation – Assisted Landfarming) for a petroleum hydrocarbons contaminated soil. *Chemosphere*, 170, 10-16. https://doi.org/10.1016/j.chemosphere.2016.11.165

Guirado, M., Garrido-Sanz, D., Pindado, O., Rodríguez-Rastrero, M., Merino-Martín, L., Sierra, M. J., Escolano, O., Rivilla, R., & Millán, R. (2021). Effectiveness of biochar application and bioaugmentation techniques for the remediation of freshly and aged diesel-polluted soils. International Biodeterioration & Biodegradation, 163, 105259. https://doi.org/10.1016/j.ibiod.2021.105259

Guo, H., Qian, Y., Yuan, G. xiang, & Wang, C. xiao. (2020). Research progress on the soil vapor extraction. *Journal of Groundwater Science and Engineering*, 8(1), 57-66. https://doi.org/10.19637/j.cnki.2305-7068.2020.01.006

Hartemink, A. E. (2016). The definition of soil since the early 1800s. *Advances in Agronomy*, 137, 73-126. https://doi.org/10.1016/bs.agron.2015.12.001

Havugimana, E., Sopan Bhople, B., Kumar, A., Byiringiro, E., Mugabo, J. P., & Kumar, A. (2017). Soil Pollution-Major Sources and Types of Soil Pollutants. *Environmental Science and Engineering*, 11, 53-86.

Heuser, Dr. I. (2022). Soil Governance in current European Union Law and in the European Green Deal. *Soil Security*, 6. https://doi.org/10.1016/j.soisec.2022.100053

Hou, D., Bolan, N. S., Tsang, D. C. W., Kirkham, M. B., & O'Connor, D. (2020). Sustainable soil use and management: An interdisciplinary and systematic approach. *Science of the Total Environment*, 729, 138961. https://doi.org/10.1016/j.scitotenv.2020.138961

Joe, M. M., Gomathi, R., Benson, A., Shalini, D., Rengasamy, P., Henry, A. J., Truu, J., Truu, M., & Sa, T. (2019). Simultaneous application of biosurfactant and bioaugmentation with rhamnolipid-producing shewanella for enhanced bioremediation of oil-polluted soil. *Applied Sciences*, 9(18), 3773. https://doi.org/10.3390/app9183773

Kafle, A., Timilsina, A., Gautam, A., Adhikari, K., Bhattarai, A., & Aryal, N. (2022). Phytoremediation: Mechanisms, plant selection and enhancement by natural and

synthetic agents. *Environmental Advances*, 8, 100203. https://doi.org/10.1016/j.envadv.2022.100203

Kalia, A., Sharma, S., Semor, N., Babele, P. K., Sagar, S., Bhatia, R. K., & Walia, A. (2022). Recent advancements in hydrocarbon bioremediation and future challenges: a review.*3 Biotech*, 12, 135. https://doi.org/10.1007/s13205-022-03199-y

Keesstra, S. D., Bouma, J., Wallinga, J., Tittonell, P., Smith, P., Cerdà, A., Montanarella, L., Quinton, J. N., Pachepsky, Y., Van Der Putten, W. H., Bardgett, R. D., Moolenaar, S., Mol, G., Jansen, B., & Fresco, L. O. (2016). The significance of soils and soil science towards realization of the United Nations sustainable development goals. *SOIL*, 2, 111-128. https://doi.org/10.5194/soil-2-111-2016

Khalid, S., Shahid, M., Niazi, N. K., Murtaza, B., Bibi, I., & Dumat, C. (2017). A comparison of technologies for remediation of heavy metal contaminated soils. *Journal of Geochemical Exploration*, 182, 247-268. https://doi.org/10.1016/j.gexplo.2016.11.021

Khan, S., Naushad, M., Lima, E. C., Zhang, S., Shaheen, S. M., & Rinklebe, J. (2021). Global soil pollution by toxic elements: Current status and future perspectives on the risk assessment and remediation strategies–A review. *Journal of Hazardous Materials*, 417, 126039. https://doi.org/10.1016/j.jhazmat.2021.126039

Kim, H., Cho, K., Purev, O., Choi, N., & Lee, J. (2022). Remediation of Toxic Heavy Metal Contaminated Soil by Combining a Washing Ejector Based on Hydrodynamic Cavitation and Soil Washing Process. *International Journal of Environmental Research and Public Health*, 19(2), 786. https://doi.org/10.3390/ijerph19020786

Koolivand, A., Saeedi, R., Coulon, F., Kumar, V., Villaseñor, J., Asghari, F., & Hesampoor, F. (2020). Bioremediation of petroleum hydrocarbons by vermicomposting process bioaugmentated with indigenous bacterial consortium isolated from petroleum oily sludge. *Ecotoxicology and Environmental Safety,* 198, 110645. https://doi.org/10.1016/j.ecoenv.2020.110645

Kumar, A., Yadav, A. N., Mondal, R., Kour, D., Subrahmanyam, G., Shabnam, A. A., Khan, S. A., Yadav, K. K., Sharma, G. K., Cabral-Pinto, M., Fagodiya, R. K., Gupta, D. K., Hota, S., & Malyan, S. K. (2021). Myco-remediation: A mechanistic understanding of contaminants alleviation from natural environment and future prospect. *Chemosphere*, 284, 131325. https://doi.org/10.1016/j.chemosphere.2021.131325

Kumar, V., Shahi, S. K., & Singh, S. (2018). Bioremediation: An eco-sustainable approach for restoration of contaminated sites. *Microbial Bioprospecting for Sustainable Development*, 115-136. https://doi.org/10.1007/978-981-13-0053-0_6

Kundu, A., Harrisson, O., & Ghoshal, S. (2022). Evaluating the impact of nutrient doses on biostimulation of petroleum hydrocarbon biodegradation in cold region soils. *ACS ES&T Engineering*, 2(12), 2287-2300. https://doi.org/10.1021/acsestengg.2c00220

Kuppusamy, S., Venkateswarlu, K., Megharaj, M., Vasudevan, N., & Lee, Y. B. (2020). Biostimulation and bioaugmentation: Modern strategies for the successful bioremediation of contaminated environments. In book: *The Handbook of Environmental Remediation: Classic and Modern Techniques* (pp.299-330) Chapter 11Publisher: The Royal Society of Chemistry

Lee, W. C., Lee, J. H., Lee, S. H., Lee, S. W., Jeon, J. H., Lee, S. H., & Kim, S. O. (2022). Revitalization of Total Petroleum Hydrocarbon Contaminated Soil Remediated by Landfarming. *Toxics*, 10(3), 147. https://doi.org/10.3390/toxics10030147

Li, Y., Liao, X., Huling, S. G., Xue, T., Liu, Q., Cao, H., & Lin, Q. (2019a). The combined effects of surfactant solubilization and chemical oxidation on the removal of polycyclic aromatic

hydrocarbon from soil. *Science of the Total Environment*, 647(10), 1106-1112. https://doi.org/10.1016/j.scitotenv.2018.07.420

Li, Y., Liao, X., & Li, W. (2019b). Combined sieving and washing of multi-metal-contaminated soils using remediation equipment: A pilot-scale demonstration. *Journal of Cleaner Production*, 212, 81-89. https://doi.org/10.1016/j.jclepro.2018.11.294

Li, Y., Wei, M., Yu, B., Liu, L., & Xue, Q. (2022). Thermal desorption optimization for the remediation of hydrocarbon-contaminated soils by a self-built sustainability evaluation tool. *Journal of Hazardous Materials*, 436, 129156. https://doi.org/10.1016/j.jhazmat.2022.129156

Lin, C., Cheruiyot, N. K., Bui, X. T., & Ngo, H. H. (2022). Composting and its application in bioremediation of organic contaminants. *Bioengineered*, 13(1), 1073-1089. https://doi.org/10.1080/21655979.2021.2017624

Lukić, B., Panico, A., Huguenot, D., Fabbricino, M., van Hullebusch, E. D., & Esposito, G. (2017). A review on the efficiency of landfarming integrated with composting as a soil remediation treatment. *Environmental Technology Reviews*, 6(1), 94-116. https://doi.org/10.1080/21622515.2017.1310310

Mihai, F. C., Plana, R., Taherzadeh, M. J., Aswathi, M. K., & Ezeah, C. (2020). Bioremediation of organic contaminants based on biowaste composting practices. *Handbook of Bioremediation: Physiological, Molecular and Biotechnological Interventions*, 2021, 701-714. https://doi.org/10.1016/B978-0-12-819382-2.00045-4

Nwankwegu, A. S., Zhang, L., Xie, D., Onwosi, C. O., Muhammad, W. I., Odoh, C. K., Sam, K., & Idenyi, J. N. (2022). Bioaugmentation as a green technology for hydrocarbon pollution remediation. Problems and prospects. *Journal of Environmental Management*, 304, 114313. https://doi.org/10.1016/j.jenvman.2021.114313

Ossai, I. C., Ahmed, A., Hassan, A., & Hamid, F. S. (2020). Remediation of soil and water contaminated with petroleum hydrocarbon: A review. *Environmental Technology and Innovation*, 17, 100526. https://doi.org/10.1016/j.eti.2019.100526

Panwar, R., & Mathur, J. (2023). Remediation of polycyclic aromatic hydrocarbon-contaminated soils using microbes and nanoparticles: A review. *Pedosphere*, 33(1), 93-104. https://doi.org/10.1016/j.pedsph.2022.06.032

Paul, O., Jasu, A., Lahiri, D., Nag, M., & Ray, R. R. (2021). *In situ* and *ex situ* bioremediation of heavy metals: The present scenario. *Journal of Environmental Engineering and Landscape Management*, 29(4), 454-469. https://doi.org/10.3846/jeelm.2021.15447

Picariello, E., Baldantoni, D., & De Nicola, F. (2020). Acute effects of PAH contamination on microbial community of different forest soils. *Environmental Pollution*, 262, 114378. https://doi.org/10.1016/j.envpol.2020.114378

Priya, A. K., Muruganandam, M., Ali, S. S., & Kornaros, M. (2023). Clean-Up of Heavy Metals from Contaminated Soil by Phytoremediation: A Multidisciplinary and Eco-Friendly Approach. *Toxics*, 11(5), 422. https://doi.org/10.3390/toxics11050422

Raffa, C. M., Chiampo, F., & Shanthakumar, S. (2021). Remediation of metal/metalloid-polluted soils: A short review. *Applied Sciences*, 11(9), 4134. https://doi.org/10.3390/app11094134

Rawat, C. D., Phian, S., Gupta, R., Verma, H., Kumar, M., Kaur, J., & Rawat, V. S. (2023). Microbial bioprocesses in remediation of contaminated environments and resource recovery. In: *Microbial Bioprocesses: Applications and Perspectives* (P. Shukla ed.) ELSEVIER, pp. 225-274. https://doi.org/10.1016/B978-0-323-95332-0.00005-3

Regulation (EU) 2019/1021 of the European Parliament and of the Council of 20 June 2019 on persistent organic pollutants (recast) (Text with EEA relevance.) http://data.europa.eu/eli/reg/2019/1021/oj

Regulation (EC) 850/2004 of the European Parliament and of the Council of 29 April 2004 on persistent organic pollutants and amending Directive 79/117/EEC http://data.europa.eu/eli/reg/2004/850/oj

Rodríguez-Eugenio, N., McLaughlin, M., Pennock. D. (2018). Soil Pollution: A Hidden Danger Beneath our Feet. *Rome: FAO*, 142 pages. ISBN: 978-92-5-130505-8, https://www.fao.org/3/I9183EN/i9183en.pdf

Sakshi, Singh, S. K., & Haritash, A. K. (2019). Polycyclic aromatic hydrocarbons: soil pollution and remediation. *International Journal of Environmental Science and Technology*, 16, 6489–6512. Center for Environmental and Energy Research and Studies. https://doi.org/10.1007/s13762-019-02414-3

Sayara, T., & Sánchez, A. (2020). Bioremediation of PAH-contaminated soils: Process enhancement through composting/compost. *Applied* Sciences, 10(11), 3684. https://doi.org/10.3390/app10113684

Schlögl, S., Diendorfer, P., Baldermann, A., & Vollprecht, D. (2023). Use of industrial residues for heavy metals immobilization in contaminated site remediation: a brief review. *International Journal of Environmental Science and Technology*, 20, 2313-2326. https://doi.org/10.1007/s13762-022-04184-x

Setiadi, T., Harimawan, A., Sumampouw, G. A., & Indarto, A. (2022). The mixture of agricultural pesticides and their impact on populations: Bioremediation strategies. In: *Emerging Contaminants in the Environment: Challenges and Sustainable Practices* (H. Sarma, D. C. Dominguez, W.-Y. Lee eds.), ELSEVIER, pp. 511-546. https://doi.org/10.1016/B978-0-323-85160-2.00011-1

Sharma, P., Singh, S. P., Iqbal, H. M., & Tong, Y. W. (2022). Omics approaches in bioremediation of environmental contaminants: An integrated approach for environmental safety and sustainability. *Environmental Research*, 211, 113102. https://doi.org/10.1016/j.envres.2022.113102

Sharma, S., Tiwari, S., Hasan, A., Saxena, V., & Pandey, L. M. (2018). Recent advances in conventional and contemporary methods for remediation of heavy metal-contaminated soils. *3 Biotech*, 8(216), 1-18. https://doi.org/10.1007/s13205-018-1237-8

Silver, W. L., Perez, T., Mayer, A., & Jones, A. R. (2021). The role of soil in the contribution of food and feed. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 376(1834), 20200181. https://doi.org/10.1098/rstb.2020.0181

Song, P., Xu, D., Yue, J., Ma, Y., Dong, S., & Feng, J. (2022). Recent advances in soil remediation technology for heavy metal contaminated sites: A critical review. *Science of the Total Environment*, 838(3), 156417. https://doi.org/10.1016/j.scitotenv.2022.156417

Sörengård, M., Lindh, A. S., & Ahrens, L. (2020). Thermal desorption as a high removal remediation technique for soils contaminated with per- And polyfluoroalkyl substances (PFASs). *PLoS ONE*, 15(6), e0234476. https://doi.org/10.1371/journal.pone.0234476

Stewart, L., Lutes, C., Truesdale, R., Schumacher, B., Zimmerman, J. H., & Connell, R. (2020). Field Study of Soil Vapor Extraction for Reducing Off-Site Vapor Intrusion. *Groundwater Monitoring and Remediation*, 40(1), 74-85. https://doi.org/10.1111/gwmr.12359

Teng, D., Mao, K., Ali, W., Xu, G., Huang, G., Niazi, N. K., Feng, X., & Zhang, H. (2020). Describing the toxicity and sources and the remediation technologies for mercury-contaminated soil. *RSC Advances*, 10(39), 23221-23232. https://doi.org/10.1039/d0ra01507e

Udume, O. A., Abu, G. O., Stanley, H. O., Vincent-Akpu, I. F., Momoh, Y., & Eze, M. O. (2023). Biostimulation of Petroleum-Contaminated Soil Using Organic and Inorganic Amendments. *Plants*, 12(3), 431. https://doi.org/10.3390/plants12030431

Ukaogo, P. O., Ewuzie, U., & Onwuka, C. V. (2020). Environmental pollution: Causes, effects, and the remedies. In: *Microorganisms for Sustainable Environment and Health* (Chowdhary, P., Raj, A., Verma, D., Akhter, Y. eds.). ELSEVIER, pp. 419-429. https://doi.org/10.1016/B978-0-12-819001-2.00021-8

Usman, M., Hanna, K., & Faure, P. (2018). Remediation of oil-contaminated harbor sediments by chemical oxidation. *Science of the Total Environment*, 634, 1100-1107. https://doi.org/10.1016/j.scitotenv.2018.04.092

Van Liedekerke, M., Prokop, G., Rabl-Berger, S., Kibblewhite, M., Louwagie, G. (2014). Progress in the Management of Contaminated Sites in Europe. *EUR 26376*. Luxembourg (Luxembourg), Publications Office of the European Union. JRC85913. https://doi.org/10.2788/4658

Varjani, S. J. (2017). Microbial degradation of petroleum hydrocarbons. *Bioresource technology*, 223, 277-286. https://doi.org/10.1016/j.biortech.2016.10.037

Vidonish, J. E., Zygourakis, K., Masiello, C. A., Sabadell, G., & Alvarez, P. J. J. (2016). Thermal Treatment of Hydrocarbon-Impacted Soils: A Review of Technology Innovation for Sustainable Remediation. *Engineering*, 2(4), 426-437. https://doi.org/10.1016/J.ENG.2016.04.005

Wu, M., Dick, W. A., Li, W., Wang, X., Yang, Q., Wang, T., Xu, L., Zhang, M., & Chen, L. (2016). Bioaugmentation and biostimulation of hydrocarbon degradation and the microbial community in a petroleum-contaminated soil. *International Biodeterioration & Biodegradation*, 107, 158-164. https://doi.org/10.1016/j.ibiod.2015.11.019

Wu, M., Wu, J., Zhang, X., & Ye, X. (2019). Effect of bioaugmentation and biostimulation on hydrocarbon degradation and microbial community composition in petroleum-contaminated loessal soil. *Chemosphere*, 237, 124456. https://doi.org/10.1016/j.chemosphere.2019.124456

Xu, D. M., Fu, R. B., Wang, J. X., Shi, Y. X., & Guo, X. P. (2021). Chemical stabilization remediation for heavy metals in contaminated soils on the latest decade: Available stabilizing materials and associated evaluation methods—A critical review. *Journal of Cleaner Production*, 321, 128730. https://doi.org/10.1016/j.jclepro.2021.128730

Yadav, M., Singh, G., & Jadeja, R. N. (2021). Bioremediation of organic pollutants: A sustainable green approach. In: *Sustainable Environmental Clean-up: Green Remediation* (V. K. Mishra, A. Kumar, eds.) ELSEVIER, pp. 131-147. https://doi.org/10.1016/B978-0-12-823828-8.00006-2

Yang, K., Fan, M., Han, X., & Wang, Y. (2021). An effective thermal treatment of toluene-contaminated soil by uniform heating from microwave coupled infrared radiation (MCIR). *Journal of Soils and Sediments*, 21, 1329-1336. https://doi.org/10.1007/s11368-020-02855-1

Yap, H. S., Zakaria, N. N., Zulkharnain, A., Sabri, S., Gomez-Fuentes, C., & Ahmad, S. A. (2021). Bibliometric analysis of hydrocarbon bioremediation in cold regions and a review on

enhanced soil bioremediation. *Biology*, 10(5), 354. https://doi.org/10.3390/biology10050354

Ye, S., Zeng, G., Wu, H., Zhang, C., Dai, J., Liang, J., Yu, J., Ren, X., Yi, H., Cheng, M., & Zhang, C. (2017). Biological technologies for the remediation of co-contaminated soil. *Critical Reviews in Biotechnology*, 37(8), 1062-1076. https://doi.org/10.1080/07388551.2017.1304357

CHAPTER 2

Background, Previous Experiments, and Scope

2.1. Background.

The increased pollution and all that it entails makes it necessary to seek solutions to put an end to this problem, which, if left unresolved, could have very serious consequences for the environment and living organisms, with many of the current consequences already irreversible. The Food and Agriculture Organisation of the United Nations (FAO) underlines the global importance of soil pollution as a hidden reality, with both diffuse and acute pollution coming from both natural sources and anthropogenic activities (FAO & ITPS, 2015). Therefore, FAO calls for concrete solutions to address the causes and impacts of soil pollution, underlining the need for urgent actions with integrated and science-based approaches to soil governance and management. Currently, the European Union does not have a specific directive on soil, although there are legal instruments that refer to certain threats to soil, mainly soil contamination (Heuser, 2022). However, it should be noted that the European Union commitment to tackle increasing soil contamination is evident in its policy framework, including the European Green Deal and the EU Soil Strategy 2030, which the objectives of promote healthy soils for people, food, environment, and the climate. As stated in existing European Union legislation, as well as in the European Green Deal, the EU creates a comprehensive approach, reflecting the recognition of soil contamination as a critical environmental problem that requires effective legal and regulatory measures to protect and sustainably manage soil resources. Furthermore, according to the European Green Deal, soils play a significant role in achieving Europe's major goal of a climate-neutral European Union by 2050 (Montanarella & Panagos, 2021). This will also lead to the implementation of Sustainable Development Goal (SDG) number 15, where SDG 15.3 reflects the need to address soil degradation and other relevant soil related targets (Heuser, 2022).

In addition, the European Union is making an important effort by contributing large amounts of money to projects that investigate and look for solutions to the enormous problem of pollution, because there are more and more polluted sites in the world, and it is something that must be stopped. In 2022, more than 2.8 million suspected contaminated sites were dated in Europe alone, of which 69% of the sites have been confirmed as potentially contaminated, meaning that at least 2 million of the suspected

contaminated sites will be contaminated (European Environmental Agency, 2022). However, the number of suspected contaminated sites may be even higher than indicated in the previous study.

This doctoral thesis arises from the need to find effective solutions to this emerging pollution, specifically to soil contamination by hydrocarbons and other xenobiotic pollutants and has therefore been carried out as part of the European Project GREENER H2020 (Figure 2).

Figure 2. GREENER Project logo.

2.1.1. GREENER Project.

The GREENER project acronym stands for *InteGRated systems for Effective ENvironmEntal Remediation*, which gives a general idea of the purpose of the project. GREENER project proposes the development of green, sustainable, efficient, and low-cost solutions for soil/sediment and water bioremediation that, integrating several remediation strategies with innovative bio-electrochemical technologies, will effectively accelerate the remediation time of a range of organic and inorganic pollutants of high concern, while producing end-products of interests, such as bioelectricity and/or harmless metabolites of industrial interest.

GREENER project has a consortium of researcher belonging to different universities, research centres and companies, with a total of 16 European partners and 4 partners from China (Figure 3). All the work was carried out under the coordination of the University of Burgos.

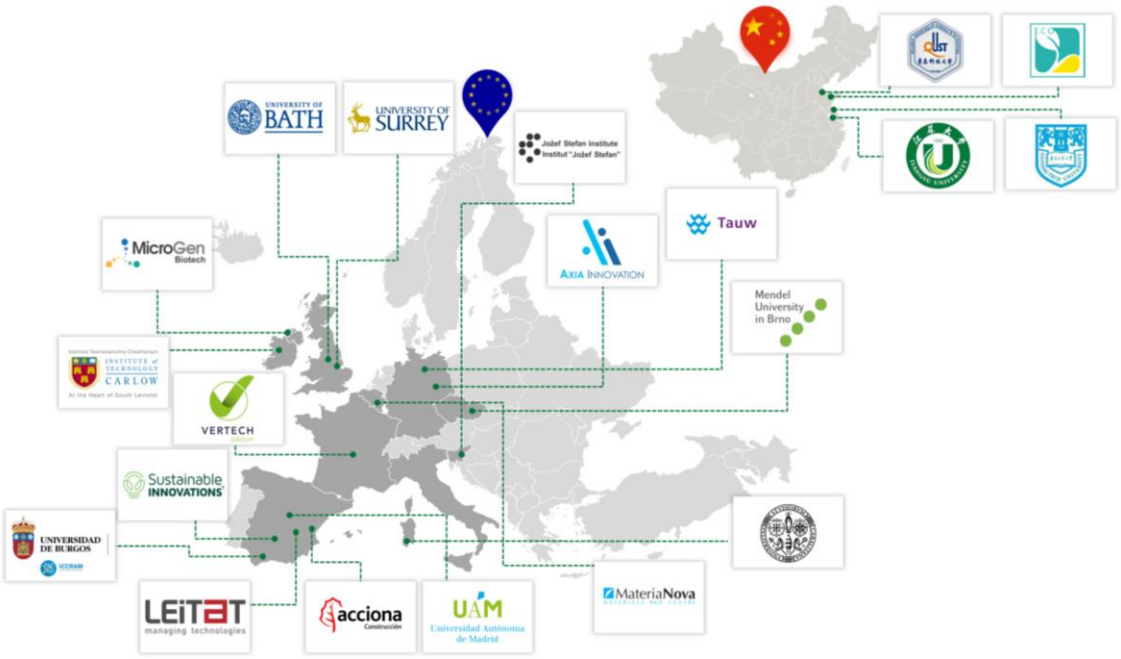


Figure 3. GREENER Project partners consortium.

Each partner has developed several of the tasks proposed in each work packages, and the University of Burgos has participated in all the packages with specific tasks and also in the whole coordination. To understand the work carried out during the development of this doctoral thesis, work packages 3, 5 and 6 of the GREENER Project must first be explained, as final results have been achieved by carrying out the tasks proposed in them.

2.1.2. GREENER work packages.

Soil bioremediation activities are addressed by GREENER Project in Work Packages 3, 5 and 6. **Work Package 3**, is centred in the **characterisation of selected contaminated sites and in the identification of the best available bioremediation techniques**. The University of Burgos had an important role in it and the following work was developed:

Task 3.1. Study and selection of the contaminated soils/sediments and waters.

A list of point source contaminated soils was proposed from both the European and Chinese partners, based in previous analysis, to find out what sort of contaminants they had and to choose which kind of soil bioremediation each

project partner was going to work with, in order to develop different technologies according to the type of contamination.

Task 3.2. Sampling of contaminated soil/sediment and surface/ground water and determination of contaminant concentrations.

Once the most adequate polluted soils had been selected by each partner of the consortium, a large sampling campaign was developed in the different contaminated areas and distributed to the selected laboratories with the objective of doing an exhaustive characterization of contaminant profiles using common and standardized methodologies.

Task 3.3. Physicochemical characterization of soil/sediment and surface/ground water samples.

Soil physicochemical analyses were also carried out that may influence the effectiveness of the remediation technology to be studied, such as pH, electrical conductivity, texture, water retention capacity, organic matter content, carbonate content, cation exchange capacity, total carbon and nitrogen, and elemental composition of both macro and microelements.

Task 3.4. In-depth analysis of the metabolic potential of microbial communities through metagenomic, metaproteomic and biochemical analysis for optimal degradation of selected contaminants.

Metagenomic, metaproteomic and biochemical analyses, in terms of hydrolytic enzyme activities, microbial biomass content and basal soil respirometry (BSR), prior to the implementation of the bioremediation techniques were carried out by different members of the consortium.

Task 3.6. Microbial studies and isolation.

The isolation and composition of the microbial community from three selected soils polluted with petroleum hydrocarbons were carried out at the Universidad Autónoma de Madrid (UAM) according to the methodology developed by Garrido-

Sanz et al., (2019). These initial consortia were thereafter used in the bioremediation activities of TPHs contaminated soils by the University of Burgos, the South-East Tecnological University (SETU) of Carlow (Ireland) and the Ecology Institute Shandong Academy of Science (China) well as microbiological tests necessary for the development of the thesis.

Finally, as other important objective of the Project GREENER, the UAM group designed a synthetic microbial community, removing pathogenic and inefficient strains, that was applied both at laboratory scale and at real scale, with the aim of obtaining efficient and safe technologies for bioremediation activities.

Task 3.7. Key performance indicators for the selection of best bioremediation techniques.

Once all the above tasks were completed, key performance indicators (KPIs) were defined for the selection of the best bioremediation technology depending on the type of soil, environment, and its contaminants profile.

Work Package 5, was centred in the optimization of the bio**remediation technologies** and was the central part of the research work developed in this thesis that has being developed according to the following tasks:

Task 5.1. Improvement of biostimulation/bioaugmentation technologies for soil remediation.

Laboratory-scale trials were conducted to test the biostimulation and bioaugmentation technologies by both the microbial consortium previously isolated from the contaminated soil under study and a synthetic community, in which selected microorganisms from the initial consortium are combined, were used. The nutrient solution, that provides soil enough inorganic resources, was also defined. The soil was also enriched to improve its properties or even improve the bioavailability of the pollutants by adding organic amendments such as vermicompost or different organic additives: biochars, or biological surfactants.

Task 5.4. Definition of operation conditions for the scale-up for soil remediation technologies.

Once the optimal operative conditions that have provided the best results on a laboratory scale were known, a Life-Cycle Assessment (LCA) and a Life-Cycle Cost Analysis (LCCA) were carried out to find out whether it was feasible to replicate these technologies both on a pilot scale and on a real scale.

Finally, in **Work Package 6**, **the technology scale-up and field testing** has been carried out by performing the following tasks:

Task 6.1. Scale-up of the optimal technologies.

A discussion of all tested technologies was held based on the bioremediation efficiency obtained in Work Package 3 and each partner decided on the best technology to replicate at pilot scale.

Task 6.2. Definition of the physicochemical techniques for the application of the technology at pilot and real scale.

The physicochemical properties and operational conditions were defined for TPHs biodegradation using both biostimulation and bioaugmentation technologies, alone or in combination with other hybrid processes such is a passive bioelectrochemical system (BES).

Task 6.4. Pilot scale experiments for soil technologies.

A study was carried out on a pilot scale, using mesocosms of one tonne of soil in adapted containers under real environmental conditions. In the case of the University of Burgos, the bioaugmentation technologies were tested by adding vermicompost as an organic amendment, nutrients, and inoculation with the microbial consortium and a passive bioelectrochemical system. This pilot scale trial is described in detail in chapter 5.

Once the pilot scale was established and the results were obtained, a methodology was developed for the selection of the most environmentally friendly and best

performing technologies to be taken to real scale. For this purpose, a multicriteria analysis was carried out comparing the KPIs (key performance indicators) of the GREENER project, taking into account technical oriented KPIs such as pollutant concentration, pollutant reduction, decontamination cost and time, as well as sustainability oriented KPIs, relating waste generation, material and energy requirements and emissions generated. After decision-making, a technology was chosen to be taken to full scale.

Task 6.7. Field testing of the developed technologies in contaminated soil.

The technologies chosen by the different partners were scaled up to real experiments. In the case of the University of Burgos, it was scaled up to 10 tonnes of the contaminated soil with TPHs, making biopiles, one of the biopiles was a used as a control and the other with the optimized bioaugmentation treatment.

2.1.3. Contaminated sites.

GREENER Project selected 13 contaminated sites around Europe and China as best candidates to implement bioremediation technologies and hybrid combinations of them in polluted soils and waters, both surface of groundwater. These contaminated sites were due to different causes and with very different contamination levels, are located in very different sites such as river Salle (Germany), a hospital in Netherlands, nine industries located in different places of Spain, Ireland, Germany and China, a coastal aquifer in Belgium, and an agricultural land in Germany. The contaminated sites can be observed in Figure 4.

Three selected soils characterized at the University of Burgos were affected by mixed contamination of TPHs or crude oil spills and heavy metals and correspond to the following sites:

- **Site 001:** The source of contamination were fuel and engine oil leaks from a construction machinery park, in Toledo (Spain). The main contaminants of this soil are TPHs, PAHs and potentially toxic metals and metalloids. In the initial contaminant characterization study, aliphatic compounds were predominant, with

about 1,677 mg kg^{-1}, compared to 937 mg kg^{-1} of aromatics, mostly composed of TPHs fractions larger than C12. The main metal contamination was due to the high concentration of iron (72,120 mg kg^{-1}), arsenic (57.7 mg kg^{-1}), copper (15.2 mg kg^{-1}), chromium (7.35 mg kg^{-1}), lead (327 mg kg^{-1}) and zinc (623 mg kg^{-1}).

- **Site 002:** The source of contamination was heavy fuel from a former sugar factory located in Carlow (Ireland). The contaminants in this soil were TPHs and low levels of potentially toxic metals and metalloids. Several samples were taken from this site and in this case, aromatic compounds (14,526 mg kg^{-1}) were more abundant than aliphatic compounds (12,167 mg kg^{-1}). PAHs were very abundant in some of the samples with up to 50 mg kg^{-1}total PAHs; in other locations they were in the range between 24 and 10 mg kg^{-1}. The most predominant compounds were phenanthrene, followed by pyrene and fluoranthene. Metal contamination was also present with copper (71 mg kg^{-1}), chromium (172 mg kg^{-1}), and nickel (33.7 mg kg^{-1}) as main contaminants.

- **Site 013:** The source of contamination were fuel and engine oil leaks from a former refinery, in Gudao (China). Soil had mainly high concentrations of TPHs, PAHs and salinity. Several samples were taken at the contaminated site, whereby total aliphatic hydrocarbons ranged from 3,900 to 15,600 mg kg^{-1} and total aromatic hydrocarbons ranged from 2,760 to 11,040 mg kg^{-1}.

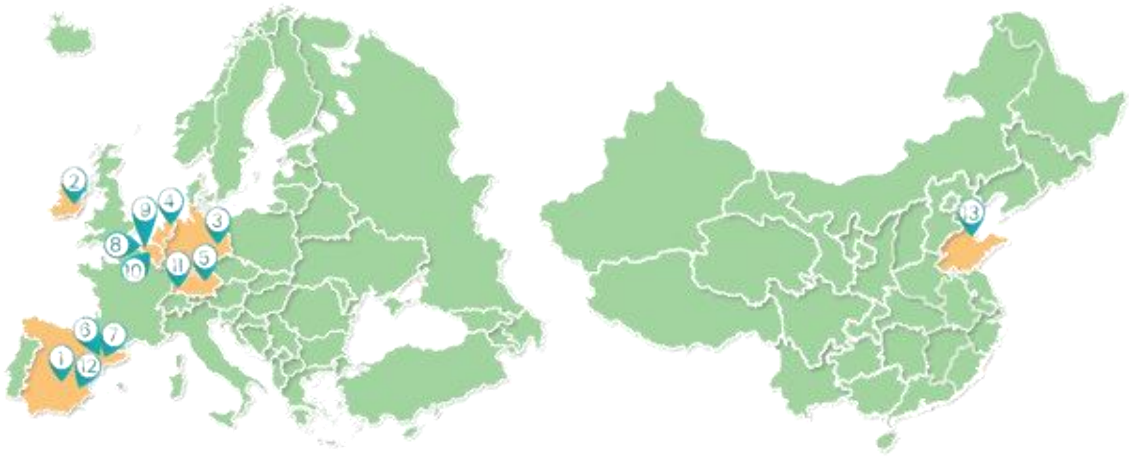


Figure 4. GREENER Contaminated sites.

All these soils were studied and characterised in during the development of Work Package 3. Finally, for Work Packages 5 and 6, we decided to centre our study in Site 001 due to the proximity of the sample and the combination of contaminants with the presence of TPHs and trace metals in a soil depleted of nutrients, that constitutes a challenge for our bioremediation experiment. The physicochemical characteristics of the soil are shown in the **¡Error! No se encuentra el origen de la referencia.**, and the characterization of hydrocarbons in soil are in **¡Error! No se encuentra el origen de la referencia.**.

Table 1. Physicochemical characterization of the soil from Site 001.

Physicochemical parameters		Results
Electrical conductivity (1:5 w/v, 25ºC)		0.801 dS m^{-1}
pH (1:5 w/v)		7.18
Water retention capacity		25.33 %
Texture	Clay	11.8 %
	Silt	29.8 %
	Sand	58.3 %
Organic Carbon		2.59 %
Total Nitrogen		0.02 %
Nutrients	PO_4-P	0.10 mg kg^{-1}
	NO_3-N	0.14 mg kg^{-1}
	NH_4-N	2.99 mg kg^{-1}
Exchangeable Calcium		198,822 mg kg^{-1}
Aluminium		11,147 mg kg^{-1}
Iron		31,758 mg kg^{-1}
Trace elements	As	77.3 mg kg^{-1}
	Cd	7.8 mg kg^{-1}
	Cr	14.9 mg kg^{-1}
	Cu	8.5 mg kg^{-1}
	Ni	9.9 mg kg^{-1}
	Pb	339.2 mg kg^{-1}
	Zn	680.5 mg kg^{-1}

Table 2. Characterization of hydrocarbons presented in soil from Site 001.

Number of Carbons	Aliphatics	Aromatics
C5-C6	Not detectable	Not detectable
C6-C8	< 5	Not detectable
C8-C10	< 5	Not detectable
C10-C12	<5	< 5
C12-C16	74	43
C16-C21	33	61

C21-C35	1570	833
Total C5-C35	1677	937

The topography of the contaminated area is mainly flat and there is no vegetation, as it is shown on Figure 5. The contaminated site selected is mainly formed by two soil horizons which are explained below, but not visible in the Figure 5:

- Level 0: Anthropic landfills. Formed by a mixture of rubble and materials usually from excavations and/or a soil surface alteration. It has an average thickness of 0 – 0.40 m.

- Level 1: Micritic or biomicritic limestones of gray or beige tones, massive, tobacic, sandy or clayey, arranged in 0.30 – 1.00 m thick banks, with abundant stretches of reddish sandy clays and loamy clays.

Figure 5. Contaminated soil Site 001 in Noblejas, Toledo (Spain).

The mean annual temperature there is 13.5 °C, and the mean rainfall is 444 mm. The climate in the site is warm and is classified as *Csa* by the Köppen-Geiger system. *Csa* denotes a hot-summer Mediterranean climate, with at least a month of average temperature exceeding 22 °C, the coldest month with an average temperature above 0 °C or −3 °C, and at least four months average temperature being above 10 °C.

Possible bioremediation techniques were studied for the selected soil type, the contaminants contained and the site characteristics, and it was decided to study bioaugmentation and biostimulation techniques.

2.1.4. Soil technologies developed.

During the development of this doctoral thesis and the GREENER project, the aim has been to improve the different bioremediation technologies tested and go from laboratory scale to pilot scale and finally, to real scale, with the construction of biopiles. For this purpose, small microcosms were developed in the laboratory from the beginning, in which 200 g of soil were developed to define the real conditions that we would have to face on a large scale, where many types of treatments could be tested and those that performed best could be chosen for further development. At pilot scale, treatments were again defined in the form of 500 kg mesocosms, where real conditions could be tested without the amount of soil being extremely large, allowing us to work in a relatively simple way. Finally, at real scale, a more realistic study of both the conditions and the target soil can be carried out in the form of 10 tonne biopiles, which are large soil piles that remediated large quantities of soil by reducing the number of contaminants contained in them.

In this work, biostimulation, bioaugmentation and bioelectrochemical technologies have been tested, modifying different factors to improve hydrocarbon degradation in the soils studied.

2.1.5. Impacts.

The most significant impact of bioremediation can be assessed by measuring the reduction in the concentration of hydrocarbons and other contaminants present in the soil over time. To convert pollutants into less hazardous forms, bioremediation requires minimal chemical and energy input in an environmentally friendly way (Kalia et al., 2022). Comparing bioremediation with chemical remediation, e.g. chemical oxidation through the addition of a strong oxidant such as permanganate, it can be seen that as a natural process it has a low environmental impact compared to non-biological treatments (Barbato & Reynolds, 2021).

Although the use of biological substances does not have the same impact as the use of chemical substances, it can also pose a risk due to their bioactivity, as surfactants present in large amounts in soil have an adverse effect on ecosystems (da Silva et al., 2020). Adding organic substances to the soil can put ecosystems at risk and pose an environmental problem, as harmful by-products or metabolites can be released. Therefore, non-invasive methods are often used, such as bioelectrochemical systems in which graphite rods are inserted *in situ*, without the input of chemical agents, which provide efficient and environmentally friendly results (Wang et al., 2020).

There is also a direct environmental impact on soil properties, so the search for less aggressive technologies may be a key aspect to reduce its effects. Bioelectrochemical remediation, a technique mentioned above for its non-invasive characteristic, has a lower impact on soil properties, which makes it more attractive for practical applications in the future (Lan et al., 2023). Changes in soil properties cause significant problems in the following areas: the environment, due to scarcity or unavailability of arable land; agriculture, due to decreased soil fertility and nitrogen fixation; urban areas, due to waste management and public health problems; agriculture, due to decreased agricultural production; and agriculture, due to increased erosion and nutrient depletion, sludge storage and imbalance between plant and animal life in the soil (Hernández-Soriano, 2014).

Another important impact is the economic cost of soil decontamination, so the search for less expensive techniques is one of the key factors in their choice to be economically viable. *Ex situ* bioremediation techniques are more expensive due to the excavation and transport of the soil from the contaminated site to the treatment site, whereas *in situ* techniques do not have this cost overrun but pose an additional challenge in their choice (Alori et al., 2022). *In situ* techniques provide significant economic savings especially in large contaminated areas (Lan et al., 2023). It reduces the economic cost of treatment, but also reduces the environmental impact by not carrying out excavation and transport, reducing the pollution caused by this machinery and the visual impact of contaminated areas. Not only does transport increase costs, but the use of traditional chemical and physical remediation

technologies has proven to be economically costly and sometimes ineffective and can cause secondary contamination (Ren et al., 2018).

The use of these biological technologies instead of the physical or chemical ones commonly used in the past has resulted in lower environmental and economic impacts, more efficient pollutant degradation results and easier application of the treatments.

2.2. Previous experiments.

To understand the next three chapters and the conclusions and final remarks presented in chapter 6, an overview of the work carried out during the development of this doctoral thesis has been explained. Although some of the most significant results are presented in the chapters found throughout the manuscript of this thesis, it is necessary to mention some details to understand the order of development and the methodology applied in the chapters 3, 4 and 5.

Prior to the laboratory experiment corresponding to the incubation detailed in chapter 3, some analyses were carried out to find out the general properties of the soils under study, so that the most appropriate strategies could be chosen according to the previous knowledge we had of the soils, as well as to choose the soil with which we were going to develop the different techniques that would be studied later. Therefore, once the properties of the soils were known, the first incubation strategy was carried out with the soil from Site 001, Noblejas (Toledo), from GREENER project. The biostimulation technology (BS) was tested in this soil to determine the capacity of the indigenous soil microorganisms in the biodegradation of hydrocarbons with the application of a standard BHB (Bushnell Haas Broth) - $FeCl_3$ nutrient solution as shown in Table 3. In addition, a control treatment (CT) was developed at the same time to study the natural attenuation of hydrocarbons without any external addition. As this was the first experiment to be carried out, a laboratory scale set up was proposed and a microcosm incubation was prepared in airtight jars with 200 g of soil inside (Figure 6). The experiment was carried out during 90 days in darkness, in a climatic chamber with controlled conditions of temperature (22 ± 0.5 °C) and humidity, moistening the soil with

deionised water until it reached 40% of its water retention capacity. In addition, the soil was aerated twice a week to maintain aerobic conditions and to allow the growth of soil microorganisms. All strategies were studied in quadruplicate, in order to have enough samples for statistical calculations. Samples were taken throughout the incubation to monitor the evolution of microcosms and the degradation of total petroleum hydrocarbons (TPHs) and polycyclic aromatic hydrocarbons (PAHs).

Table 3. Composition of the BHB - $FeCl_3$ nutrient solution of the first incubation experiment.

Compound	Amount (g kg^{-1})	g 200 g^{-1}	Solution (g L^{-1})
$MgSO_4$	0.0612	0.01224	1.224
$CaCl_2$	0.0061	0.00122	0.61
KH_2PO_4	0.3058	0.06116	6.116
K_2HPO_4	0.3058	0.06116	6.116
$(NH_4)NO_3$	0.3058	0.06116	6.116
$FeCl_3$	0.0153	0.00306	1.53
Total	1	0.2	

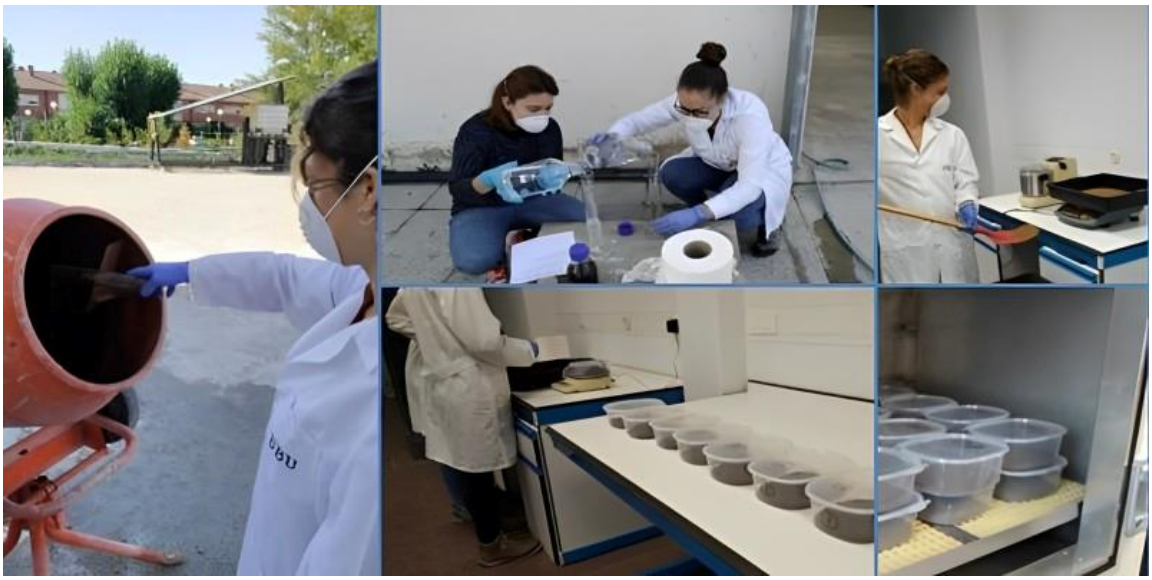


Figure 6. Preparation of the first incubation experiment.

After this first incubation and the subsequent study of the soil characteristics, it was observed that the hydrocarbons extracted from the soil were predominantly aliphatic and aromatic hydrocarbons of high molecular weight, with carbon chain lengths higher

than 22 carbon atoms, which showed us a soil with difficult characteristics for bioremediation treatments, due to the low availability of a large part of the pollutants contained in it. At the same time, using previous results of the soil under study, it was verified that the time elapsed between the collection of the soil and the execution of the experiment in the laboratory caused the attenuation of the levels of hydrocarbons, probably due to the degradation and volatilisation of the lower carbon fractions, which were more volatile and less retained in the soil components, mainly clays and humic substances. Analysis of the evolution of the hydrocarbons contained in the soil throughout the experiment showed that the biostimulation treatment resulted a slight decrease on the concentration of Extractable Petroleum Hydrocarbons (EPHs), with the most notable decrease in the case of high molecular weight PAHs, as shown in Table 4 and Table 5.

Table 4. Extractable Petroleum Hydrocarbon (EPH) contents in the samples at 2 days of incubation and after 90 days of incubation. Treatments: CT, control soil; BS, biostimulation treatment. Mean values ± standard deviation.

Treatment	C10-C12	C13-C16	C17-C21	C22-C35	>C35	EPHs
CT-2	2 ± 0	117 ± 18	112 ± 1	2,661 ± 88	208 ± 56	3,100 ± 124
BS-2	2 ± 0	90 ± 18	110 ± 1	2,785 ± 88	287 ± 56	3,275 ± 126
CT-90	26 ± 23	169 ± 9	129 ± 7	3,076 ± 166	242 ± 34	3,641 ± 207
BS-90	5 ± 1	145 ± 23	107 ± 21	2,833 ± 211	234 ± 7	3,324 ± 248

Table 5. Polycyclic Aromatic Hydrocarbons (PAHs) contents in the samples at 2 days of incubation and after 90 days of incubation. Treatments: CT, control soil; BS, biostimulation treatment. Mean values ± standard deviation.

Treatment	C10-C12	C13-C16	C17-C21	C22-C35	>C35	PAHs
CT-2	15 ± 1	11 ± 3	111 ± 29	340 ± 48	146 ± 13	623 ± 32
BS-2	16 ± 1	7 ± 3	70 ± 29	272 ± 48	128 ± 13	493 ± 32
CT-90	45 ± 39	15 ± 2	76 ± 21	344 ± 24	113 ± 49	590 ± 203
BS-90	10 ± 4	7 ± 1	56 ± 4	280 ± 39	109 ± 6	460 ± 302

However, the differences between the control, in which natural attenuation was tested, and biostimulation treatment were not statistically significant, as the variability observed between replicates was high, which led us to see that the treatment did not

work effectively. Regarding the analysis of biological parameters, basal soil respiration (Figure 7.a) showed a higher activity during the first days of the biostimulation treatment, an expected result due to the addition of the nutrient solution, which improved the activity of indigenous soil microorganisms. However, these respiration levels were not maintained and progressively decreased, and on the day 15 of incubation very low values were observed, which showed that despite maintaining the humidity and temperature conditions, biological activity was not maintained. The lack of soil biological activity may have been affected by the decrease in nitrogen (Figure 7.b) observed by nutrient analysis, because other nutrients such as potassium or phosphorus (Figure 7.c or Figure 7.d) remained highly bioavailable throughout the entire treatment. In contrast, the nitrogen source was found to be rapidly depleted during the first 15 days of treatment, indicating that the nutrient solution was not sufficient to maintain the biostimulation of indigenous soil microorganisms.

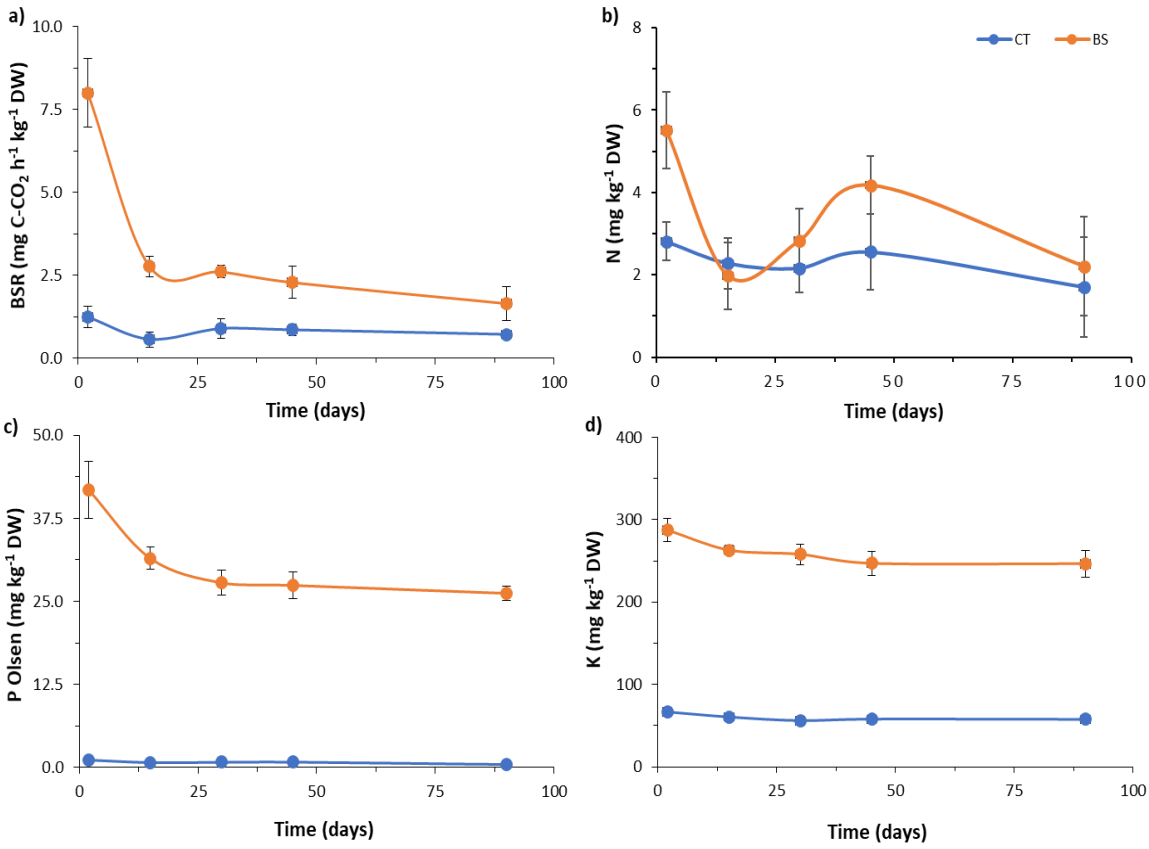


Figure 7. Evolution of chemical soil properties during incubation: a) Basal soil respiration (BSR); b) Extractable nitrogen; c) Available P; d) Available K. Treatments: CT, control soil; BS, biostimulation treatment. Mean values ± standard deviation.

Finally, the main conclusions of this first laboratory scale incubation strategy were that the application of the biostimulation treatment alone was not effective in improving the decontamination of the soil under study, whose levels were still far from the targets set by the legislation for TPHs and PAHs permitted at the soil site under study. Therefore, considering the conclusions obtained in this first strategy, new treatments and their characteristics were designed to continue the research. The composition of the BHB-$FeCl_3$ nutrient solution was modified by increasing the nitrogen concentration to avoid its quick depletion. A bioaugmentation treatment was also designed using an indigenous soil microbial consortium previously isolated from the soil under study. This experimental design led to the incubation detailed in chapter 3, which was published in the journal Chemosphere (Impact Factor = 8.8, Q1), under the title "Evaluation of biostimulation, bioaugmentation, and organic amendments application on the bioremediation of recalcitrant hydrocarbons of soil".

The third chapter, as indicated above, shows the study published in the journal Chemosphere, detailing the second incubation, in which new bioremediation strategies were tested. In this chapter, the same strategies of humidity, temperature, and sample amount were used to carry out the incubation, and natural attenuation as a control, biostimulation with the application of a new improved formula of the BHB-$FeCl_3$ nutrient solution, according to the needs of the soil, and biostimulation with the application not only of this improved nutrient solution, but also of the microbial consortium previously isolated from the soil under study, were tested. On the other hand, all strategies were also improved by the application of a vermicompost as an organic amendment, to improve soil properties and allow better growth of the microbial consortium. In addition, a second inoculation of the microbial consortium was carried out in the bioaugmentation treatment, as well as another addition of the nutrient solution, and in the biostimulation treatment the nutrient solution was also added, reaching in all treatments a 50% moisture content above the water retention capacity of the soil. From these two incubations, a way of working was established for the next incubations where the steps to be followed are shown in Figure 8.

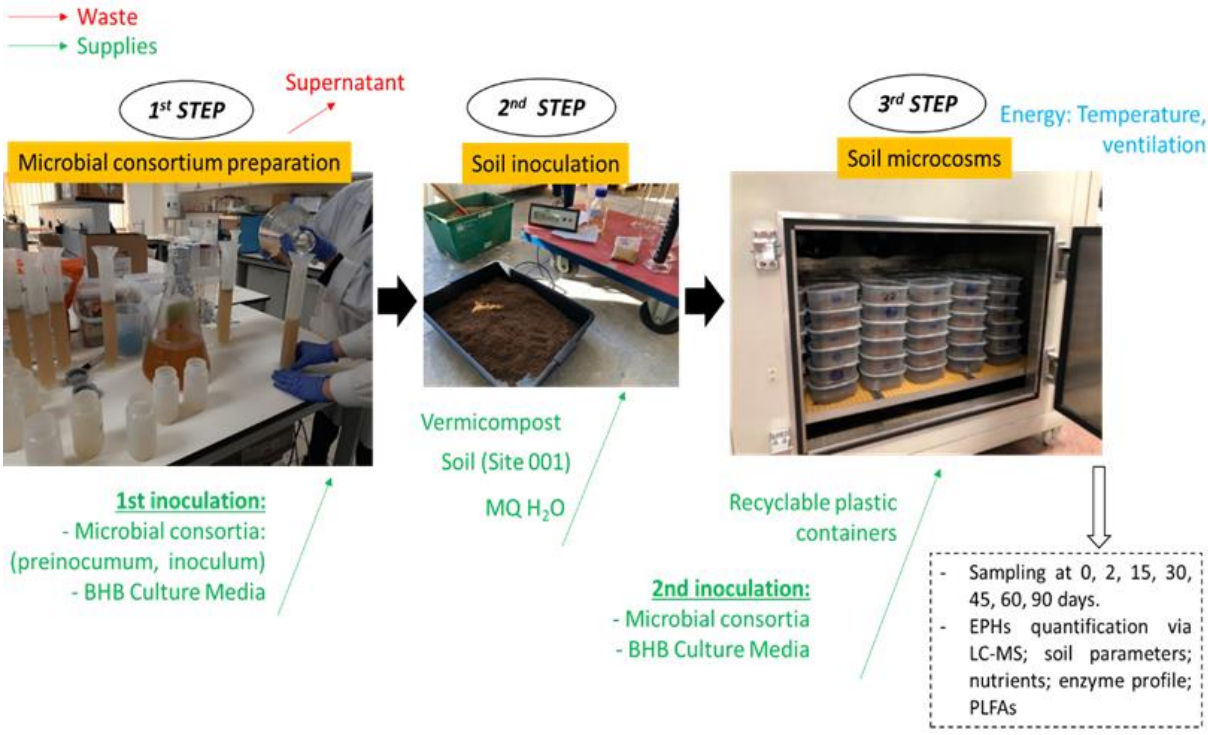


Figure 8. Steps to set-up of microcosm experiments.

The results obtained in relation to the biodegradation of hydrocarbons showed improvements in relation to the previous incubation. Since the soil used corresponded to an aged soil contaminated with hydrocarbons and which had been undergoing natural attenuation for a long period of time at the site of the contamination, the hydrocarbons with lower carbon chains and volatile hydrocarbons had already been degraded. Therefore, the degradation obtained during this experiment focused on compounds that were still bioavailable as linear, alkanes and polar, and reached even aromatic compounds and branched alkanes, while recalcitrant and heavy hydrocarbons such as hopanes remained practically intact. The results obtained showed the effectiveness of the application of vermicompost, which increased the amount of available phosphorus by a factor of five and the amount of exchangeable potassium by a factor of two. In addition, microbial activity increased with soil acidification, which allowed the solubility of P and other micronutrients. And according to the results of the phospholipid fatty acids (PLFAs), there was also a variation in the microbial groups throughout the experiment, with an increase in alkaline phosphatases (AlkPA and AcPA) and proteases (LeuAMP) related to bacterial growth. This increase in microbial metabolism was directly related to the degradation of TPHs, reaching degradation percentages for the

vermicompost treatments of 32.5% in the biostimulation treatments and 34.4% in the bioaugmentation treatments. Remarkable results were also observed for PAHs, which reduced their presence in the soil, especially phenanthrene, which had a higher presence. Although hydrocarbon degradation results were better than the degradation obtained for the first strategy, recalcitrant and hydrophobic hydrocarbons remained in the soil with little alteration, indicating that their bioavailability would be the limiting factor for bioremediation.

These results led us to consider that perhaps the problem of bioavailability could also be because the soil moisture was not sufficient for degradation to be effective, so a third incubation was carried out, where control, biostimulation, and bioaugmentation treatments were tested with different percentages of moisture on the water retention capacity to check whether moisture was a limiting factor. The evolution of the soil characteristics of the different microcosms at 40%, 60% and 80% of the soil water retention capacity was studied for 30 days. The results for hydrocarbon degradation obtained at the end of this strategy showed no significant differences between the microcosms at different humidities as shown in Figure 9. Therefore, it was decided to continue with the next incubations at 40%, because when taking the technology developed in the laboratory to a larger scale, the increase in water implies a higher economic cost and unnecessary waste of water, which is less sustainable for the environment.

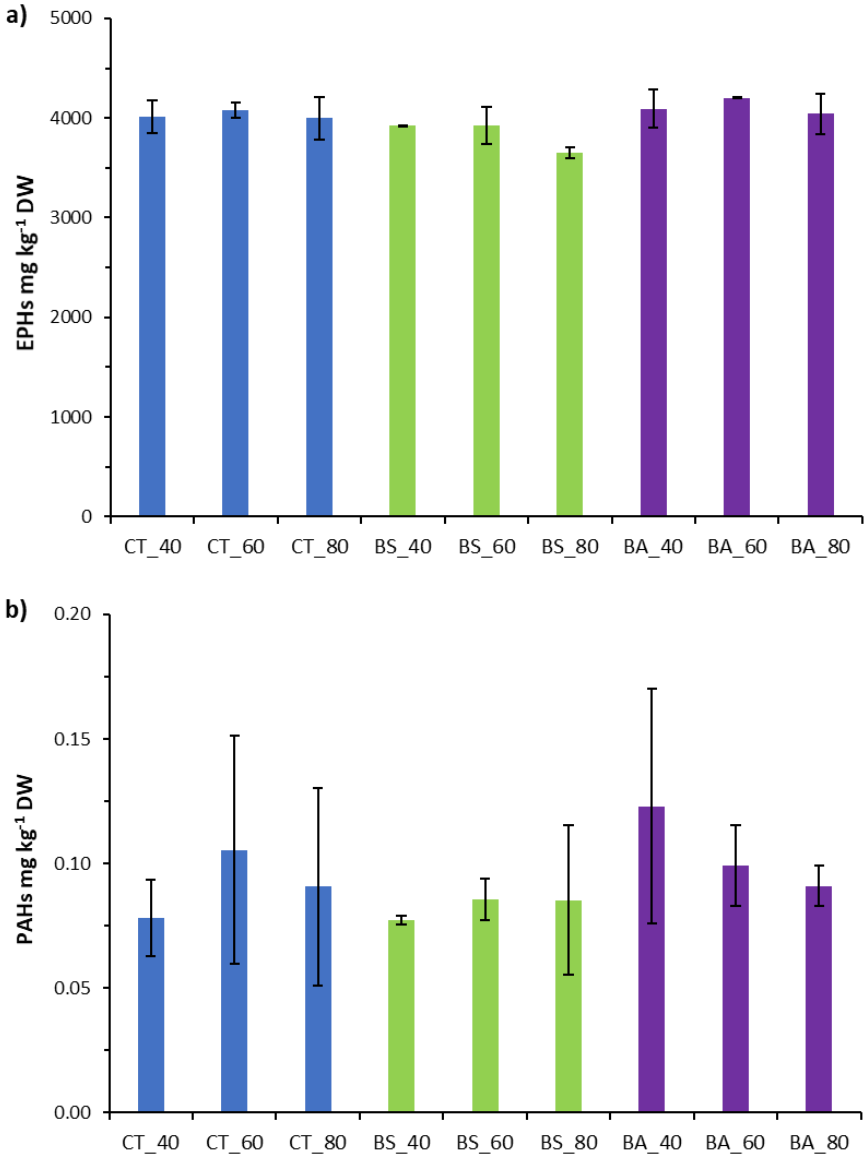


Figure 9. a) Content of Extractable Petroleum Hydrocarbons (EPHs); and b) Content of Polycyclic Aromatic Hydrocarbons (PAHs) at the end of the 30 days incubation period and different percentages of the soil water retention capacity (40%, 60% and 80%).

The knowledge gained in relation both to the soil under study and the bioremediation strategies allowed further development of bioremediation strategies to improve the results obtained previously. Therefore, chapter 4 develops the fourth incubation, which is currently under review for publication in the journal Environmental Research (Impact Factor = 8.3, Q1) under the title "Unveiling capacity of bioaugmentation application, in comparison with biochar and rhamnolipid for TPHs degradation in aged hydrocarbons polluted soil". During the development of this strategy the aim was to improve the bioavailability of the pollutants in the soil. For this purpose, new bioremediation

strategies were studied in which the organic amendments added to the soil were different, and it was decided to test two biochars (BH1, BH2) obtained from apricot seeds but pyrolyzed at different temperatures (450 °C and 650 °C, respectively), so that the characteristics of the biochars were different. In addition, during this incubation, the addition of commercial rhamnolipids (RML) was tested to determine their capacity as organic additives with the capacity to biostimulate both the indigenous microorganisms of the soil under study and the microorganisms previously added as part of the bioaugmentation. The experiment was conducted with the characteristics of the previous incubations, but due to the magnitude of these incubations, it was decided to dispense of a replicate, so it was carried out with the treatments in triplicate, as it was a sufficient number to be able to carry out statistical analyses. In addition, specific analyses were carried out to observe the growth of bacteria on the biochars and how the composition of both biochars and rhamnolipids changed with the presence of the microbial consortium. This study showed significant differences between the biostimulation and bioaugmentation treatments, with higher hydrocarbon degradation occurring in those in which an organic additive had been added. Although rhamnolipids have not been useful without the addition of the bioaugmentation, in the case of biochars they have been significant but in a limited way, which indicates that without the use of the microbial consortium they will not be completely useful in soils with such a high number of hydrocarbons, but that they can be useful and economically viable for soils with less contamination and with short chain hydrocarbons, that is, with fractions lower than C21. In addition, it was concluded that the treatments showed more significant results for fractions C21-30 and C30-35, with higher differences being observed in the latter group for the treatments in which an amendment was added in addition to bioaugmentation (BABH1, BABH2 and BARML). In the case of the use of rhamnolipids as an organic additive together with bioaugmentation, it has shown significant results like those of both biochars, with the degradation percentages of the bioaugmentation treatments being 27.5% with BH1, and 29.8% with BH2 or RML. However, the high cost of rhamnolipids as raw material will make us rule out the use of this amendment, or at least the use of commercial rhamnolipids, pending the testing of rhamnolipids produced by us in the laboratory. Although the addition of the microbial consortium previously isolated from the soil under study increased the biological activity

of the soil itself, it only achieved a limited improvement in the bioavailability of the target contaminants, because these contaminants have been retained for many years, and most of the hydrocarbons now present in the soil are long chain fractions with more than 22 carbon atoms, making them less accessible to microorganisms due to their recalcitrant nature.

The incubations developed showed good results and it was observed that the bioremediation capacities were improving with the adaptation of the strategies to the treated soil. Therefore, in order to continue with this progression and seeking to improve the techniques, an international stay was carried out at the Jožef Stefan Institute in Ljubljana (Slovenia), where two experiments were carried out which are not yet published but are in an advanced stage of writing to be submitted for publication in scientific journals.

On the one hand, and after the second incubation developed in chapter 3, it was considered interesting to study more specifically the microorganisms contained in the microcosms of the different treatments. To do this, firstly, DNA was extracted from the soil of all the microcosms, to analyse the microbial diversity present in the soil. After extraction, the samples were purified to eliminate possible inhibiting molecules such as humic acid and other contaminants. The DNA extracted from the different soil samples was analysed using techniques such as quantitative PCR amplification and 16S ribosomal DNA analysis to study the bacterial diversity and the specific microorganisms present in the soil. The aim of these analyses was to understand the composition of the microbial community throughout the experiment, to understand more precisely the mechanisms of hydrocarbon degradation. These mechanisms were known through knowledge of the proliferation of the microbial community contained in the soils as shown in Figure 10, understanding that those with the highest growth were those with the highest degradation capacities.

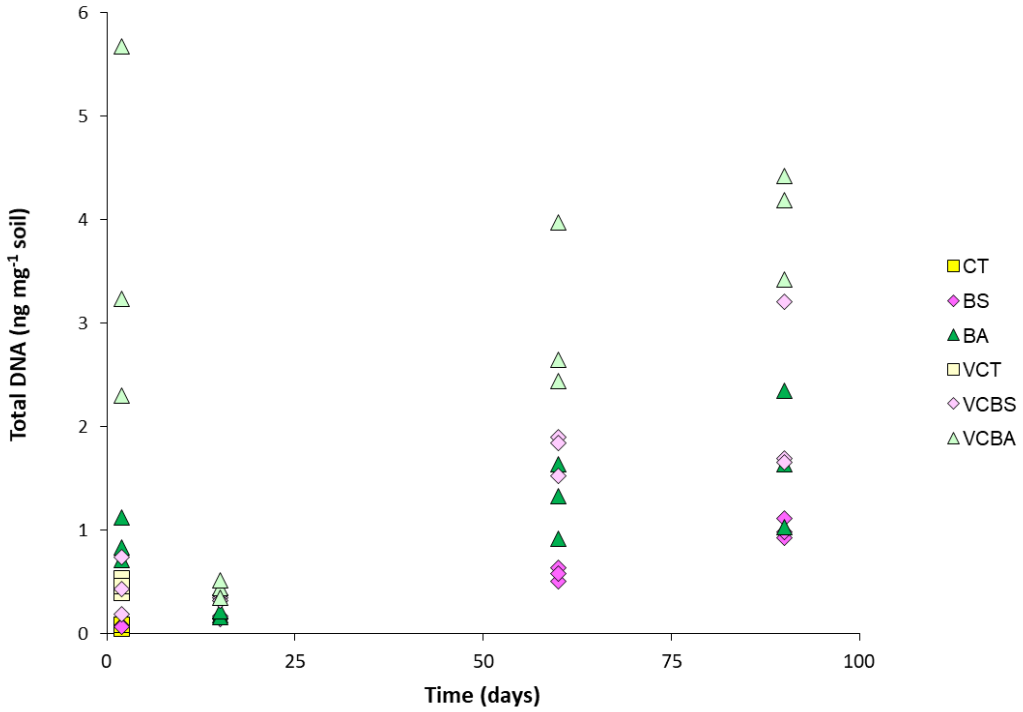


Figure 10. Total DNA according to the proliferation of the microbial community. Treatments: CT, control; BS, biostimulation; BA, bioaugmentation; VCT, control with vermicompost; VCBS, biostimulation with vermicompost; VCBA, bioaugmentation with vermicompost.

The results were combined with those previously obtained from physicochemical, biological and hydrocarbon content evolution to develop a pattern with the evolution of the microbial community. Currently, a machine learning study is being carried out to guide future applications, in which, knowing the soil characteristics, synthetic microbial communities can be developed according to the needs of soil pollutant degradation.

On the other hand, during the international stay, an incubation experiment was also carried out, in which the knowledge acquired in the previous incubations carried out at the University of Burgos was combined with the microbiological knowledge of the Slovenian researchers. This work is also unpublished, but the manuscript is in an advanced stage, to be submitted once finalised to a scientific journal. In this work, an incubation was carried out in which four organic carriers such as a biochar, cellulose, hay, and wood sawdust were tested, as well as four different bacterial strains with the ability to degrade different compounds such as oil degrading consortia, and auxiliary bacterial strains (ligninolytic, cellulolytic and diazotrophic). In this experiment, 44

different treatments were tested with the main objective of making longer chain hydrocarbons more bioavailable, testing three different combinations of bacteria: hydrocarbon degrading bacterial consortia, auxiliary bacteria, and hydrocarbon degrading consortia with auxiliary bacteria. As nutrient availability had previously been shown to be a key factor in bioremediation efficiency, ligninolytic and cellulolytic bacteria were used to decompose the organic carriers and thus provide the soil with additional available nutrients, as well as diazotrophic bacteria that served as a source of nitrogen. In addition, half of the treatments were supplemented with urea hydrogen peroxide as an additional source of nitrogen and oxygen for the bacteria. In addition, because low substrate availability was a problem previously shown in hydrocarbon bioremediation studies, alginate was introduced on the surface of the carriers with bacteria immobilised on them, for the development of hydrophobicity on the carriers, due to the bioavailability of the substrate. To develop these bacterial carriers, bacteria were first embedded in the alginate matrix, which was achieved by the addition of ZrOCl2, and nutrients necessary for bacterial growth were also added where appropriate. Then, the mixture of bacteria was added to the different carriers and the metabolic activity tests were carried out, after which the bacteria were mixed with the soil in the different pots to develop the treatments to be studied. The carriers with the bacteria would have an image similar to the picture shown in Figure 11.

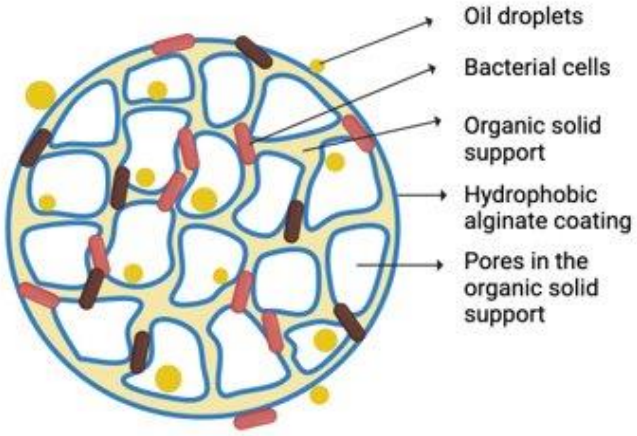


Figure 11. Representation of a bacterial carrier used in bioaugmentation.

The results obtained have shown higher degradation percentages than those previously obtained, which shows that this technique has significantly improved the bioavailability of the recalcitrant hydrocarbons, which can be subsequently degraded.

Figure 12 shows the best results obtained in relation to the degradation of the TPHs, where the four tested carriers have worked in different ways by varying the bacteria present in them, as well as with the urea hydrogen peroxide supplement. In addition, for a better understanding of the graph, the degradation obtained in the control treatment has been added, in which only natural attenuation has acted in the polluted soil without additions and has reached a degradation percentage of 19.19%. For the treatments in which the carriers were cellulose or biochar, the best results were obtained with the treatment in which the hydrocarbon degrading consortium and the auxiliary bacteria (ligninolytic, cellulolytic and diazotrophic) were added together, obtaining percentages of 59.25% and 91.71% respectively. However, for the treatments in which the carriers were hay or wood sawdust, the best results were obtained with the hydrocarbon degrading consortium and the urea hydrogen peroxide supplement, obtaining degradation percentages of 93.85% and 90.55% respectively. It should also be noted that the hay, in addition to achieving the highest percentage of degradation, was also the treatment with the most homogeneous results for all replicates, so the standard deviation is lower, which indicates that it has worked more efficiently.

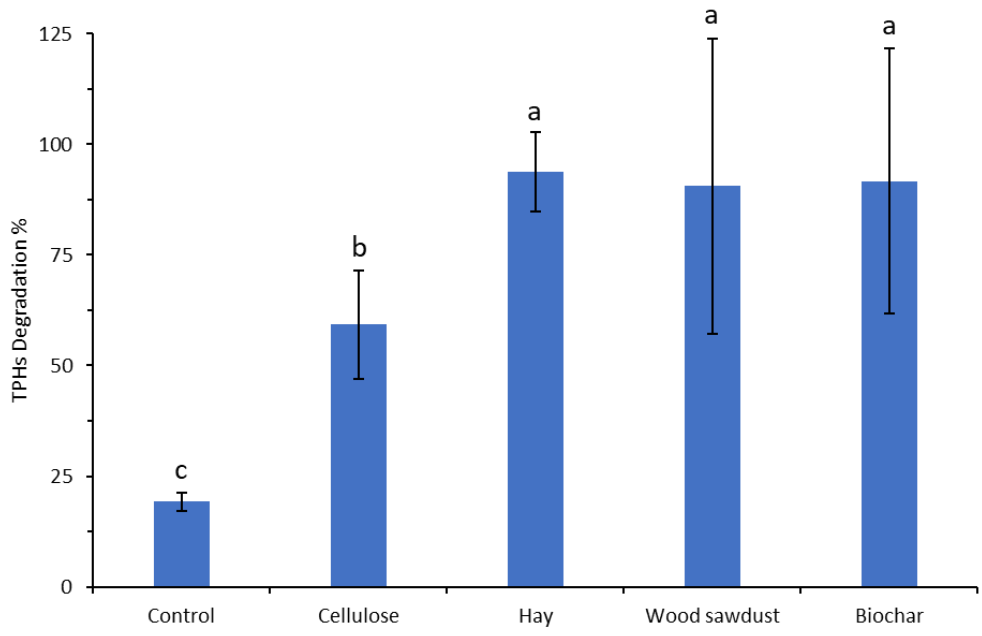


Figure 12. Improved degradation rates of TPHs obtained with carrier treatments. Columns with different letters displayed significant statistical differences (one-way ANOVA, followed by Tukey's post-hoc test with significance defined at $p<0.05$).

Once back from the international stay at the Jožef Stefan Institute, the GREENER project gave us another important challenge, we had to make the step from laboratory scale to pilot scale, in which we went from small microcosms, normally of 200 g of soil in a climatic chamber, with totally controlled conditions, to mesocosms of 500 kg, in which, although humidification and turning was carried out to avoid anaerobic conditions and the death of microorganisms, conditions such as temperature could not be totally controlled, as they were located in the street, in the facilities of another of our project partners, ACCIONA, in Alcobendas (Madrid). This study is developed in chapter 5 and is currently under review to be published in a special publication issue corresponding to the 7th International Symposium on Environmental Biotechnology and Engineering (7ISEBE) held in Marseille (France), for the journal Environmental Science and Pollution Research (Impact Factor = 5.8, Q1) under the title "Hydrocarbon Bioremediation: Scaling Up from Lab to Field for Petroleum-Contaminated Soils". To set up this experiment, three mesocosms were prepared in which a control, a bioaugmentation treatment with vermicompost using as a reference the results obtained in the incubation explained in chapter 3 (BAVC), and a bioaugmentation treatment with vermicompost in which graphite tubes were inserted as part of a bioelectrochemical system to try to improve the bioavailability of hydrocarbons and the degradation capacity of soil microorganisms (BESBAVC). The results obtained for the bioaugmentation treatments after 90 days of experience were satisfactory to achieve green, sustainable, efficient, and low-cost solutions for soil bioremediation. The degradation of soil TPHs achieved very promising results with significance between the bioaugmentation treatments and the control treatment. No significant differences were observed between the BAVC and BESBAVC treatments, reaching similar biodegradation values 90.29% and 86.77%, respectively, compared to 15% for the control. Therefore, it was decided that the bioaugmentation technique with vermicompost would be used for the pilot scale experiment, which obtained very promising results, with a lower economic cost than all the previously tested techniques, and which was also proven to work both at laboratory and pilot scale. But after 90 days of experience, we still observed that there was a higher concentration of long-chain polluting petroleum hydrocarbons, such as C21-C30, followed by the C30-C35 fraction. This is because the soil, in addition to petroleum hydrocarbons, being a soil collected from contamination stains from a machinery park, also contains motor oils and

lubricants, which are much more difficult to degrade than the hydrocarbons present in petrol or diesel. In order to understand the degradation processes, a microbial study was also carried out, which showed us that the soil contained an important microbial population capable of degrading hydrocarbons and that it was also metabolically very active, which was ideal both for the biostimulation of the indigenous bacteria and for the isolation of this population and its subsequent bioaugmentation.

With the extra knowledge acquired about the microorganisms present in the soil, a new incubation was carried out with the same objective as the previous one but using a synthetic microbial community with the capacity to degrade the most recalcitrant hydrocarbon compounds, designed by the Autonomous University of Madrid as part of our partners in the European GREENER project. It was a synthetic community with biological risk factor 1, which allows its direct application to the environment and composed of six bacterial strains: *Pseudomonas putida*, *Rhodococcus jialingiae*, *Rhodococcus WAY-2*, *Achromobacter aegrifaciens*, *Delftia acidovorans*, and *Novosphingobium silvae*. In addition, this research focused on the use of chemicals, such as stronger oxidants or surfactants, which improve the mobility of heavy hydrocarbon fractions. This is because the addition of surfactants can counteract the hydrophobic nature of petroleum hydrocarbons that makes them unavailable for bioremediation, but this type of chemicals is able to disperse the oily substrate in the soil and thus facilitate its accessibility to microorganisms. The use of a BHB-$FeCl_3$ nutrient solution was also modified according to the soil mixtures used. For the development of the incubation, different types of chemical surfactants (Triton X-100, Tween 20, Tween 80, and Sodium Dodecylsulphate (SDS)) and others surfactants with biological origin (commercial Rhamnolipids, EMULSAN II SP, SOILACT, CONTEROL and REMSURF) were previously tested, and once it was decided which were the ones with the best emulsifying index in contact with hydrocarbons, the ones with the best results were chosen, although finally only one of a chemical nature was used, not only because of the results, but also because of the sustainable and environmentally friendly objective. The experiment in which the emulsification index was studied showed how the mixture of the hydrocarbons with the surfactant studied was formed, of an immiscible nature, as can be seen in Figure 13. Emulsification has varied for the different types of surfactants and percentages of

addition as shown in Figure 14, which has allowed us to make a choice of the most efficient surfactants and the amount in which they have been applied.

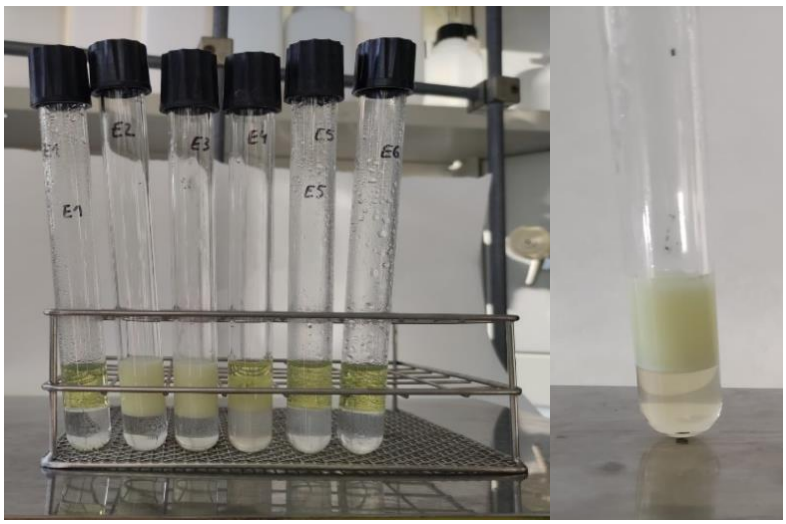


Figure 13. Emulsification index experiment for surfactant selection.

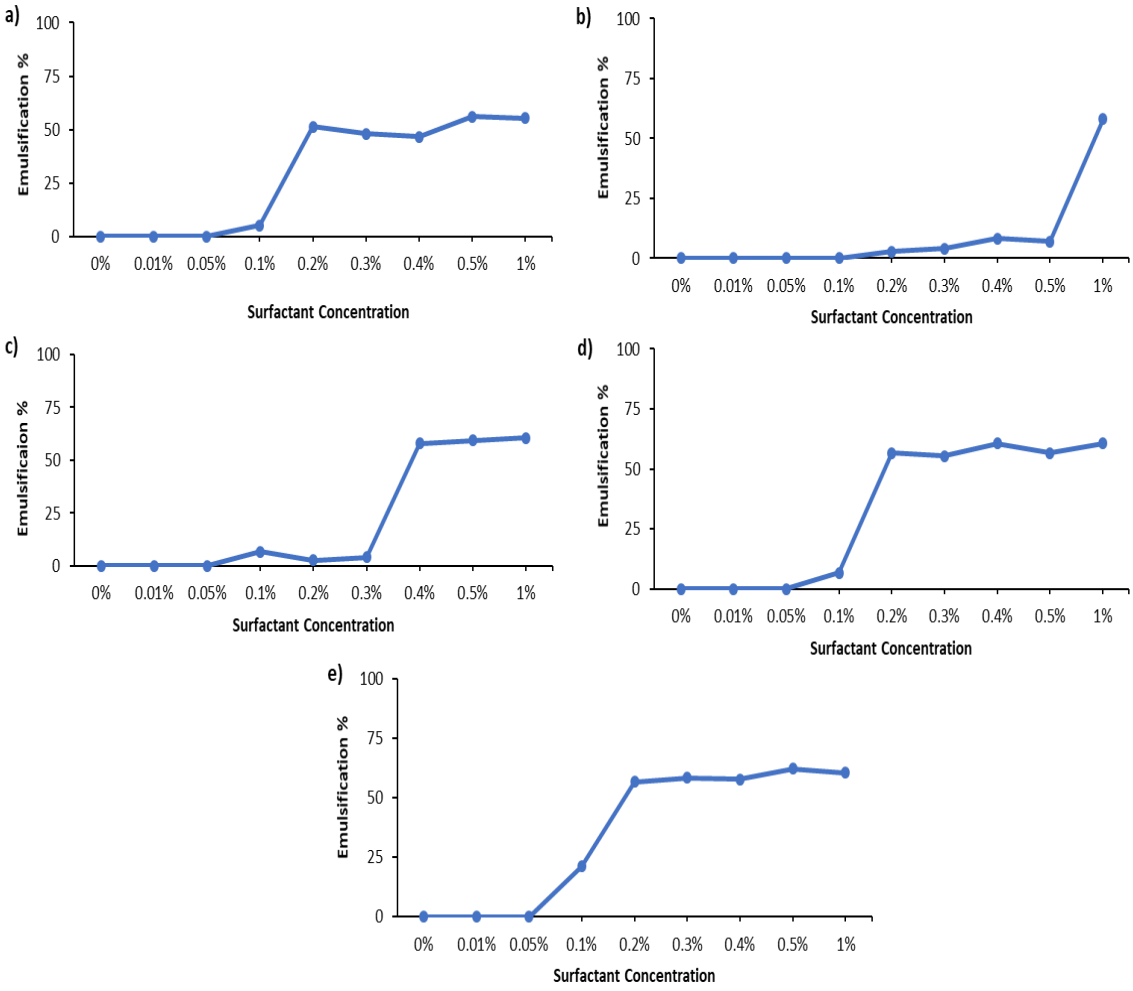


Figure 14. Emulsification index experiment for the chosen biosurfactant a) Rhamnolipids (RML); b) SOILACT; c) CONTEROL; d) REMSURF; and the only chemical surfactant e) Sodium Dodecylsulphate (SDS).

This incubation was carried out in the same way as the previous ones carried out at the University of Burgos, therefore, 200 g microcosms were prepared in which seven different treatments were developed, although the experiment was reduced to 60 days and a second bacterial inoculation was not introduced, in order to find out whether the addition of surfactants would be sufficient to meet the planned objective. This work is not described in this doctoral thesis, because some of the results are still pending analysis, but we hope to have results at the beginning of 2024 and publish them to increase scientific knowledge with the aim of advancing the elimination of long chain hydrocarbons in the environment. However, despite the usefulness of surfactants, they are in many cases synthetic and costly to manufacture, especially when used on a large scale.

2.3. Scope.

The scope is related to the results obtained from the application of the studied bioremediation techniques on hydrocarbon contaminated soils and their effectiveness.

2.3.1. Contamination type.

Soil bioremediation techniques are applied to very diverse types of contaminants, such as many types of hydrocarbons and heavy metals. Therefore, the studied bioremediation technologies can be adapted and improved to degrade the specific soil contaminants (hydrocarbons), based on its properties and environmental impact.

2.3.2. Contamination site.

The contamination site determines the chosen technique, because depending on this there are techniques that can be applied, and others that cannot. This makes it possible to treat soils with different natures and properties both *in situ* and *ex situ.*

2.3.3. Properties evolution.

The evolution of properties during the bioremediation process, to evaluate the progress and effectiveness of the applied technologies. By monitoring contaminant

concentrations, microbial activity, nutrient availability, ecotoxicity, and other relevant soil parameters. and minimizing potential environmental and health risks.

2.3.4. Remediation technologies.

Depending on the remediation technology developed, the complete degradation or mineralization of hydrocarbons, the immobilization or transformation of heavy metals, the reduction of contaminant concentrations to levels accepted by legislation, the restoration of specific soil functions and the reduction of environmental and human health risks associated with the types of contaminants treated by the technologies.

2.3.5. Remediation hybrid systems.

Bioremediation techniques can be integrated with other remediation techniques, creating hybrid systems to improve the effectiveness of the techniques. This involves studying the compatibility and integration of all possible technologies.

2.3.6. Feasibility.

Bioremediation techniques have a wide scope due to their economic feasibility and economic profitability compared to traditional remediation methods, since many of them can be applied *in situ*, minimizing the costs of excavation and transportation of contaminated soils.

2.3.7. Regulation and Stakeholders.

The scope covers compliance with applicable legal regulations, obtaining the necessary permits to develop the technologies and attention to the considerations of interested parties, such as the owners of contaminated sites, regulatory bodies, the communities where the contaminated site is located and other interested parties. In general, the scope of the techniques is broad and covers different types of contaminants, site conditions and properties, bioremediation technologies and hybrid systems, feasibility, and regulatory considerations. This extensive scope allows for wide flexibility and adaptation, allowing techniques to be implemented effectively to obtain better results.

2.4. References.

Alori, E. T., Gabasawa, A. I., Elenwo, C. E., & Agbeyegbe, O. O. (2022). Bioremediation techniques as affected by limiting factors in soil environment. *Frontiers in Soil Science*, 2, 937186. https://doi.org/10.3389/fsoil.2022.937186

Barbato, R. A., & Reynolds, M. C. (2021). Bioremediation of contaminated soils. *Principles and Applications of Soil Microbiology*, 607–631. https://doi.org/10.1016/B978-0-12-820202-9.00022-8

da Silva, I. G. S., de Almeida, F. C. G., da Rocha e Silva, N. M. P., Casazza, A. A., Converti, A., & Sarubbo, L. A. (2020). Soil bioremediation: Overview of technologies and trends. *Energies*, 13(18), 4664. https://doi.org/10.3390/en13184664

European Environmental Agency (2022). Progress in the management of contaminated sites in Europe. https://www.eea.europa.eu/ims/progress-in-the-management-of?utm_source=EEASubscriptions&utm_medium=RSSFeeds&utm_campaign=Generic (Accessed on 2/01/2024).

FAO & ITPS. 2015. *Status of the World's Soil Resources (SWSR) - Main Report*. Rome, Italy, Food and Agriculture Organization of the United Nations and Intergovernmental Technical Panel on Soils. (also available at http://www.fao.org/3/ a-i5199e.pdf)

Garrido-Sanz, D., Redondo-Nieto, M., Guirado, M., Jiménez, O. P., Millán, R., Martin, M., & Rivilla, R. (2019). Metagenomic Insights into the Bacterial Functions of a Diesel-Degrading Consortium for the Rhizoremediation of Diesel-Polluted Soil. *Genes*, 10(6), 456. https://doi.org/10.3390/GENES10060456

Hernández-Soriano, M. C. (2012). Soil Health and Land Use Management. *IntechOpen.* https://doi.org/10.5772/2516

Heuser, Dr. I. (2022). Soil Governance in current European Union Law and in the European Green Deal. *Soil Security*, 6, 100053. https://doi.org/10.1016/j.soisec.2022.100053

Kalia, A., Sharma, S., Semor, N., Babele, P. K., Sagar, S., Bhatia, R. K., & Walia, A. (2022). Recent advancements in hydrocarbon bioremediation and future challenges: a review. *3 Biotech*, 12, 135. https://doi.org/10.1007/s13205-022-03199-y

Lan, J., Wen, F., Ren, Y., Liu, G., Jiang, Y., Wang, Z., & Zhu, X. (2023). An overview of bioelectrokinetic and bioelectrochemical remediation of petroleum-contaminated soils. *Environmental Science and Ecotechnology*, 16, 100278. https://doi.org/10.1016/j.ese.2023.100278

Montanarella, L., & Panagos, P. (2021). The relevance of sustainable soil management within the European Green Deal. *Land Use Policy*, 100, 104950. https://doi.org/10.1016/j.landusepol.2020.104950

Ren, X., Zeng, G., Tang, L., Wang, J., Wan, J., Wang, J., Deng, Y., Liu, Y., & Peng, B. (2018). The potential impact on the biodegradation of organic pollutants from composting technology for soil remediation. *Waste Management*, 72, 138-149. https://doi.org/10.1016/j.wasman.2017.11.032

Wang, A. J., Wang, H. C., Cheng, H. Y., Liang, B., Liu, W. Z., Han, J. L., Zhang, B., & Wang, S.-S. (2020). Electrochemistry-stimulated environmental bioremediation: Development of applicable modular electrode and system scale-up. *Environmental Science and Ecotechnology*, 3, 100050. https://doi.org/10.1016/j.ese.2020.100050

CHAPTER 3

Evaluation of biostimulation, bioaugmentation, and organic amendments application on the bioremediation of recalcitrant hydrocarbons of soil[1]

[1] Curiel-Alegre, S., Velasco-Arroyo, B., Rumbo, C., Khan, A. H. A., Tamayo-Ramos, J. A., Rad, C., Gallego, J. L. R., & Barros, R. (2022). Evaluation of biostimulation, bioaugmentation, and organic amendments application on the bioremediation of recalcitrant hydrocarbons of soil. *Chemosphere*, 307, 135638. https://doi.org/10.1016/j.chemosphere.2022.135638

3.1. Introduction.

Modern economies are characterized by a strong dependence on fossil fuels (Sima et al., 2019). However, increasing the frequency of accidental oil spills and the release of petroleum derivatives cause significant environmental impacts and pose substantial hazards to human health (Hussain et al., 2022; Khan et al., 2019). Despite numerous studies focused on decontamination strategies, it remains crucial to gain new, innovative, and sustainable techniques to reduce, degrade, and remove diverse pollutants in soils, sediments, and water bodies. Conventional technologies for cleaning-up contaminated soils (excavation, landfilling, and soil washing) and waters (treatment with activated carbon or ion exchange resins) are generally energy-intensive, expensive, time-consuming, and waste-producing (Yousaf et al., 2022). These technologies are not efficient for treating moderate to low-level polluted sites, which still pose human health and environmental risk. Bioremediation for oil-contaminated soils and sediments is a cost-effective and sustainable clean-up technology (Hussain et al., 2018). These include landfarming, bio-piling, phytoremediation, biostimulation and bioaugmentation (Mair et al., 2013). Biostimulation (BS) and bioaugmentation (BA) have unveiled good results based on improving hydrocarbons biodegradation. The difference between these two methods is that, in the case of bioaugmentation, the basis is the inoculation of exogenous degrading microorganisms into the soil (Wu et al., 2017). At the same time, biostimulation consists of the promotion of the degradation capacity of the autochthonous microbial communities by the addition of nutrients' optimized formulas, carrier materials, and amendments to stimulate the metabolic functions of the microorganisms that can use the contaminants present in the soils as "feeding" sources (Khan et al., 2017). Applying soil amendments with organic residues raises the soil's organic matter (OM), a crucial property intimately linked to soil fertility (Iqbal et al., 2020). The OM helps stimulate microbial activities, mineralize resistant materials, and eliminate xenobiotic compounds by promoting biotic and abiotic processes that rely on biodegradation and adsorption mechanisms (Mushtaq et al., 2020). Composting polluted soils and organic wastes or adding compost to the soil is a commonly used soil bioremediation economic method (Chen et al., 2015). The aerobic bio-decomposition of organic waste reduces biowaste volume by 40–50% and provides a product that acts as

a soil conditioner or biofertilizer along with polluted soil remediation (Iqbal et al., 2020; Wu et al., 2017). Vermicomposts (product of the decomposition process using various species of worms) have significantly larger nutrients than composts and result in higher microbial population size and activity (Das & Deka, 2021). The hydrocarbon remediation process is highly dependent on the pollutant load. An increased pollutant load can delay the profits of remediation using microorganisms (Hussain et al., 2022). Apart from this, the hydrocarbon-impacted soil remediation process is highly dependent on aeration, nutrients content, organic matter, CN ratio and the presence of the hydrocarbon-degrading microbial population (Yousaf et al., 2022). Even if all these factors are maintained to optimal, the biggest challenge to treatment is associated with aged or weathered hydrocarbon-contaminated soils. The aging of hydrocarbon in the soil makes the treatment process much more complex, leading to the heterogeneous spatial distribution of the pollutants, soil structure deterioration, nutrient imbalance, and hydrophobicity. The increased hydrophobicity leads to high sorption of recalcitrant PAHs fractions between soil pores. It causes soil aging, makes the hydrocarbon less bio-accessible, and reduces the soil moisture and water retention capacity (Hussain et al., 2018). Despite all these known issues, much of the focus in the previously published research work has remained on the spiked contaminated soils that lack the aging hydrocarbon effect, while the aged hydrocarbon contaminated soil has always remained a grey area in research and requires due attention. Hence the objective of the current study has been to encounter new bioremediation solutions to optimize the treatment of soils contaminated with total petroleum hydrocarbons (TPHs), including polycyclic aromatic hydrocarbons (PAHs), in the presence of potentially toxic metals and metalloids. Accordingly, specifically designed biostimulation and bioaugmentation strategies have been tested and compared to quantify the efficacy of the hydrocarbon remediation strategy. The targeted soil matrix has been challenging since it harbours a contamination history showing around recalcitrant long-chain hydrocarbons (C21-30), exhibiting a low bioavailability after a long natural attenuation lasting for years.

3.2. Materials and methods.

3.2.1. Chemicals and reagents.

For chromatographic separation quality aliphatic hydrocarbons: Alkanes-Mix 12, 100 µg mL^{-1} in toluene (C_7H_8), C8–C40 (pair no. of C, 17 HC) and polyaromatic hydrocarbons: PAHs-Mix 9, in cyclohexane (100 µg mL^{-1}) were supplied by LGC standards (details in supplementary material). The certified reference CRM-357 and CRM-359 for TPH–sandy loam and TPH–clay was used, respectively (Sigma-Aldrich). Isolute Sorbent EPH (25 mL 5 g^{-1}) extraction cartridges for sample extraction and elution were used (Biotage) in the SPE-24G column processor (JT Baker). Extraction solvents, GC grade acetone (C_3H_6O), dichloromethane (DCM), internal standard nonadecanoic acid (C19:0), and hexane (hx), and reagents for phospholipid fatty acids (PLFAs) determination, the analytical grade for chromatography; bacterial acid methyl ester (BAME) mix and Supelco 37 component fatty acid methyl esters (FAME) mix (CRM47885), were provided by Sigma Aldrich.

3.2.2. Nutrient solutions and inoculum preparation.

Modified BHB (Bushnell Haas Broth) solutions were prepared, either for biostimulation or bioaugmentation (BHB-2) or for the treatments with VC (BHB-3), in which the amount of N supplemented by the VC was subtracted from the nutrient solution, considering a mineralization coefficient of 30% (Khan et al., 2016a). BHB-2 was prepared by mixing individual solutions to reach the following added amounts (g kg^{-1} dry soil): $CaCl_2$ 0.0061; KH_2PO_4 0.3058; K_2HPO_4 0.3058; $MgSO_4$ 0.0612; NH_4NO_3 3.5425; $FeCl_3$ 0.0153. the $FeCl_3$ was added separately to avoid precipitation. BHB-3 had the same saline composition, except NH_4NO_3 3.0454 (g kg^{-1} dry soil basis). The consortium used as inoculant was isolated from the same soil used in this study by serial enrichment cultures, using diesel (10 g/L) as the sole carbon and energy source (Garrido-Sanz et al., 2019). The consortium harbours around 50 OTUs and is composed by E*nterobacteriaceae* (33,56%), followed by *Pseudomonadaceae* (26,61%) *Burkholderiaceae* (24,35%), *Moraxellaceae* (3,64%), *Xantomonadaceae* (4,40%), *Rhizobiaceae* (2,42%), *Sphingomonadaceae* (1,87%) and

Rhodanobacteriaceae (1,00%). All other families are below 1%. An aliquot of this consortium from glycerol stock was inoculated in a 20 mL minimal medium with gas oil (1% v:v) as a carbon source and incubated in a rotary shaker at 150 rpm at 28 ºC overnight. After this incubation, the culture was centrifuged and resuspended in 20 mL of minimal medium. To prepare the pre-inoculum, 2 mL of this culture were inoculated in 200 mL of minimal medium with gas oil and cultured for 2 days in the mentioned conditions. This pre-inoculum was used to inoculate six Erlenmeyer flasks containing 2 L of minimal medium with gas oil, which was incubated for 4 days in the same conditions (20 mL of pre-inoculum per flask). Cultures were then centrifuged at 3,800 g and 8 ºC for 30 min. Supernatants were discarded, and pellets were resuspended in a minimal medium, divided into 2 equal halves, and centrifuged again to remove the remnants of the culture medium with gasoil used for consortium growth. Finally, one of the obtained pellets was resuspended in 200 mL of BHB-2, while the other was resuspended into 200 mL of BHB-3. Both samples were transferred to a 250 mL volumetric flask. Aliquots of these suspensions were collected for CFU (Colony Forming Unit) counting and inoculum preparation.

3.2.3. Soil properties and characterization.

The soil under study corresponds to a machinery park area in Noblejas (Toledo, Spain), contaminated with different hydrocarbons, mineral oils, and heavy metals. Air-dried soil samples were sieved at 2 mm and characterized in their physicochemical properties according to standard methods. Soil pH (H2O, 1:5, w:v) was determined using 5 g of soil sample and 25 mL deionized water after 30 of agitation at 60 RPMs using a pH meter (GLP21, Crison). After pH determination, the soil suspension was centrifuged for 20 min at 2,000 g, filtered, and the electrical conductivity (EC) was measured using a conductivity meter (GLP31, Crison). Soil available P was assessed with the help of the Olsen method. Briefly, 2 g of dry soil was extracted with 40 mL 0.5 M $NaCO_3$ at pH 8.5 after shaking for 30 min at 60 RPMs. After centrifugation, the extract was analysed and quantified using the molybdenum blue method for orthophosphate on an autoanalyzer (San++, Skalar). Available macronutrients and trace elements were analysed using Mehlich 3 method (Mehlich, 1984). Briefly, 20 mL of extraction solution (0.2 M CH_3COOH, 0.001 M EDTA, 0.013 M HNO_3, 0.015 M NH_4F,

and 0.25 M NH_4NO_3) were mixed with 2 g dry soil (1:10, w:v), agitated for 5 min at 120 RPMs, centrifuged at 2,000 g for 10 min and filtered (Whatman No. 42). Nutrients and trace elements were determined in the extracts using an ICP-OES (Genesis, Spectro). Soil organic carbon and nitrogen were measured using 0.5 M K_2SO_4 (1:8, w:v) as extractant. These extracts were centrifuged at 60 RPMs for 45 min and filtered. Then the extracts were subjected to organic C and total N analysis via a TOC-V CSN autoanalyzer (Shimadzu). Soluble ammonium and nitrate were also determined in these extracts on an autoanalyzer using the indophenol blue method and Griess reaction after reducing nitrate in a copperized-cd column, respectively (San++ Skalar, Breda). Basal soil respiration (BSR) was articulated as mg CO_2–C kg^{-1} dry soil h^{-1}. For this activity, frozen soil samples (20 g) were attemperated for 72 h at 22 ± 0.5 °C and placed in 1-Liter jars with hermetic sealing caps. The jar was provided with a beaker, acting as a gas trap, containing 4 mL of 0.5 M NaOH. After 24 h of incubation, 2 mL of 0.5 M $BaCl_2$ were added, which led to the barium carbonate-adsorbed CO2 precipitation. Using 0.1 M HCl through an automatic titrator (718 Stat Titrino, Metrohm), the remaining NaOH was quantified.

3.2.4. Experimental setup and microcosms preparation.

The study was conducted at a laboratory scale in microcosms conditions for 90 days. Six treatments have been tested, including natural attenuation as a control, biostimulation with an improved nutrient formula, biostimulation with vermicompost (VC), and the same treatments with bioaugmentation. In the case of the bioaugmentation treatments, the microbial consortia inoculated corresponded to the native microbial population previously isolated from the soil site under research, which has been characterized and cultured as part of the tasks developed within the framework of the European GREENER project. In the presence and absence of VC, six different incubation treatments were prepared using biostimulation and bioaugmentation strategies. These included control soil (abbreviated as CT), biostimulation of soil (added with BHB-2, abbreviated as BS), bioaugmentation of soil (inoculated with consortium suspended in BHB-2, abbreviated as BA), vermicompost amended control soil (abbreviated as VCT), VC aided BS of soil (VC + BHB-3, abbreviated as VCBS), and VC aided BA of soil (VC + consortium suspended in BHB-3,

abbreviated as VCBA). The CT was a control of natural attenuation effects over the bioremediation soil. Prior to the initiation of the experiments, the polluted soil was sieved to 2 mm and watered to reach 40% of soil water holding capacity (SWHC); to highlight, on day 43rd, when the second consortia inoculation was carried out, the SWHC was increased to 50%, using this volume of water to introduce the inoculum and nutrient solutions. Each treatment was prepared by mixing soil, amendments, and solutions using a concrete mixer and immediately distributed by weighing 200 g of dried soil into 1 L hermetic containers. A final concentration of 10^{11} CFU kg^{-1} soil was applied for the respective BA treatments. The same procedure was repeated for the second inoculation (on the 43rd day), but the final suspension was made in 100 mL of nutrient solutions. Samplings were taken 2, 15, 30, 45, 60, and 90 days of treatment. Four experimental replicates for each sampling point and treatment were maintained. The incubation was conducted in a chamber under controlled temperature conditions (22 ± 0.5 °C). Individual microcosms were opened and mixed weekly (twice) to control the moisture and soil aeration. At the end of each sampling point, soil samples were divided to perform different types of analysis: 70 g was dried in an oven at 30 °C for 48 h, and the remaining fraction was frozen at − 20 °C for subsequent experiments. Control samples of day 0 for each treatment were also included.

3.2.5. EPH quantification, LC fractionation and GC-MS qualitative study.

Extractable petroleum hydrocarbons (EPH) were extracted from 1 g soil samples (dried at 30 °C) and 20 mL of mixture acetone: hexane (hx) (1:1, v:v) in a microwave extraction oven (Ethos X, Milestone, Sorisole, Italy) at 150 °C for 20 min. The cold mixture was subjected to centrifugation for 30 min at 2,500 g. The resulting supernatant was filtered (though 0.22 µm filter) and evaporated to a volume of 1 mL in a rotary evaporator (SAVANT SPD111V, Thermo). Before sample loading, the extraction cartridges were conditioned with 30 mL hx, preventing the drying of cartridges. After loading the sample at ambient pressure, elution was performed using 12 and 20 mL of hx and DCM (dichloromethane). The two fractions produced were evaporated above 1 mL and injected into a Varian 3900 gas chromatography (GC) instrument provided with a flame ionization detector (FID) device and a Varian

CP8907 capillary column (25 m, 0.25 mm inner diameter, with the film thickness of 0.25 mm). Spitless injection mode was performed with 250 °C of injection temperature and 3 µL of injection volume. The oven operating conditions were: initial temperature 80 °C, raising to 200 °C at 7 °C min^{-1}, then reaching 300 °C at 11 °C min^{-1}, which was maintained for 17 min. Helium was the carrier gas (74 kPa). The FID operated at 325 °C and 20 Hz. The detected aliphatic and aromatic fractions were defined based on carbon or equivalent ranges. Equivalent carbon numbers denote an assigned value to a petroleum mixture fraction, empirically derived from the fraction normalized boiling point compared to the boiling point of n-alkanes or the n-alkanes retention time in a boiling point GC column. Hydrocarbon extraction was performed with 90-day samples for all tested treatments. An additional fingerprint study was carried out for some samples using GC-MS after LC (Liquid Chromatography) fractionation. In a Soxtherm system, using 3:1 DCM to methanol (MeOH), the soil samples (5 g) were extracted (Gerhardt analytical systems). Rotary evaporation was performed to concentrate the extracts. Extract aliquots were subjected to fractionation and gravimetrically quantified using open column LC. Using hx and DCM as eluents, maltenes and asphaltenes were segregated using 0.45 µm filters. Maltenes were further fractionated into three fractions using LC columns, filled with silica gel and alumina. Aliphatic hydrocarbons (Fraction 1) through hx, aromatic hydrocarbons (fraction 2) with DCM: hx (7:3, v:v), while polar compounds (fraction 3) using DCM: MeOH (1:1, v:v), were eluted. LC fractions were analysed with GC-MS (QP-2010 Plus, Shimadzu). Helium as carrier gas at 1 mL min^{-1} was used in the capillary column (DB-5 ms). The specification of the capillary column was 60 m × 0.25 mm i.d. × 0.25 µm film (Agilent Technologies), packed with phenyl and dimethylpolysiloxane at 5:95%, respectively. The initial temperature of the oven was maintained at 50 °C for 2 min. Later the temperature was ramped at 2.5 °C min^{-1} up to 310 °C and maintained for 45 min. The GC-MS worked in electron ionization (EI) mode at 70 eV, with autotuning calibrations performed using perfluorotributylamine. The full-scan mode was used to obtain the chromatograms, with mass range for acquisition ranging from 45 to 500 m z^{-1}. NIST 2014 Mass Spectral Library (NIST 2014/EPA/NIH) was used to identify the compounds.

3.2.6. Soil enzyme activities.

Enzyme activities were measured using fluorogenic MUF or AMC substrates in 96-microtiter plates (Marx et al., 2001). The studied activities included acid phosphatases (EC 3.1.3.2 – AcPA), alkaline phosphatase (EC 3.1.3.3 – AlkPA), α-glucosidases (EC 3.2.1.20 – aGA), β-glucosidases (EC 3.2.1.21 – bGA), N-acetyl-β-glucosaminidase (EC 3.2.1.30 - bNAG), β-xylosidase (EC 3.2.2.27 - bXyl), leucine-aminopeptidase (EC 3.4.11.1 - LeuAMP) and sulfatase (EC 3.1.6.1 - AS). These activities were analyzed using amino-4-methylcoumarin (AMC) and 4-methylumbelliferone (MUF) derivatives as substrates. A frozen sample (1 g oven-dry) was suspended in 20 mL of deionized water in sterilized conditions. For homogenous suspension, soil samples (ice cold) were subjected to pulsed sonication (40 W) for 2 min. Aliquots of 50 µL were used for analysis with eight analytical replicates. 50 µL Modified universal buffer (MUB) at 500 µM was used to analyse each enzymatic activity. Specifically, MUB for AcPH and bGA has a pH of 5, for the activities of aGA, AS, bNAG, and bXyl pH was maintained at 6, while pH 9 and 10 were kept for AlkPA and LeuAMP, respectively. Plates were sealed to prevent evaporation and kept at 30 °C for 180 min under agitation at 150 RPMs. Tris buffer (50 µL) at pH 12 was added to stop the reaction and immediately analysed using a fluorometric plate-reader (GENios, TECAN) with 360 and 450 nm excitation and emission filters. Fluorescence was converted into an amount of MUB or AMC according to calibration standards (0–1,500 pmol) prepared on each plate to consider the degree of fluorescence quenching through soil particles and OM.

3.2.7. Determination of phospholipid fatty acids.

Bligh & Dyer (1959) proposed method to extract the PLFAs, was used. Chloroform ($CHCl_3$) was used to redissolution lipid fractions, and the fractions were later added to silica columns. Fractions of neutral lipid and glycolipid were eliminated using $CHCl_3$ and C_3H_6O (respectively), and phospholipids were eluted using MeOH and dried with the help of rotary evaporation. The sample was redissolved with C_7H_8 and added with an internal standard (C19:0, Sigma-Aldrich). Alkaline methanolysis was performed to make fatty acid methyl esters (FAMEs) derivates. The FAMEs were extracted using 2 mL hx twice, followed by drying and redissolution n-octane (100 µL). Agilent 6890 N

GC system was used for FAMEs quantification. The system provided FID detection with a Supelco Omegawax 320 fused silica column (30 m in length), film 0.25 µm, ID 0.32. The operation temperature program of the system was as follows 140–170 °C at 2 °C min^{-1}, maintained for 30 min, followed by 170–260 °C at 5 °C min^{-1}, maintained for 20 min. Commercial standards, BacMix and FAMEmix (Sigma Aldrich), were used for the FAME peaks identification, as proposed by Palojärvi (2006).

The individual FAMEs abundance was expressed in nmol g^{-1} of dry soil. Fatty acids i15:0, a15:0, i16:0, 10 Me16:0, i17:0, Me18:0 (PLFA Gram+) was used for the representation of Gram-positive bacteria, while c16:1w9c, cy17:0, and cy19:0 (PLFA Gram-) for Gram-negative bacteria. The sum of both represents total bacterial PLFAs (PLFA Bacteria). Fungal PLFA was considered the sum of c18:2ω6,9t and c18:2ω6,9c (PLFA Fungi), and the sum of 10Me16:0 and 10Me18:0 was considered for Actinobacteria. The sum of all fatty acids quantified total PLFA to represent total microbial biomass.

3.2.8. Statistical analysis.

The mean and the standard error of at least three independent experimental replicates were calculated for all variables. Normality and homogeneity of variances assumptions were assessed with Kolmogorov Smirnov and Levene tests, respectively, using treatment as the fixed factor, and the data were analysed with ANOVA (ANalysis Of VAriance) with a significance of $p \leq 0.05$. Then, the treatments were compared with the Tukey's or Dunnett's (only for PAHs) *post-hoc* tests. All statistical analysis was performed on a statistical package for social sciences (v22.0 for Windows).

3.3. Results.

3.3.1. Soil and organic amendment characterization.

Soil collected from the machinery park area in Noblejas was sandy-loam texture (11.8 clay, 29.8 silt, and 58.3 sand, percent respectively), with bulk density 1.51 g cm^{-3}, highest water retention capacity (HWRC) 25.33%, pH (1:5) 7.1, electrical conductivity (EC 1:5) 0.839 dS m^{-1}, loss on ignition (LOI) 3.85%, oxidizable organic carbon 2.59%,

total N 0.02%, nutrients (mg kg^{-1}): NH_4–N 2.99; NO_3–N 0.14; PO_4–P 0.10, lime content 35.08%, trace elements (mg kg^{-1}): As 77.3; Cd 7.8; Cr 14.9; Cu 8.5; Ni 9.9; Pb 339.2; Zn 680.5, and total petroleum hydrocarbons (TPHs): 4051.0 mg kg^{-1}. As an organic amendment, vermicompost (VC) from agro-industrial wastes (ROPULPAT, Spain) was used at a rate of 2% (w:w). Physicochemical VC properties were as follows: pH (1:5) 7.0, EC (1:5) 3.157 dS m^{-1}, total organic carbon (TOC) 33.3%, and total nitrogen (TN) 2.9%. VC has been included as an organic amendment since it, in principle, leads to better hydrocarbons' adsorption, filtering, and degradation.

3.3.2. Biodegradation of TPHs.

To understand and compare the potential degradation efficiency of the treatments under study, the quantity of extractable petroleum hydrocarbons (EPHs) and their correspondent aliphatic and aromatic fractions (see below for details) was analysed after 90 days of microcosms incubation. The EPHs content was reduced from the initial 4051.0 mg kg^{-1} dry soil to 3806.0 mg kg^{-1} in the case of the control soil (CT), to 3,098.5 mg kg^{-1} in the case of the biostimulation treatment (BS), and 2864.5 mg kg^{-1} after applying the microbial consortium (BA), which represents a reduction of 6.0%, 23.5% and 29.3% of the initial content, respectively (Figure 15.A). Likewise, EPHs were reduced by applying 2% (w:w) vermicompost (VCT) to 3640.0 mg kg^{-1}; this quantity was reduced to 2,733.5 mg kg^{-1} when this mixture was combined with biostimulation (VCBS) and to 2,658.5 mg kg^{-1} when it was combined with bioaugmentation (VCBA). In this last case, the results revealed a moderate increase in EPHs degradation' efficiency when compared with samples without VC addition, reaching degradation rates of 10.1% (VCT), 32.5% (VCBS), and 34.4% (VCBA) of the initial hydrocarbon levels. Concerning the 16 EPA (Environmental Protection Agency) PAHs, Figure 15.B exhibits the scenario for the total PAHs fraction (left) per treatment at the end-point experiment (90 days). The concentration of the 16 EPA PAHs from the initial contaminated soil can be seen in Supplementary Table 1. However, a decreasing trend was observed compared to the untreated control soil, either with or without VC addition. The results were not statistically significant. Specifically, the phenanthrene, the most abundant PAHs present in the soil understudy, was significantly reduced after the VCBS and VCBA treatments (Figure 15.B), from 0.052 mg kg^{-1} in the control

soil to 0.0265 mg kg^{-1} in both treated soils (BS and BA). It should be highlighted that, in this soil, EPHs corresponded to the TPHs fraction since the composition was dominated by medium and high molecular weight in both linear aliphatic and aromatic hydrocarbons (Figure 16 and Figure 17). In contrast, the volatile fraction was almost completely depleted, and this distribution did not change along with the 90-day incubation (CT). EPH fractions are displayed in different ranges as a function of the length of carbon chains and are shown in Figure 16.A and Figure 16.B for aliphatic and aromatic hydrocarbons, respectively. Aliphatic hydrocarbons (designated as Cn, where “n" is the number of C) in the ranges of C22–C35 and >C35 accounted for 79.4% of total EPHs content. For the aromatic hydrocarbons fraction (EC), EC22-EC35 and >EC35 accounted for another 15.0%. In the case of the aliphatic fraction, these long-chain hydrocarbons (C22–C35) represented the most degraded fraction during the incubation period and showed an apparent effect of soil microbial stimulation and inoculum addition while higher molecular weight hydrocarbons (>C35) were more recalcitrant against biodegradation. In the case of aromatic hydrocarbons, low molecular weight compounds (EC10-EC16) nearly disappeared in the treatments with vermicompost and microbial inoculation, whereas the highest molecular weight compounds displayed a similar pattern as described for aliphatic.

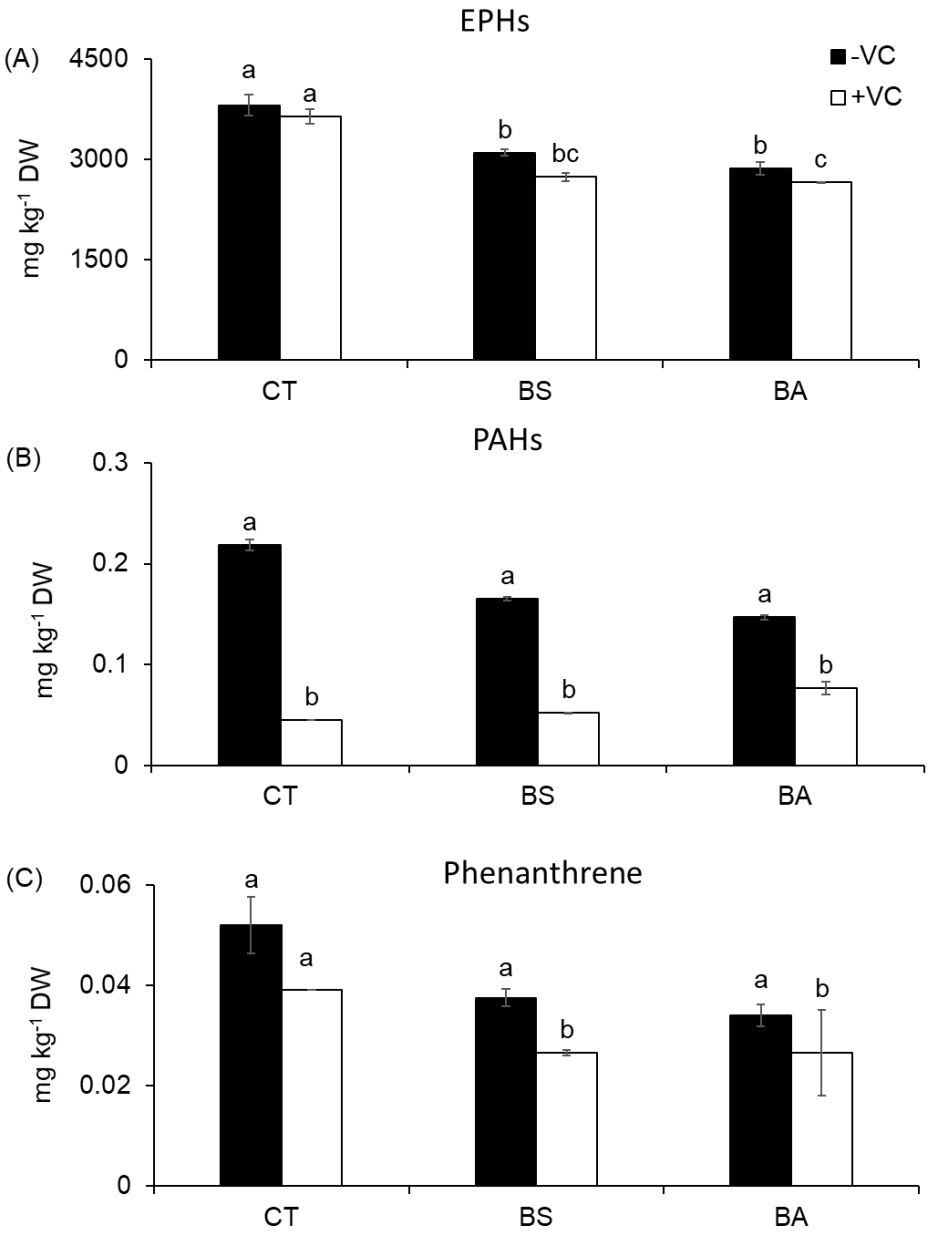


Figure 15. (A) Content of Extractable Petroleum Hydrocarbons (EPHs) at the end of the 90 days incubation period; (B) Content of Polycyclic Aromatic Hydrocarbons (PAHs); and (C) phenanthrene at the end of the 90 days incubation period. Columns with different letters displayed significant statistical differences (one-way ANOVA, followed by Tukey's *post-hoc* test with significance defined at $p<0.05$). DW: Dry Weight.

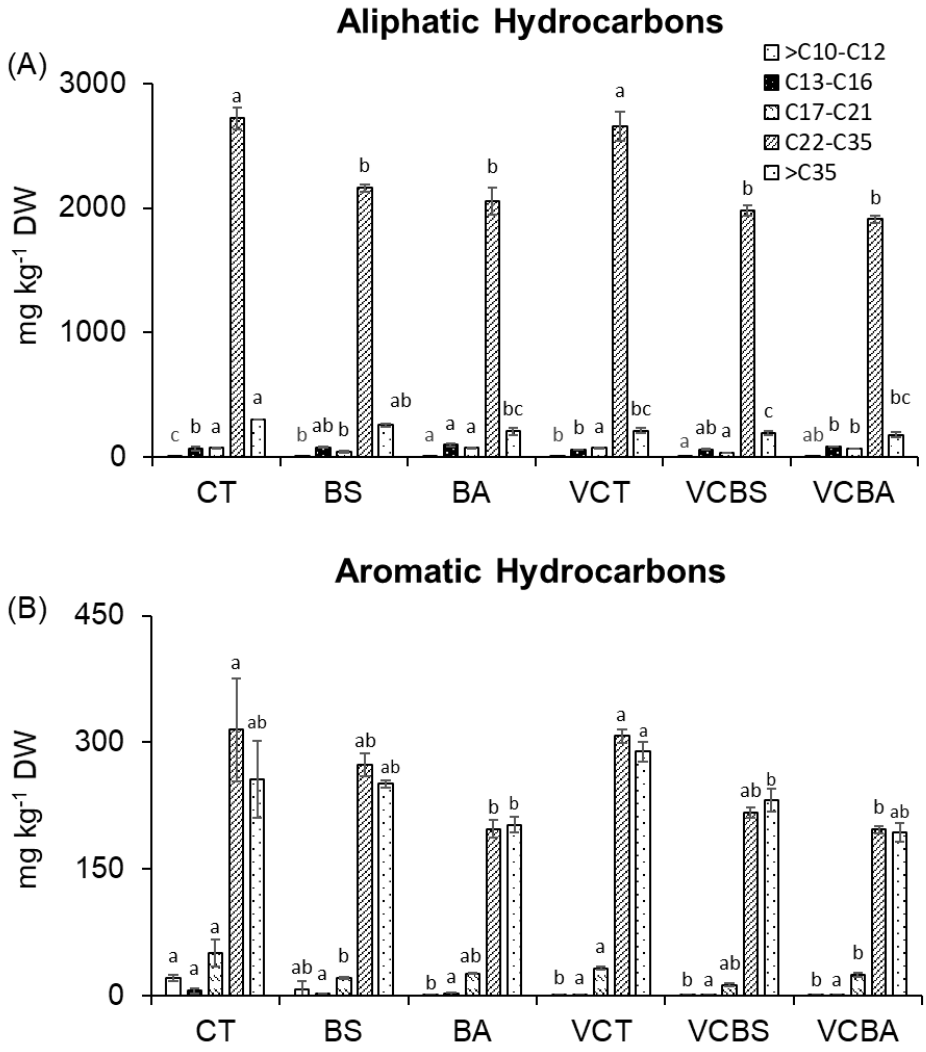


Figure 16. Molecular ranges of EPH after liquid fractionation between aliphatic (A) and aromatic (B) hydrocarbons fractions at the end of the 90 days incubation period. Columns between fractions with different letters displayed significant statistical differences among treatments (One-way ANOVA, followed by Tukey's *post-hoc* test with significance defined at $p<0.05$). DW: Dry Weight.

3.3.3. GC-MS qualitative study.

Once the concentration of EPH and its fractions were evaluated as described above, a deeper examination of the evolution of the main hydrocarbon and non-hydrocarbon compounds was carried out utilizing a qualitative GC-MS (Gas Chromatography-Mass Spectrometry) study. The samples selected to compare the initial status and the advanced states of biodegradation were control (CT) and bioaugmentation with vermicompost (VCBA) after 60 days of incubation. As a first step, the fate of the main

fractions of the contaminants was analysed after LC fractionation according to the SARA procedure (Saturates, aromatics, resins, and asphaltenes). The F1 fraction (mainly aliphatics) was initially 22.8% of the total extract and rose to 36.7% after 60 days of incubation (VCBA treatment); a similar pattern was observed with the F2 fraction (mainly aromatics), which was increased from 4.3% to 5.9%; on the contrary, the sum of F3 fraction (polar compounds) and asphaltenes diminished from 72.8% to 57.4%. This initial result revealed that many polar compounds were degraded during the experiments (Figure 17.A and Figure 17.B). The predominant compounds within this polar F3 fraction were oleochemicals. Regarding the aliphatic fraction (F1), the main differences between CT and VCBA are shown in Figure 17.C (chromatograms in SIM –Single Ion Monitoring-mode m z^{-1} = 57) and Figure 17.D (SIM, m z^{-1} = 191). In the chromatogram in Figure 17.C, the initial product displayed a fingerprint with a predominance of linear alkanes from 15 to 38 carbon atoms, enriched in heavy linear alkanes (with the maximum in 27 carbon atoms). It also shows a prevalence of linear alkanes over branched alkanes (n-C18 Phytane^{-1} ratio slightly above 1) and a moderate UCM (Unresolved Complex Mixture), thereby revealing an initial moderate degradation and weathering of the hydrocarbons in soil. On the contrary, after 60 days of incubation in the VCBA treatment (Figure 17.C), the linear alkanes were depleted entirely (n-C1 Phytane^{-1} = 0); branched alkanes such as isoprenoids, although less biodegradable, were also notably degraded (Phytane peak is almost negligible); the UCM is notable, and the predominant compounds were heavy branched alkanes, and mainly hopanes, a recalcitrant group of cycloalkanes that remained unaltered since the initial situation, as shown in Figure 17.D. Finally, although aromatics (fraction F2) were a minority fraction, alkylbenzenes were identified as the predominant compounds. As shown in Supplementary Figure 1 (see Supplementary material), after 60 days, a moderate reduction in the variety and abundance of these compounds was evidenced.

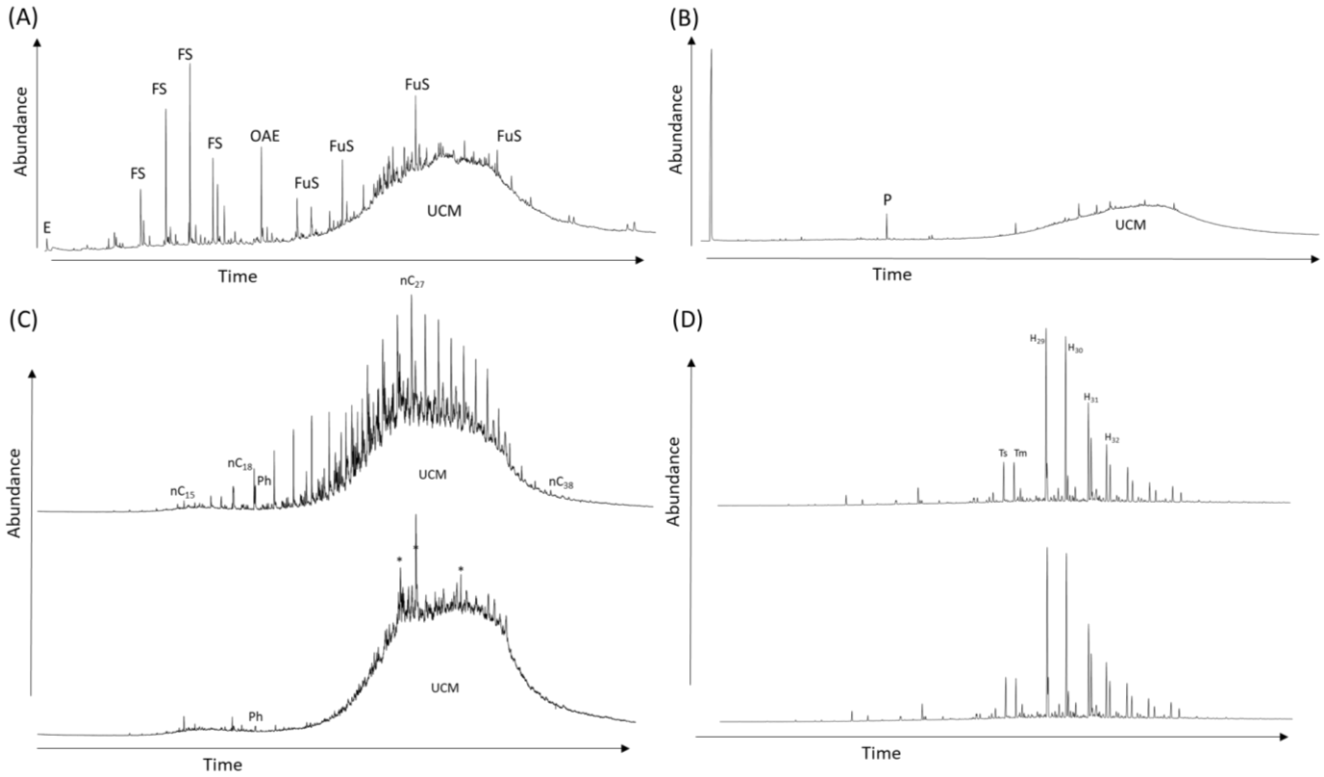


Figure 17. Total Ion Chromatograms (TIC) represented in 3.A. are 3.B. are showing representative F3 fraction fingerprints, (A): CT control initial; (B): VCBA treatment after 60 days. Note that most of the compounds identified in the control (oleochemicals) were fully degraded after 60 days, this process was accompanied with a remarkable reduction of the UCM thereby revealing an almost complete depletion of fraction F3. *E: epoxioctane, FS: Fatty acid (saturated) esters; OAE: Oleic acid ester; PUFA: Fatty acid (unsaturated) esters; P: plasticizer; UCM: Unresolved Complex Mixture.* SIM (Single Ion Chromatograms) showing representative F1 fraction fingerprints of CT control initial (top) and VCBA treatment after 60 days (down). While 3.C. shows the SIM chromatograms (m z^{-1}=57) of alkanes, and 3.D. SIM chromatograms (m z^{-1}=191) of hopanes. nC_n: Linear alkanes of 'n' carbon atoms; Ph: Phytane (branched alkane); *: Other branched alkanes; H_x: Hopanes (x is the carbon number); Ts: 18α(H)-22,29,30-trisnorhopane; Tm: 17α(H)-22,29,30-trisnorhopane; UCM: Unresolved Complex Mixture.

3.3.4. Changes in soil properties.

The most significant results on the measured soil parameters and their evolution during the incubation strategies are displayed in Table 6. Soil pH values did not change significantly in the control soil (CT) during the whole incubation period; however, clear acidification was observed in the rest of the treatments, especially during the first 15 days. This acidification is associated with the introduction of inorganic and organic nutrients in the case of BS and VC treatments, respectively, and could be related to increased CO_2 production due to OM mineralization. The second inoculation (BA and VCBA) on day 43rd only provoked a slight decrease in the pH values on day 45th as compared with the control soil (CT) or the vermicompost-soil mixture (VCT). Values of electrical conductivity (EC; Table 6) of the control soils, both with and without VC, displayed slight variations during the incubation. These values were lower than those observed for the samples subjected to biostimulation and bioaugmentation, reflecting the effect of nutrient additions. At the end of the incubation periods (before and after microbial consortia inoculation, i.e., days 0 and 43rd), a small drop in EC values was likewise observed due to the microbial consumption activity. Regarding the evolution of available P and K levels (Table 6), a different behaviour was displayed compared to the EC parameter. In this case, the application of 2% vermicompost increased by more than five and two times the amounts of available orthophosphate (P-Olsen) and exchangeable-K, respectively, as a direct consequence of the organic amendment addition. The nutrient level increase was observed between BS-BA and VCBS-VCBA. These treatments presented the highest values of exchangeable-K and available orthophosphate throughout the whole incubation, except for day 45th, where the latter's levels reached similar values in all the treatments after the nutrient's addition. Finally, the values of extractable organic C (EOC) and total extractable N (TEN), as a proxy to test the availability of C and N, are displayed in Table 7, respectively. Initial values of EOC were similar for all the treatments, being this particularly unusual in regolith soils, such is the case of the one used in this work, this being exclusively attributable to the presence of the soluble organic pollutants. For VCT samples, after an initial decrease in EOC observed during the first fifteen days, values remained consistently higher than in CT samples due to a probable priming

effect of the incubation conditions. This increase in EOC was probably due to the depolymerization of macromolecules and the concomitant release of soluble low molecular weight compounds. This effect was also responsible for the higher values of EOC in BS and BA treatments, with and without VC addition. For TEN, a different situation was observed, where the initial depletion in this soil (CT) was noted even after the VC addition (Table 7). The low amount of VC added (2% w:w), and its stability implied that most N was in organic forms, which is difficult to extract or quickly assimilated by soil biota. In contrast, the introduction of nutrient solutions was reflected in an initial increase of TEN, rapidly consumed during the first 30 days of incubation, showing a reduction of more than 30% in contrast to their initial contents. This effect disappeared after adding the second nutrient, revealing the inefficiency of introducing this surplus of nutrients in future experiments (Table 7).

Table 6. Impacts of applied treatments on the physico-chemical parameters of TPHs contaminated soil with reference to incubation intervals.

Treatments	Days	pH		EC		P		K	
		NV	V	NV	V	NV	V	NV	V
Control	2	7.58 ±0.12 ^{ab}	7.62 ±0.09 ^{a}	0.71 ±0.04 ^{a}	0.68 ±0.04 ^{c}	5.00 ±0.74 ^{a*}	23.71 ±3.37 ^{a*}	64.04 ±1.84 ^{a*}	146.09 ±5.20 ^{a*}
	15	7.53 ±0.04 ^{b*}	6.77 ±0.26 ^{d*}	0.73 ±0.02 ^{a}	0.75 ±0.02 ^{ab}	4.91 ±0.30 ^{ab*}	18.09 ±9.35 ^{ab*}	60.97 ±0.76 ^{ab*}	133.09 ±24.86 ^{a*}
	30	7.50 ±0.00 ^{b*}	6.89 ±0.03 ^{cd*}	0.74 ±0.02 ^{a}	0.75 ±0.03 ^{ab}	4.86 ±0.11 ^{ab*}	19.34 ±5.71 ^{ab*}	59.44 ±1.91 ^{bc*}	131.12 ±4.98 ^{a*}
	45	7.47 ±0.04 ^{b*}	6.99 ±0.41 ^{bc*}	0.75 ±0.02 ^{a}	0.77 ±0.01 ^{a}	4.82 ±0.21 ^{ab*}	14.65 ±5.04 ^{b*}	57.91 ±3.12 ^{bc*}	137.23 ±26.27 ^{a*}
	60	7.63 ±0.08 ^{a*}	7.13 ±0.17 ^{b*}	0.73 ±0.06 ^{a}	0.73 ±0.04 ^{ab}	4.60 ±0.59 ^{ab*}	19.74 ±1.06 ^{ab*}	60.30 ±2.39 ^{bc*}	142.44 ±6.30 ^{a*}
	90	7.67 ±0.07 ^{a*}	7.01 ±0.17 ^{b*}	0.71 ±0.04 ^{a}	0.71 ±0.04 ^{bc}	4.26 ±0.20 ^{b*}	22.35 ±2.59 ^{ab*}	56.81 ±3.04 ^{c*}	143.54 ±5.29 ^{a*}
Biostimulation	2	7.12 ±0.18 ^{a}	7.20 ±0.03 ^{a}	1.22 ±0.08 ^{c}	1.42 ±0.13 ^{b}	30.68 ±7.58 ^{b*}	56.11 ±4.58 ^{c*}	227.28 ±36.96 ^{c*}	358.85 ±34.63 ^{c*}
	15	6.58 ±0.06 ^{c}	6.58 ±0.08 ^{d}	1.30 ±0.05 ^{c}	1.41 ±0.08 ^{b}	25.35 ±0.85 ^{b*}	40.79 ±3.88 ^{d*}	243.48 ±12.46 ^{c*}	333.04 ±11.71 ^{c*}
	30	6.78 ±0.09 ^{b}	6.74 ±0.05 ^{c}	1.31 ±0.05 ^{c}	1.36 ±0.05 ^{b}	24.44 ±2.24 ^{b*}	45.67 ±4.39 ^{cd*}	254.41 ±18.00 ^{c*}	317.32 ±13.14 ^{c*}
	45	6.80 ±0.05 ^{b}	6.84 ±0.05 ^{b}	1.89 ±0.16 ^{a}	1.88 ±0.16 ^{a}	40.73 ±7.00 ^{a*}	87.02 ±18.65 ^{a*}	320.27 ±26.98 ^{b*}	488.77 ±47.90 ^{a*}
	60	7.11 ±0.04 ^{a}	7.25 ±0.04 ^{a}	1.73 ±0.12 ^{b}	1.92 ±0.10 ^{a}	47.14 ±5.58 ^{a*}	97.91 ±7.52 ^{a*}	353.84 ±9.75 ^{a*}	471.78 ±11.98 ^{ab*}
	90	6.99 ±0.07 ^{a*}	7.19 ±0.06 ^{a*}	1.74 ±0.04 ^{b}	1.78 ±0.08 ^{a}	47.80 ±5.04 ^{a*}	72.46 ±10.51 ^{b*}	358.04 ±5.98 ^{a*}	429.09 ±32.84 ^{b*}
Bioaugmentation	2	7.31 ±0.10 ^{a}	7.18 ±0.06 ^{a}	1.28 ±0.03 ^{c}	1.46 ±0.04 ^{b}	30.15 ±4.82 ^{c*}	76.81 ±13.32 ^{a*}	241.49 ±10.33 ^{c*}	375.27 ±21.28 ^{b*}
	15	6.60 ±0.04 ^{e}	6.56 ±0.02 ^{e}	1.30 ±0.09 ^{c}	1.44 ±0.06 ^{b}	21.39 ±0.51 ^{d*}	57.27 ±8.81 ^{b*}	246.51 ±11.34 ^{c*}	369.36 ±36.56 ^{b*}
	30	6.84 ±0.04 ^{d}	6.73 ±0.02 ^{d}	1.19 ±0.04 ^{c}	1.27 ±0.07 ^{b}	26.59 ±3.74 ^{cd*}	48.02 ±7.88 ^{b*}	238.56 ±10.50 ^{c*}	327.96 ±21.54 ^{c*}
	45	6.86 ±0.03 ^{d}	6.87 ±0.06 ^{c}	1.68 ±0.02 ^{b}	1.99 ±0.31 ^{a}	45.90 ±3.50 ^{b*}	77.83 ±5.64 ^{a*}	349.68 ±20.52 ^{ab*}	496.97 ±30.30 ^{a*}
	60	7.18 ±0.05 ^{b}	7.19 ±0.04 ^{a}	1.85 ±0.10 ^{a}	2.01 ±0.12 ^{a}	58.32 ±4.09 ^{a*}	74.31 ±5.40 ^{a*}	372.32 ±13.64 ^{a*}	497.39 ±28.45 ^{a*}
	90	7.10 ±0.01 ^{c}	7.07 ±0.03 ^{b}	1.70 ±0.10 ^{b}	1.85 ±0.09 ^{a}	47.70 ±5.61 ^{b*}	74.49 ±2.13 ^{a*}	344.22 ±26.27 ^{b*}	521.36 ±17.45 ^{a*}

In super script alphabets indicate significant difference between incubation days of different treatments (Control, biostimulation, bioaugmentation) with presence or absence of vermicompost, with "a" being highest followed by later alphabets, while * indicate significant difference with presence or absence of vermicompost at specific incubation time within the same treatment condition.

Table 7. Impacts of applied treatments and incubation on extractable organic carbon and nitrogen, and basal soil respiration of TPHs contaminated soil.

Treatments	Days	EOC[1]		TEN[1]		BSR[1]	
		NV	V	NV	V	NV	V
Control	2	2341.17 ±114.24 ^{a}	2331.01 ±71.94 ^{c}	27.82 ±0.22 ^{b*}	81.18 ±3.22 ^{a*}	0.54 ±4.29 ^{b}	0.69 ±0.19 ^{c}
	15	2249.15 ±61.17 ^{ab}	2119.95 ±47.94 ^{c}	22.66 ±0.19 ^{c*}	44.31 ±1.22 ^{b*}	0.50 ±2.02 ^{b*}	2.48 ±0.34 ^{a*}
	30	2203.14 ±40.09 ^{b}	2429.45 ±67.73 ^{b}	20.08 ±0.17 ^{cd*}	44.95 ±5.84 ^{b*}	0.48 ±0.95 ^{b*}	2.33 ±0.13 ^{a*}
	45	2157.13 ±33.94 ^{b*}	2491.95 ±74.60 ^{ab*}	17.50 ±0.16 ^{e*}	46.65 ±4.39 ^{b*}	0.46 ±0.59 ^{b*}	2.15 ±0.22 ^{a*}
	60	2217.72 ±97.37 ^{b*}	2561.68 ±46.40 ^{a*}	33.94 ±0.28 ^{a*}	47.15 ±2.57 ^{b*}	1.03 ±5.30 ^{a*}	2.19 ±0.17 ^{a*}
	90	2347.32 ±23.10 ^{a*}	2584.86 ±62.42 ^{a*}	27.22 ±1.84 ^{b*}	43.58 ±3.22 ^{b*}	0.78 ±0.26 ^{ab*}	1.77 ±0.31 ^{b*}
Biostimulation	2	2357.27 ±83.24 ^{c}	2473.96 ±94.53 ^{d}	1024.04 ±86.34 ^{b*}	839.28 ±78.49 ^{b*}	0.33 ±0.09 ^{e}	0.44 ±0.03 ^{d}
	15	2474.22 ±32.05 ^{c}	2389.59 ±27.68 ^{d}	841.40 ±27.66 ^{c*}	703.40 ±57.20 ^{c*}	17.64 ±1.15 ^{a*}	21.74 ±1.28 ^{a*}
	30	3157.26 ±168.32 ^{b}	2750.79 ±56.56 ^{c}	710.18 ±18.727 ^{d*}	575.01 ±33.52 ^{d*}	4.26 ±0.15 ^{b}	5.52 ±0.29 ^{b}
	45	3155.56 ±123.73 ^{b}	3009.84 ±112.01 ^{b}	1198.54 ±42.41 ^{a*}	1049.34 ±37.74 ^{a*}	3.16 ±0.13 ^{c}	3.20 ±0.87 ^{c}
	60	3353.40 ±89.55 ^{a}	3150.14 ±113.78 ^{b}	1256.01 ±8.33 ^{a*}	1073.69 ±64.19 ^{a*}	2.47 ±0.27 ^{d}	2.31 ±0.13 ^{c}
	90	3339.97 ±82.55 ^{a}	3333.55 ±154.72 ^{a}	1236.03 ±37.18 ^{a*}	1069.01 ±72.47 ^{a*}	1.77 ±0.16 ^{d}	2.45 ±0.59 ^{c}
Bioaugmentation	2	2197.27 ±59.66 ^{d*}	2532.44 ±55.81 ^{c*}	961.51 ±0.25 ^{b}	943.95 ±91.14 ^{b}	0.79 ±0.25 ^{f}	0.46 ±0.26 ^{e}
	15	2576.80 ±77.08 ^{c}	2555.47 ±113.93 ^{c}	789.35 ±0.33 ^{c}	774.34 ±50.16 ^{c}	17.84 ±0.33 ^{a}	17.36 ±1.31 ^{a}
	30	2696.98 ±57.38 ^{b*}	3029.71 ±106.40 ^{b*}	651.44 ±0.29 ^{d}	545.06 ±68.58 ^{d}	4.31 ±0.28 ^{b}	5.19 ±0.17 ^{b}
	45	2666.36 ±40.4 ^{b*}	3126.37 ±121.71 ^{ab*}	1194.71 ±0.01 ^{a}	1132.01 ±157.01 ^{a}	2.88 ±0.01 ^{c}	3.84 ±0.32 ^{c}
	60	2909.94 ±29.08 ^{a*}	3260.97 ±106.02 ^{a*}	1219.05 ±0.24 ^{a}	1234.27 ±101.89 ^{a}	2.47 ±0.23 ^{d}	2.46 ±0.45 ^{d}
	90	2926.73 ±52.63 ^{a*}	3258.03 ±94.61 ^{a*}	1164.64 ±0.27 ^{a}	1230.02 ±95.09 ^{a}	1.57 ±0.26 ^{e}	1.64 ±0.12 ^{d}

[1] EOC = Extractable organic carbon, TEN = Total extractable nitrogen, and BSR = Basal Soil Respiration.
In super script alphabets indicate significant difference between incubation days of different treatments (Control, biostimulation, bioaugmentation) with presence or absence of vermicompost, with “a” being highest followed by later alphabets, while * indicate significant difference with presence or absence of vermicompost at specific incubation time within the same treatment condition.

3.3.5. Evolution of biological parameters.

In order to complement and correlate the above-mentioned quantitative results on contaminants' degradation efficiency and soil parameters evolution, Basal Soil Respiration (BSR), enzyme profile activity, and PLFAs evolution were carefully analysed. During the first incubation (2nd day), all the samples displayed very low BSR values. In control samples (CT), these values remained constant during the whole incubation period, exhibiting a small increase only on day 45th, associated with the increase in soil humidity applied to reach 50% of the HWRC (Table 7). In contrast, the rest of the treatments evinced a substantial increase in BSR on day 15th, where VCBS samples displayed the highest values (nearly 50 times higher than the initial value), being growth lower for VCT (5 times higher than the initial value). The assimilation of the nutrients and the degradation of the most labile organic fractions, probably including low molecular weight aliphatic and aromatic hydrocarbons, could be associated with the increased values encountered for this parameter. On the other hand, despite the substantial increment in nutrients, such as the associated release of available N, P, and K, the second inoculation or nutrient addition was only responsible for a limited rise in BSR. The absence of labile organic fractions to support the microbial activity of a microbial consortium specialized in diesel degradation, as in the current study, could explain the observed lack of microbial growth. The evolution of soil microbial activity is complemented by assessing the evolution of the enzymatic activity profile for the samples at four different times: 2, 15, 45, and 90 days. The results are presented in Table 8. After 2 days of incubation, the soil enzyme profile for the control soil reflected the lowest microbial activity in the polluted soil. This profile did not significatively vary with the addition of the microbial consortium, but more significant changes were observed for BS and the samples with VC. In these samples, all activities were increased, mainly phosphatases (AlkPA but also AcPA), chitinases (bNAG), and proteases (LeuAMP), which supposed an increase in their catabolic capacities. This feature was maintained on day 15th, where a general increase in all the enzyme profiles was observed, and it definitively changed on day 45th, after the second inoculation. At this time, a marked increase of bNAG and, to a lesser extent, in bGA (mainly for BA, BS, and VCBA samples) was observed. This

enzyme profile was also observed at 90 days of incubation, except for AlkPA and bNAG activities, which showed a lower trend. This substantial increase of bNAG, one of the enzymes involved in the final process of chitin degradation, could be associated with the initial and severe fungal development observed during the first 15 incubation days and considering that the mycelia were mixed with the soil during the periodic soil aerations of microcosms (Table 8). The analysis of PLFAs, as an indirect determination of the soil microbial biomass, was carried out at 15 and 90 days of incubation. According to BSR results (Table 7) and the final composition of the bioremediation mixtures, these time points were selected as representatives of the highest picks in microbial activity. The results for total PLFAs (Figure 18.A) reflect a trend of the microbial biomass from the initial moments of the incubation, in which samples with vermicompost displayed the highest values of microbial biomass till the end of the incubation when BS and BA exhibited higher microbial numbers than the rest of the treatments. Comparing the different bacterial microbial groups (Figure 18.B, Figure 18.C, and Figure 18.D), Gram-positive bacteria (Figure 18.C) were predominant, with a higher prevalence in VCBA and VCBS at the beginning of the experience (15 days). On the contrary, for Gram-negative bacteria (Figure 18.D) and actinobacteria (Figure 18.E), results displayed higher values at the end of the experience than at the beginning in most of the cases, reflecting the changes in the microbial community happening along the incubation process, as a consequence of nutrient, consortium, and vermicompost additions. Fungal components (Figure 18.F) were higher in both BA and VCBA samples than in each respective control on day 15th; this proportion was the opposite at the end of the experience when a higher presence of these fungal components was observed in BA and BS samples.

Table 8. Enzymatic profile of TPHs containment soil with different applied treatments and incubation durations.

Treatments	Days	Control		Biostimulation		Bioaugmentation	
		NV	V	NV	V	NV	V
Acid phosphatase	0	13.56 ±0.28 ^{a*}	71.76 ±1.49 ^{a*}	23.02 ±0.48 ^{a*}	72.13 ±1.49 ^{a*}	20.49 ±0.42 ^{c*}	85.52 ±3.54 ^{a*}
	15	10.53 ±2.04 ^{ab*}	90.24 ±17.95 ^{a*}	22.2 ±4.31 ^{a*}	66.83 ±18.17 ^{a*}	43.04 ±10.65 ^{a*}	83.02 ±23.04 ^{a*}
	45	7.49 ±3.98 ^{c*}	69.65 ±10.2 ^{a*}	17.62 ±1.62 ^{a*}	67.06 ±28.5 ^{a*}	27.51 ±16.47 ^{ab*}	48.07 ±14.18 ^{b*}
	90	11.23 ±5.32 ^{ab*}	72.6 ±43.46 ^{a*}	25.44 ±11.04 ^{a*}	62.67 ±30.47 ^{a*}	13.3 ±4.87 ^{c*}	55.06 ±22.97 ^{b*}
α-glucosidase	0	1.43 ±0.03 ^{c*}	17.82 ±0.37 ^{c*}	2.36 ±0.05 ^{b}	14.99 ±0.31 ^{c*}	3.15 ±0.07 ^{c*}	11.50 ±0.48 ^{b*}
	15	5.05 ±2.05 ^{bc*}	108.12 ±15.87 ^{a*}	39.77 ±1.72 ^{b}	106.21 ±19.84 ^{b*}	89.43 ±21.06 ^{b*}	170.55 ±46.85 ^{a*}
	45	8.66 ±4.09 ^{b*}	60.45 ±13.65 ^{b*}	152.49 ±23.76 ^{a}	203.96 ±48.55 ^{a*}	132.05 ±38.19 ^{a*}	188.9 ±72.62 ^{a*}
	90	16.87 ±6.17 ^{a*}	47.2 ±10.72 ^{b*}	143.29 ±82.21 ^{a}	146.94 ±41.37 ^{b}	126.13 ±16.61 ^{a*}	196.65 ±24.64 ^{a*}
β-glucosidase	0	0.87 ±0.03 ^{b*}	3.91 ±0.08 ^{c*}	2.59 ±0.46 ^{a*}	4.57 ±0.09 ^{a*}	2.78 ±0.06 ^{a}	2.42 ±0.10 ^{a}
	15	0.89 ±0.03 ^{b*}	4.22 ±0.64 ^{c*}	2.65 ±0.47 ^{a*}	3.57 ±0.79 ^{a*}	1.92 ±0.86 ^{a*}	5.14 ±2.91 ^{a*}
	45	1.77 ±0.05 ^{a*}	6.64 ±2.06 ^{b*}	2.56 ±0.47 ^{a*}	4.93 ±1.98 ^{a*}	1.61 ±0.51 ^{a*}	3.38 ±1.58 ^{a*}
	90	1.39 ±0.69 ^{ab*}	10.64 ±1.51 ^{a*}	2.49 ±1.11 ^{a*}	4.68 ±1.88 ^{a*}	1.79 ±0.21 ^{a*}	4.18 ±1.27 ^{a*}
β-xylosidase	0	0.43 ±0.01 ^{a*}	5.89 ±0.12 ^{c*}	1.19 ±0.02 c*	7.98 ±0.17 ^{b*}	4.23 ±0.09 ^{b}	4.51 ±0.19 ^{c}
	15	1.05 ±0.66 ^{a*}	12.64 ±1.09 ^{a*}	9.11 ±2.06 ^{b*}	20.95 ±5.45 ^{a*}	11.08 ±2.34 ^{a*}	26.14 ±6.21 ^{a*}
	45	1.67 ±1.31 ^{a*}	10.34 ±3.12 ^{ab*}	13.25 ±1.36 ^{a*}	20.49 ±3.29 ^{a*}	11.02 ±2.16 ^{a*}	19.73 ±7.14 ^{ab*}
	90	2.34 ±1.12 ^{a*}	9.28 ±1.68 ^{b*}	9.62 ±4.4 ^{ab}	12.36 ±2.75 ^{b}	8.90 ±2.83 ^{a*}	12.55 ±2.68 c*
N-acetyl-β-glucosaminidase	0	2.86 ±0.06 ^{b*}	26.36 ±0.55 ^{b*}	2.59 ±0.05 ^{b*}	19.48 ±0.40 ^{n*}	5.60 ±0.12 ^{c*}	15.47 ±0.64 ^{c*}
	15	13.38 ±8.42 ^{b*}	161.91 ±11.38 ^{a*}	51.24 ±20.98 ^{b}	44.18 ±22.87 ^{b}	159.36 ±30.54 ^{b*}	137.43 ±76.32 ^{b*}
	45	23.9 ±16.83 ^{b*}	127.66 ±33.69 ^{a*}	553.91 ±59.10 ^{a*}	307.25 ±137.39 ^{a*}	621.91 ±112.39 ^{a*}	293.44 ±71.72 ^{b*}
	90	90.1 ±37.01 ^{a*}	138.71 ±35.07 ^{a*}	466.27 ±190.45 ^{*a}	316.96 ±116.45 ^{a*}	537.67 ±94.66 ^{a*}	419.85 ±53.38 ^{a*}
Sulfatase	0	0.65 ±0.03 ^{b*}	3.71 ±0.08 ^{a*}	1.54 ±0.27 ^{a*}	4.71 ±0.10 ^{a*}	2.66 ±0.06 ^{a}	2.91 ±0.12 ^{a}
	15	0.67 ±0.03 ^{b*}	3.93 ±0.55 ^{a*}	1.59 ±0.27 ^{a*}	3.12 ±0.63 ^{a*}	1.12 ±0.89 ^{b}	2.48 ±1.17 ^{a}
	45	1.33 ±0.06 ^{a*}	4.45 ±0.99 ^{a*}	1.60 ±0.65 ^{a*}	4.04 ±1.64 ^{a*}	1.32 ±0.24 ^{b*}	2.61 ±1.40 ^{a*}
	90	0.26 ±0.19 ^{c*}	3.44 ±1.42 ^{a*}	1.61 ±0.92 ^{a*}	4.36 ±1.70 ^{a*}	1.02 ±0.17 ^{b*}	3.56 ±1.13 ^{a*}
Alkaline phosphatase	0	8.99 ±0.19 ^{b*}	179.2 ±3.71 ^{b*}	31.32 ±0.65 ^{b*}	42.44 ±0.88 ^{b*}	12.18 ±0.25 ^{b*}	66.80 ±2.77 ^{c}
	15	50.95 ±35.37 ^{*a}	341.46 ±105.24 ^{a*}	93.02 ±45.41 ^{ab*}	125.54 ±28.58 ^{a*}	251.38 ±115.58 ^{a}	248.44 ±74.54 ^{a}
	45	92.90 ±70.6 ^{a*}	489.12 ±109.78 ^{a*}	92.53 ±8.49 ^{ab*}	164.42 ±62.07 ^{a*}	226.97 ±124.81 ^{a*}	161.93 ±54.41 ^{ab*}
	90	64.82 ±20.74 ^{a*}	372.87 ±144.3 ^{a*}	178.24 ±103.45 ^{a}	153.78 ±35.42 ^{a}	76.41 ±15.14 ^{ab*}	246.5 ±78.89 ^{a*}
Leucine aminopeptidase	0	7.48 ±0.15 ^{a*}	15.44 ±0.32 ^{b*}	31.29 ±0.65 ^{a*}	13.34 ±3.28 ^{a*}	6.96 ±0.14 ^{c*}	17.9 ±0.74 ^{a*}
	15	7.35 ±0.43 ^{a*}	34.68 ±4.65 ^{a*}	17.54 ±6.84 ^{b*}	25.35 ±6.88 ^{a*}	30.16 ±7.27 ^{a}	30.69 ±16.47 ^{a}
	45	7.23 ±0.86 ^{a*}	38.70 ±15.2 ^{a*}	17.60 ±2.86 ^{b}	19.22 ±6.86 ^{a}	21.24 ±6.94 ^{b}	16.41 ±7.67 ^{a}
	90	7.82 ±4.18 ^{a*}	26.81 ±6.96 ^{ab*}	16.83 ±6.77 ^{b}	22.38 ±5.57 ^{a}	15.83 ±4.76 ^{b}	21.38 ±11.44 ^{a}

In super script alphabets indicate significant difference between incubation days of different treatments (Control, biostimulation, bioaugmentation) with presence or absence of vermicompost, with "a" being highest followed by later alphabets, while * indicate significant difference with presence or absence of vermicompost at specific incubation time within the same treatment condition.

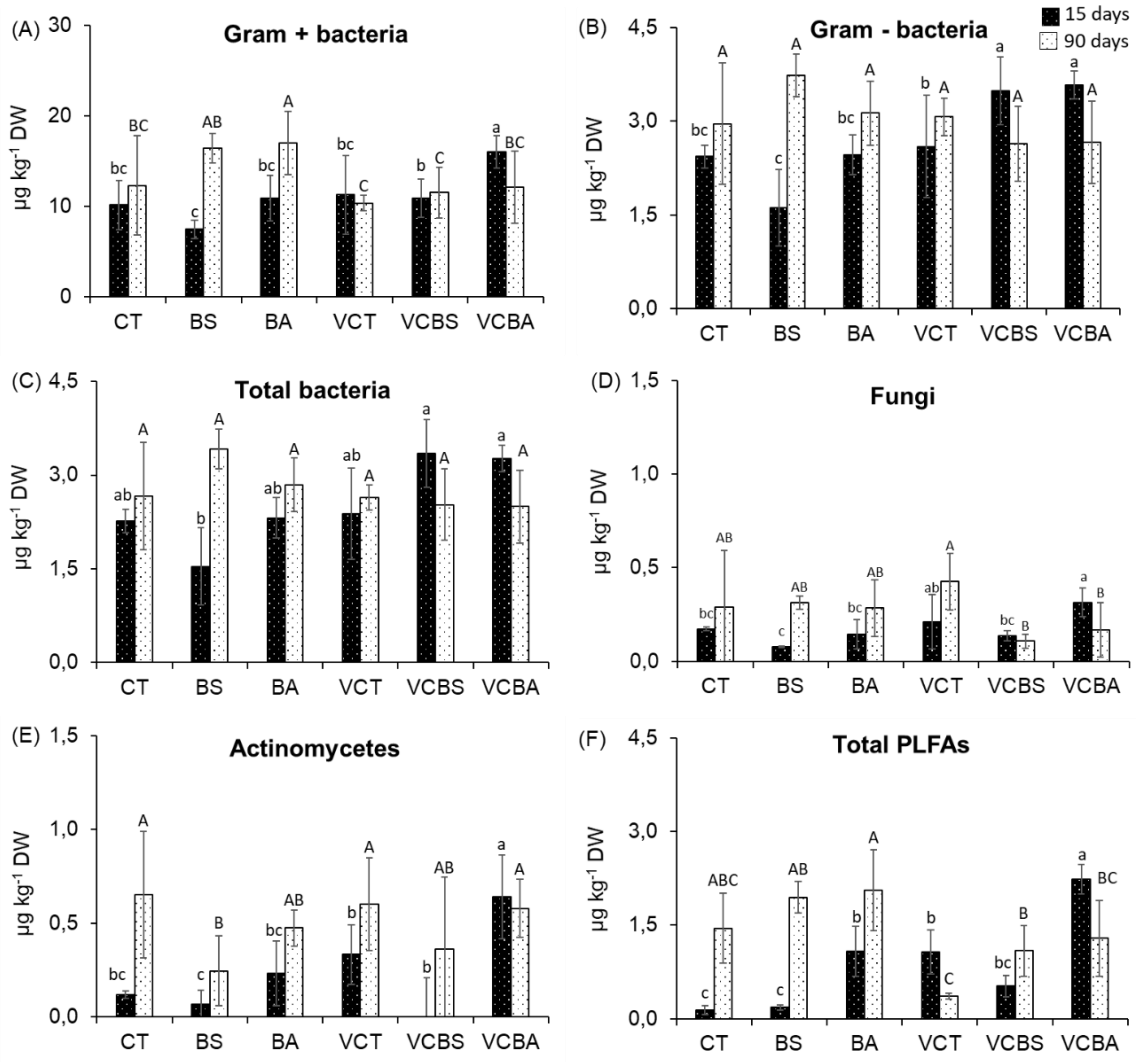


Figure 18. Changes in soil microbial community measured using PLFAs as biomarkers: (A) Gram+ bacteria, (B) Gram- bacteria, (C) Total bacteria, (D) Fungi, (E) actinomycetes and (F) Total PLFAs. Different letters (capital and small letters, for 15 and 90 days, respectively) displayed significant statistical differences between treatments at the two incubation times (15 and 90 days). One-way ANOVA, followed by Tukey's *post-hoc* test with significance defined at $p<0.05$. DW: Dry Weight.

3.4. Discussion.

Traditionally, three bioremediation strategies for TPHs pollution have been used: natural attenuation, biostimulation, and bioaugmentation (Khan et al., 2016b; Hussain et al., 2022). Natural attenuation, one of the most straightforward ways to treat soil pollution, uses the intrinsic degradation capability of the autochthonous microorganisms to degrade contaminants; however, it has a long period to achieve successful results due to

the low population size of indigenous degrading microorganisms, or the adverse soil physical-chemical conditions (Yousaf et al., 2022). In the present study, control samples did not significantly change TPHs concentrations, neither aliphatic nor aromatic fractions, even though soil incubation was carried out under optimal temperature, aeration, and available water for microbial growth. It illustrates the real and limiting possibilities of natural attenuation and the boundaries of its implementation to decrease TPHs' concentration below the established threshold values for polluted soils. In our case, TPHs remained 100 times over the values of generic reference levels, according to the Spanish legislation (Pinedo et al., 2013). PAHs were reduced, especially in the case of the most abundant compound detected in the sample, the phenanthrene, whose decrease was statistically significant both in VCBS and VCBA treatments in the presence of vermicompost, indicating the possible adsorption and sequestration mechanisms occurring in the matrix of the organic amendment mixed with the soil. Although more results are needed to understand PAHs alleviation in soils via bioremediation, this problem was not the main challenge to address in the current study since the original PAHs values were not high in terms of toxicity and threshold limits. The polluted soil used in this experience is a regolith more than a proper soil, as it is comprised of pickling layers of surface soils affected by diesel and oil spills in a machinery park. Organic matter was close to 4%, comprising the targeted organic pollutants and, consequently, is challenging to use as substrates for microbial growth. The contents of other vital nutrients, such as N or P, are depleted in this soil. Applying a BHB-culture media had a clear response in activating microbial activity. It was reflected in the increase in BSR observed in the BS treatment after 15 days of incubation. This increased microbial activity was also reflected in the soil enzyme profile, which displayed an increment in the activity of the APA and proteases, clearly linked to bacterial growth in the first days of the incubation period. The stimulation of microbial metabolism was correlated with the observed degradation of TPHs. In this work, a consortium isolated from the polluted soil was grown in a culture media in which diesel oil was added as a sole carbon source. The isolated consortium had similar features to a previously isolated consortium from another diesel-polluted soil (Garrido-Sanz et al., 2019), and its microbiome was dominated by bacterial species of the genus *Pseudomonas*, *Achromobacter*, *Cupriavidus*, *Comamonadaceae*, and *Sphingomonadaceae*. Metagenomic data identified redundant genes encoding enzymes

implicated in the initial oxidation of alkanes: alkane 1-monooxygenase (AlkB), *long-chain alkane monooxygenase* (*LadA*), *cytochrome P450 alkane hydrolase* (*CYP153* family), and a variety of *hydroxylating and ring-cleavage dioxygenases*, involved in aromatic and polyaromatic hydrocarbon degradation that assured an efficient degradation of complex mixtures, such our polluted soil (Garrido-Sanz et al., 2019). Our results displayed an increase in the degradation capacity of TPHs promoted by the specific microbial enrichment of the polluted soil. However, this was insufficient to degrade high molecular weight aliphatic and aromatic hydrocarbons. The immobilization of recalcitrant TPHs, such as branched aliphatic, PAHs and substituted aromatic hydrocarbons on some soil constituents like mineral clays or humified organic materials, reducing their bioavailability to soil microorganisms, is a critical limiting factor in the bioremediation of an aged and polluted soil (Hussain et al., 2022). In the present study, the microbial activity increased after compost and nutrient addition due to acidification of soil solution, resulting in more than one pH unit variation, which seems to be responsible for an essential effect in the solubility of P and other micronutrients and the predominance of different microbial groups. However, the second inoculation had a lower effect on soil respiration and nutrient consumption such as N and P. Many studies have already reported the effectiveness of bioaugmentation with microorganisms, either individually or in a consortium (Wu et al., 2017). However, adding different microbial communities does not always have an additive effect, and sometimes, microbial competition for soil resources or changes in nutrients' ratio (C:N:P) leads to microbial inhibition (Hussain el at., 2022; Khan et al., 2016ab). Degradation of hydrocarbons is often the result of a community-interacting microbial population, either structurally or functionally, and bioremediation's potential depends on these organisms' ability to adapt to new environmental conditions (Mishra et al., 2021). In this work, bioaugmentation and biostimulation increased soil TPHs' degradation capacity. Nevertheless, no statistically significant differences were observed with the biostimulation treatments either with or without vermicompost addition. A second inoculation or nutrient addition on day 43rd of the incubation did neither display an apparent effect in soil microbial activity; accordingly, it seemed like the second introduction of nutrients would be unnecessary with an evident accumulation of some of them, such as N and P. A comprehensive understanding of how bioremediation influences the diversity of the soil microbial

community is key to getting better insights into the behaviour and function of these populations and correlating this with pollutants degradation in every situation (Narendrula-Kotha & Nkongolo, 2017). PLFAs were used as valuable viable or active microbial biomass biomarkers in our study. These are membrane lipids rapidly metabolized and decomposed outside the cell, as demonstrated by (Lewe et al., 2021). The functional adaptation of the soil microbial community reflected in the dynamics of individual hydrocarbons was mirrored by structural adaptation reflected in PLFA dynamics expressed by a site-specific PLFA ratio (Narendrula-Kotha & Nkongolo, 2017). Mair et al. (2013) reflected the correlation between PLFA determinations and TPH degradation in a hydrocarbon-contaminated soil from an Alpine former military site, testing the effects of temperature and biostimulation. Their data demonstrated the suitability of PLFA analysis for profiling microbial communities in hydrocarbon-contaminated soils. Chen et al. (2015) showed that temperature significantly influenced fungal to bacterial PLFA ratios and Gram-positive to Gram-negative bacterial ratios in pyrene-contaminated soil bioremediation with compost. The PLFA pattern in several pans of a site contaminated with PAHs, in which landfarming with biostimulation and bioaugmentation was tested, also evinced the enhancement of Gram-negative Pseudomonas spp. at the end of the experience (George and Wan, 2020). Gram-positive bacteria were predominant in the current study, with a higher amount present in the treatment with vermicompost (VCBA and VCBS) at the beginning of the experience (15 days). Whereas fungal components were initially higher in both BA and VCBA, the trend changed at the end of the incubation, as fungal PLFAs increased in BS and VCBS. Gram-negative bacteria and actinobacteria groups were mainly in higher abundance at the end of the experience. Again, these results prove the variations of the microbial community's predominant groups in every situation. It depends on the treatment and remediation time. Petroleum components are classified into bulk groups of saturates, olefins, aromatics, resin (including a wide variety of compounds containing sulphur, oxygen, and nitrogen), and asphaltenes. Initial contamination presented a profile enriched in heavy linear alkanes suggesting a previous moderate weathering (Gallego et al., 2011). The SARA fractionation procedure is commonly used to identify which fractions of the polluted soil are degraded during the remediation experience. Despite the good microbial growth in respiration, enzymatic activity stimulation, and nutrient

consumption, results suggested that recalcitrant and hydrophobic petroleum compounds remained unchanged, revealing that mobility is linked to the bioavailability of these pollutants, the probable limiting step for soil recovery. The GC-MS study revealed a typical fingerprint of lubricant oils (Yang et al., 2016), including a mixture of aged hydrocarbons. Polar fractions, mainly composed of non-hydrocarbon oleochemicals, were in coherence with the activities in the study site (machinery park area) and rapidly consumed along with the experiments. Similar findings were reported by Soni & Agarwal (2014). Also, linear alkanes were depleted entirely, whereas branched alkanes such as isoprenoids and aromatics were moderately degraded in coherence with the reduction of medium-weight hydrocarbons observed in the quantitative study (Figure 16). Consequently, the predominant compounds were hopanes after the treatments, a recalcitrant heavy-weight group of cycloalkanes typically abundant in severely weathered and biodegraded samples (Gallego et al., 2011). Finally, volatilization and similar abiotic degradation and removal mechanisms can be discarded to have occurred during the microcosm incubations. A comprehensive study focusing on understanding the specific microbial species, biomass changes, and interactions involved in soil microbial remediation will be very interesting in identifying the potential functional microbial community. One such study was performed by Garrido-Sanz et al. (2019), in which they isolated and characterized the indigenous soil aerobic bacterial consortium growing on diesel as a sole carbon source. Garrido-Sanz et al. (2019) identified that the microbial consortium, using metagenomic analysis, capable of degrading hydrocarbon was primarily composed of *Pseudomonas*, *Aquabacterium*, *Chryseobacterium*, and *Sphingomonadaceae*. It is proposed that further research is needed for a better and more comprehensive understanding of the underlying mechanism of both BS and BA and to make better and more accurate informed decisions on implementing a given bioremediation strategy by considering not only the environment but also the rest of the inherent factors. While biostimulation might, in principle, result in better cost-effective options since the stage of cultivation of microorganisms is not necessary, more promising effects are a priori assigned when observing bioaugmentation results, mainly concerning the rate and time of degradation of HC, as derived from the current work.

3.5. Conclusions.

In general lines, it can be stated that both strategies have had significant improvements, as observed in the biodegradation rates. After long-term in situ natural attenuations, the main biodegradable contaminant fractions were depleted. From that initial situation, the treatments applied were able to eliminate the remaining bioavailable compounds (linear, alkanes, and polars) and deplete fewer biodegradable compounds (aromatics and branched alkanes), whereas recalcitrant and heavy hydrocarbon families (hopanes) remained intact. Although there have been remarkable differences between and among the biostimulation and bioaugmentation treatments, most of them are not sufficiently significant to discriminate and select one over the other, for instance, in the case of upscaling the strategy (like for bio-pile). Significant differences were observed in the microcosms treatments with the added vermicompost amendment, which can be a cost-effective technique to increase hydrocarbons' biodegradation when applied to large-scale bio-pile treatments. Similarly, using other co-application techniques also potentially speed up the hydrocarbon remediation process. These methods included the biofortification using microalgal biomass, amendments with soil conditioners, and other remediation methods (biosnorkeling, bioelectrokinesis, and mycoremediation). It will be interesting to study the evolution of microbial communities to design a functional hydrocarbon-degrading microbial pollution. Lastly, it will be important to quantify the level of soil toxicity followed by any remediation techniques adopted.

3.6. References.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology,* 37(8), 911-917. https:/doi.org/10.1139/o59-099

Chen, M., Xu, P., Zeng, G., Yang, C., Huang, D., & Zhang, J. (2015). Bioremediation of soils contaminated with polycyclic aromatic hydrocarbons, petroleum, pesticides, chlorophenols and heavy metals by composting: Applications, microbes and future research needs. *Biotechnology Advances*, 33(6), 745–755. https://doi.org/10.1016/j.biotechadv.2015.05.003

Das, D., & Deka, H. (2021). Vermicomposting of harvested waste biomass of potato crop employing *Eisenia fetida*: changes in nutrient profile and assessment of the maturity of the end products. *Environmental Science and Pollution Research,* 28, 35717-35727. https://doi.org/10.1007/s11356-021-13214-z

Gallego, J. L. R., Sierra, C., Permanyer, A., Peláez, A. I., Menéndez-Vega, D., & Sánchez, J. (2011). Full-Scale Remediation of a Jet Fuel-Contaminated Soil: Assessment of Biodegradation, Volatilisation, and Bioavailability. *Water, Air, & Soil Pollution*, 217, 197–211. https://doi.org/10.1007/s11270-010-0579-6

Garrido-Sanz, D., Redondo-Nieto, M., Guirado, M., Jiménez, O. P., Millán, R., Martin, M., & Rivilla, R. (2019). Metagenomic Insights into the Bacterial Functions of a Diesel-Degrading Consortium for the Rhizoremediation of Diesel-Polluted Soil. *Genes*, 10(6), 456. https://doi.org/10.3390/genes10060456

George, S. E. & Wan, Y. (2020). Advances in characterizing microbial community change and resistance upon exposure to lead contamination: Implications for ecological risk assessment. *Critical Reviews in Environmental Science and Technology*, 50(21), 2223-2270. https://doi.org/10.1080/10643389.2019.1698260

Hussain, F., Hussain, I., Khan, A. H. A., Muhammad, Y. S., Iqbal, M., Soja, G., Reichenauer, T. G., & Yousaf, S. (2018). Combined application of biochar, compost, and bacterial consortia with Italian ryegrass enhanced phytoremediation of petroleum hydrocarbon contaminated soil. *Environmental and Experimental Botany*, 153, 80-88. https://doi.org/10.1016/j.envexpbot.2018.05.012

Hussain, F., Khan, A. H. A., Hussain, I., Farooqi, A., Muhammad, Y. S., Iqbal, M., Arslan, & Yousaf, S. (2022). Soil conditioners improve rhizodegradation of aged petroleum hydrocarbons and enhance the growth of *Lolium multiflorum*. *Environmental Science and Pollution Research*, 29, 9097-9109. https://doi.org/10.1007/s11356-021-16149-7

Iqbal, A., Mushtaq, M. U., Khan, A. H. A., Nawaz, I., Yousaf, S., & Iqbal, M. (2020). Influence of *Pseudomonas japonica* and organic amendments on the growth and metal tolerance of *Celosia argentea* L. *Environmental Science and Pollution Research*, 27, 24671-24685. https://doi.org/10.1007/s11356-019-06181-z

Khan, A.H.A., Anees, M., Arshad, M., Muhammad, Y.S., Iqbal, M., & Yousaf, S. (2016b). Effects of illuminance and nutrients on bacterial photo-physiology of hydrocarbon degradation. *Science of The Total Environment*, 557-558, 705-711. https://doi.org/10.1016/j.scitotenv.2016.03.068

Khan, A. H. A., Ayaz, M., Arshad, M., Yousaf, S., Khan, M. A., Anees, M., Sultan, A., Nawaz, I., & Iqbal, M. (2019). Biogeochemical cycle, occurrence and biological treatments of polycyclic

aromatic hydrocarbons (PAHs). *Iranian Journal of Science and Technology, Transactions A: Science*, 43, 1393-1410. https://doi.org/10.1007/s40995-017-0393-8

Khan, A. H. A., Tanveer, S., Alia, S., Anees, M., Sultan, A., Iqbal, M., & Yousaf, S. (2017). Role of nutrients in bacterial biosurfactant production and effect of biosurfactant production on petroleum hydrocarbon biodegradation. *Ecological Engineering*, 104, 158-164. https://doi.org/10.1016/j.ecoleng.2017.04.023

Khan, A. H. A., Tanveer, S., Anees, M., Muhammad, Y. S., Iqbal, M., & Yousaf, S. (2016a). Role of nutrients and illuminance in predicting the fate of fungal mediated petroleum hydrocarbon degradation and biomass production. *Journal of Environmental Management*, 176, 54-60. https://doi.org/10.1016/j.jenvman.2016.03.040

Lewe, N., Hermans, S., Lear, G., Kelly, L. T., Thomson-Laing, G., Weisbrod, B., Wood, S. A., Keyzers, R. A., & Deslippe, J. R. (2021). Phospholipid fatty acid (PLFA) analysis as a tool to estimate absolute abundances from compositional 16S rRNA bacterial metabarcoding data. *Journal of Microbiological Methods*, 188, 106271. https://doi.org/10.1016/j.mimet.2021.106271

Mair, J., Schinner, F., & Margesin, R. (2013). A feasibility study on the bioremediation of hydrocarbon-contaminated soil from an Alpine former military site: Effects of temperature and biostimulation. *Cold Regions Science and Technology*, 96, 122–128. https://doi.org/10.1016/j.coldregions.2013.07.006

Marx, M. C., Wood, M., & Jarvis, S. C. (2001). A microplate fluorimetric assay for the study of enzyme diversity in soils. *Soil Biology and Biochemistry*, 33(12-13), 1633–1640. https://doi.org/10.1016/S0038-0717(01)00079-7

Mehlich, A. (1984). Mehlich 3 soil test extractant: A modification of Mehlich 2 extractant. *Communications in Soil Science and Plant Analysis*, 15(12), 1409–1416. https://doi.org/10.1080/00103628409367568

Mishra, S., Lin, Z., Pang, S., Zhang, W., Bhatt, P., & Chen, S. (2021). Recent advanced technologies for the characterization of xenobiotic-degrading microorganisms and microbial communities. *Frontiers in Bioengineering and Biotechnology*, 9, 632059. https://doi.org/10.3389/fbioe.2021.632059

Mushtaq, M. U., Iqbal, A., Nawaz, I., Mirza, C. R., Yousaf, S., Farooq, G., Ali, M. A., Khan, A. H. A., & Iqbal, M. (2020). Enhanced uptake of Cd, Cr, and Cu in *Catharanthus roseus* (L.) G. Don by *Bacillus cereus*: application of moss and compost to reduce metal availability. *Environmental Science and Pollution Research*, 27, 39807-39818. https://doi.org/10.1007/s11356-020-08839-5

Narendrula-Kotha, R., & Nkongolo, K. K. (2017). Bacterial and fungal community structure and diversity in a mining region under long-term metal exposure revealed by metagenomics sequencing. *Ecological Genetics and Genomics*, 2, 13-24. https://doi.org/10.1016/j.egg.2016.11.001

Palojärvi, A. (2006). Phospholipid Fatty Acid (PLFA) Analyses, in: Bloem, J., Hopkins, D.W., Benedetti, A. (Eds.), *Microbiological Methods for Assessing Soil Quality.* CABI International, Wallingford, UK, 204–211.

Pinedo, J., Ibáñez, R., Lijzen, J. P. A., & Irabien, Á. (2013). Assessment of soil pollution based on total petroleum hydrocarbons and individual oil substances. *Journal of Environmental Management,* 130, 72–79. https://doi.org/10.1016/j.jenvman.2013.08.048

Safdari, M. S., Kariminia, H. R., Rahmati, M., Fazlollahi, F., Polasko, A., Mahendra, S., Wilding, W. V., & Fletcher, T. H. (2018). Development of bioreactors for comparative study of natural attenuation, biostimulation, and bioaugmentation of petroleum-hydrocarbon

contaminated soil. *Journal of Hazardous Materials*, 342, 270–278. https://doi.org/10.1016/j.jhazmat.2017.08.044

Sima, N. A. K., Ebadi, A., Reiahisamani, N., & Rasekh, B. (2019). Bio-based remediation of petroleum-contaminated saline soils: Challenges, the current state-of-the-art and future prospects. *Journal of Environmental Management*, 250, 109476. https://doi.org/10.1016/j.jenvman.2019.109476

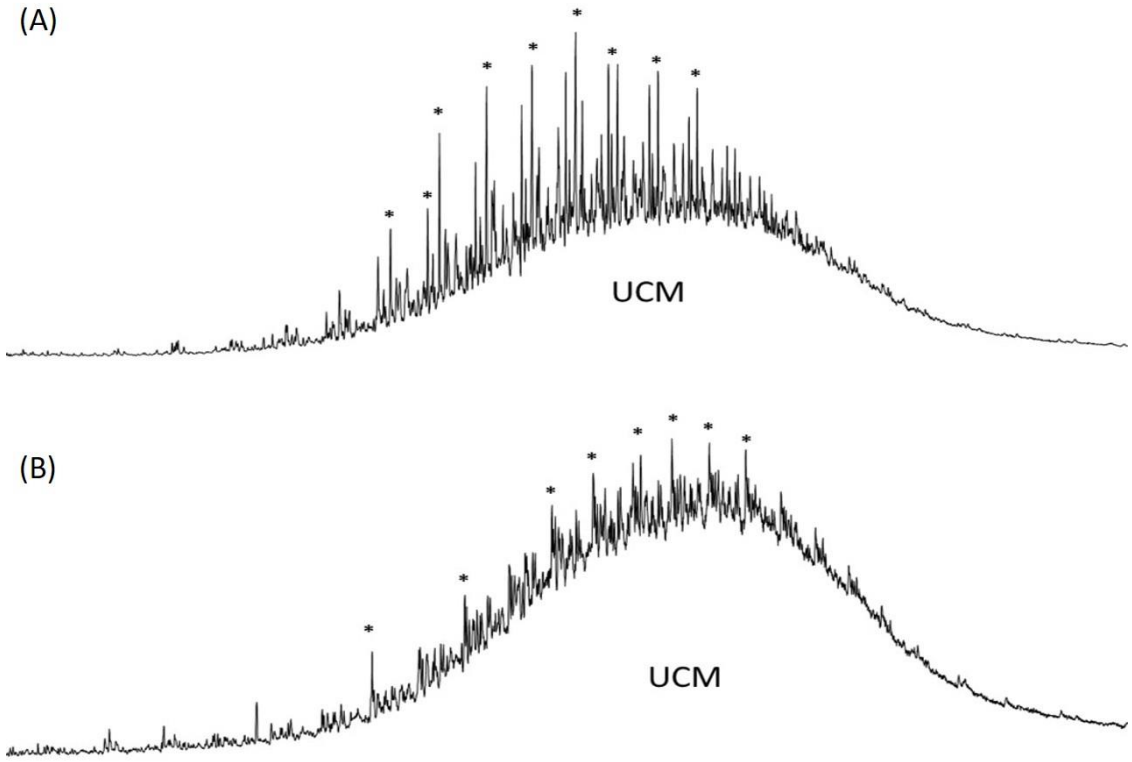
Soni, S., & Agarwal, M. (2014). Lubricants from renewable energy sources – a review. *Green Chemistry Letters and Reviews*, 7(4), 359-382. https://doi.org/10.1080/17518253.2014.959565

Wu, M., Li, W., Dick, W. A., Ye, X., Chen, K., Kost, D., & Chen, L. (2017). Bioremediation of hydrocarbon degradation in a petroleum-contaminated soil and microbial population and activity determination. *Chemosphere*, 169, 124–130. https://doi.org/10.1016/j.chemosphere.2016.11.059

Yang, C., Yang, Z., Zhang, G., Hollebone, B., Landriault, M., Wang, Z., Lambert, P., & Brown, C. E. (2016). Characterization and differentiation of chemical fingerprints of virgin and used lubricating oils for identification of contamination or adulteration sources. *Fuel*, 163, 271–281. https://doi.org/10.1016/j.fuel.2015.09.070

Yousaf, U., Khan, A. H. A., Farooqi, A., Muhammad, Y. S., Barros, R., Tamayo-Ramos, J. A., Iqbal, M., & Yousaf, S. (2022). Interactive effect of biochar and compost with *Poaceae* and *Fabaceae* plants on remediation of total petroleum hydrocarbons in crude oil contaminated soil. *Chemosphere*, 286, 131782. https://doi.org/10.1016/j.chemosphere.2021.131782

3.7. Supplementary material.



Supplementary Figure 1. IM (Single Ion Mode, m/z=133) chromatograms showing representative F2 fraction fingerprints. The comparison between the initial situation (A) and the degraded sample (B) of VCBA treatment after 60 days reveals a partial degradation of the most abundant alkyl-benzenes (labelled *) and an increase of the UCM (Unresolved Complex Mixture).

Supplementary Table 1. Concentration of 16 EPA PAHs from the initial contaminated soil.

16 EPA PAHs	mg kg^{-1} DW
Naphthalene	0.010 ± 0.000
Acenaphthylene	0.010 ± 0.000
Acenaphthene	0.010 ± 0.000
Fluorene	0.010 ± 0.000
Phenanthrene	0.047 ± 0.001
Anthracene	0.010 ± 0.000
Fluoranthene	0.016 ± 0.007
Pyrene	0.018 ± 0.003
Benzo(a)anthracene	0.010 ± 0.000
Chrysene	0.010 ± 0.000
Benzo(b)fluoranthene	0.010 ± 0.000
Benzo(k)fluoranthene	0.010 ± 0.000
Benzo(a)pyrene	0.010 ± 0.000
Dibenzo(ah)anthracene	0.010 ± 0.000
Benzo(ghi)perylene	0.010 ± 0.000
Indeno(123cd)pyrene	0.010 ± 0.000
TOTAL	**0.211**

CHAPTER 4

Unveiling the capacity of bioaugmentation application, in comparison with biochar and rhamnolipid for TPHs degradation in aged hydrocarbons polluted soil [2]

[2] Curiel-Alegre, S., Velasco-Arroyo, B., Martínez, A., Rumbo, C., Tamayo-Ramos, J. A., Khan, A. A., Rad, C., & Barros, R. (2022a). Application of a microbial consortium immobilized onto a biochar for the remediation of a polluted soil with hydrocarbons. *Revista de Ciências Agrárias*, 45(4), 295–299. https://doi.org/https://doi.org/10.19084/rca.28440
The extended version of the work has been submitted to the journal *Environmental Research* and is under revision (Ref. No. ER-23-15078).

4.1. Introduction.

Pollution with petroleum hydrocarbons is a serious environmental problem with worldwide effects (Haider et al., 2021; Yousaf et al., 2022). This pattern of influence seems no to be affected in the recent future, even today petroleum is the dominant energy source, with many dependent production activities contributing to the hydrocarbon spills, during the wide array of anthropogenic activities (Khan et al., 2016a). Soil contamination due to total petroleum hydrocarbons (TPHs) remains one of the most widespread forms of pollution, mainly affected by their weathering products (Hussain et al., 2022; Manzoor et al., 2016). Theses weathered and aged products persist in soil, due to increased hydrophobicity, linked with the increased adsorption to soil microscopic pores, with reduction photo-biotransformation and volatilization of TPHs (Khan et al., 2018).

Therefore, to counter the TPHs induced menace, numerous studies focused to decontaminate the TPHs affected areas, looking for effective and economically viable remediation techniques. Thus, it is important to take advantage of the capabilities of natural microorganisms to degrade oil (Ellis et al., 2022). The use of microorganisms for this purpose is the foundation of bioremediation, which represents an economical, environmentally friendly, and low-cost approach compared to other traditional remediation methods (Shahsavari et al., 2019). Bioremediation, based on bioaugmentation and biostimulation approaches, is a promising strategy to remediate contaminated soils (Saeed et al., 2022; Udom et al., 2023). It is based on the use of living microorganisms to break down and metabolize hydrocarbons into non-toxic substances (upon complete mineralization into carbon dioxide and water). This process can occur naturally through native soil microorganisms; however, it can also be facilitated by adding specific bacterial or fungal strains to contaminated soil that assist in the degradation of contaminants (Khan et al., 2019; Khan et al., 2016b). Bioremediation depends on the pollutant type and bioavailability, as well as the indigenous microbial community having capacity for bioremediation residing in the contaminated natural environment (Shahsavari et al., 2019). Nevertheless, oil contaminated soils are generally reported to have low indigenous microbial diversity, low porosity, few nutrients, and

reduced moisture due to hydrophobicity, all these factors limiting the degradation efficiency (Zhang et al., 2019).

The TPHs biodegradation can be improved by adding strong adsorbents, such as activated carbon (AC) and biochar, right at the time of contamination, as they link hydrophobic compounds to strong adsorbing sites, avoiding environmental dispersion of TPHs and decreasing exposure risks (Igun et al., 2019). However, it is not always possible to treat the contaminated area at the time of contamination; sometimes it takes years before it can be treated. Therefore, numerous studies now indicate that pyrolyzed agricultural wastes can be used to immobilize microorganisms that are capable of degrading pollutants, that can be used as the microbial population micro-factories, providing habitat to hydrocarbon resistant and degrading microorganism, in such a harsh environment (Lu et al., 2021; Zhang et al., 2019). Biochar is the solid product of biomass pyrolysis that is obtained from the conversion of organic materials through thermochemical processes (Zahed et al., 2021). The use of biochar as an immobilization carrier for biotechnological soil applications is already prospered and established (Saravanan et al., 2023; Bolan et al., 2023). During pyrolysis organic materials are heated to a temperature range of 250 to 850 °C in the absence of oxygen or in the presence of a limited amount of oxygen (Zahed et al., 2021). Depending on the process parameters of biochar production, specific surface area increases and contributes to the adsorption capacity of pollutants (Ding et al., 2017). Another amendment can be biosurfactants; as they are biological origin amphiphilic compounds with a hydrophilic (amino acids or peptides, disaccharides or polysaccharides, anions, or cations) and a hydrophobic (saturated or unsaturated fatty acids) moiety that could be used to increase the degradation of oil contaminants (Bera et al., 2021). Special attention should also be given to rhamnolipids, which are glycolipid biosurfactants with a potential effect on the dissolution, bioavailability, and biodegradation of a wide range of contaminants in soils (Taccari et al., 2012).

In this work, an aged, polluted soil with significant levels of long-chain refractory hydrocarbons (C21-30), due to prolonged natural attenuation over years, and the presence of potentially harmful metal and metalloids, was used to study the impacts of

new bioaugmentation approaches, by adding two types of biochar as microbial immobilization carriers and a commercial rhamnolipid biosurfactant to increase hydrocarbon bioavailability. For 90 days, eight different approaches have been evaluated at a small-scale microcosm, including natural attenuation as a control, biostimulation using an improved nutrient formulation, biostimulation using two types of pyrolyzed biochar (BH1 and BH2), and rhamnolipids (RML), and the same approaches but with bioaugmentation. For this purpose, and as a part of the objectives of the European GREENER project (https://www.greener-h2020.eu/en/normal/home), a microbial consortium was isolated from the native microbial population of the soil, characterized, cultured, and therefore inoculated in the four treatments in which bioaugmentation was implemented.

4.2. Materials and methods.

4.2.1. Chemicals and reagents.

Aliphatic hydrocarbons: Alkanes-Mix 12, 100 µg mL-1 in toluene (C_7H_8), C8-C40 (pair no. of C, 17 HC) and polyaromatic hydrocarbons: PAHs-Mix 9, 100 µg mL-1 in cyclohexane (C_6H_{12}); 16 PAHs (EPA Method 610: acenaphthene, acenaphthylene, anthracene, benzo(*a*)anthracene, benzo(*a*)pyrene, benzo(*b*)fluoranthene, benzo(k)fluoranthene, benzo(*g,h,i*)perylene, chrysene, dibenzo(*a,h*)anthracene, fluoranthene, fluorine, indeno(*1,2,3-c,d*)pyrene, naphthalene, phenanthrene, and pyrene at 100 mg mL^{-1}) were purchased for chromatographic separation by LGC Standards Ltd (Teddington, UK). The certified reference materials CRM-357 (TPH–sandy loam), CRM-359 (TPH–clay) were acquired from Sigma-Aldrich (Burlington, Massachusetts, USA). Paraformaldehyde was used for scanning electron microscopy, produced by Sigma-Aldrich (P6148).

4.2.2. Soil description.

Contaminated soil samples were obtained from a machinery park in Noblejas (Toledo, Spain), at N 39° 58' and W 3° 24'. The soil under study was contaminated with a

mixture of hydrocarbons, mineral oils, and heavy metals. Once the samples were collected, they were placed in clean, labelled containers and transported to the laboratory. Samples were air-dried and sieved at 2 mm for physicochemical characterization, using methods described Subsection 4.2.7 - Analytical methods. The results obtained for the initial soil were showed in Table 9.

Table 9. Basic properties of incubated soil at the beginning of the experience.

Basic properties	Incubated Soil
Humidity (%)	0.47
Field capacity (%)	20.60
Bulk density (g cm^{-3})	1.6830
Total carbon (%)	6.4477
Total nitrogen (%)	0.0213
Electrical conductivity (dS m^{-1})	0.801
pH (1:5 w/v H_2O)	7.18
pH (1:5 w/v KCl)	7.39
Organic matter (%)	4.95
Clay (%)	11.8
Silt (%)	29.8
Sand (%)	58.3
N-NO_3 (mg Kg^{-1})	0.15
N-NH_4 (mg Kg^{-1})	2.9900
P-PO_4 (mg Kg^{-1})	0.1033

4.2.3. Microbial consortia.

The microorganisms used in this study were isolated from the contaminated soil, using the protocol outlined by Garrido-Sanz et al., (2019) and Curiel-Alegre et al., (2022). Briefly, the microbial consortium was pre-inoculated in minimal medium (0.1 g L^{-1} NaCl, 0.1 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1g L^{-1} K_2HPO_4, 0.5 g L^{-1} KH_2PO_4, and 1 g L^{-1} $(NH_4)_2SO_4$), supplemented with phosphate-buffered mineral medium salts (PAS) (2.5-times concentrated, 19.5 g L^{-1} $MgSO_4$, 5 g L^{-1} $KMnO_4.H_2O$, 1 g L^{-1} $FeSO_4.H_2O$, and 0.3 g L^{-1} $CaCl_2$), 0.05% pre-filtered yeast extract (0.2 µm), 1% diesel, and incubated at 30 °C for 24 h at 180 RPMs. With the aim of studying the effect of microbial consortia on the hydrocarbon degradation, and the interaction between the organisms and the

biochar (BH1 and BH2) or rhamnolipids (RML), the above protocol continued as detailed below.

To know how the pool of bacteria and biochar/rhamnolipids, individually or altogether, enhance the hydrocarbon degradation the suspension was pelleted (10 min at 2,000 g) and diluted 1:100 for grew in 200 mL of minimal medium for 4 days. Once the pellets had been obtained from the pre-inoculum, it was resuspended in a modified Bushnell Haas Broth solution (BHB2). A final concentration of approximately 10^{11} CFU kg^{-1} soil was applied. This procedure was repeated twice just to perform two inoculations, on days 0 and 43. On the other hand, to analyse the interaction between bacteria and BH/RML, the flasks were then centrifuged at 4,500 rpm for 10 min and diluted 1:100 again in 1,000 mL minimal medium and growth at 180 rpm 30 °C for 4 days. The culture (2.5 mL, 10^7 CFU per mL) was mixed with each BH and the RML (concentration equivalent to 1 gram of soil). After 24 h at 100 rpm, it was procedure the FTIR-ATR analysis.

4.2.4. Nutrient solution.

A modified version of BHB2 solution was prepared for both biostimulation and bioaugmentation purposes according to Curiel-Alegre et al. (2022). To achieve the desired nutrient levels, individual solutions were mixed, resulting in the final following amounts (g kg^{-1} dry soil): $MgSO_4$ 0.0612, $CaCl_2$ 0.0061, KH_2PO_4 0.3058, K_2HPO_4 0.3058, NH_4NO_3 3.5425, and $FeCl_3$ 0.0153, the latest was added separately to prevent precipitation.

4.2.5. Organic supports and additives.

Two types of biochar both obtained from apricot stones by pyrolysis at different temperatures, were provided and characterized by the AIT Austrian Institute of Technology GmbH: Biochar 1 at 450 °C (BH1) and Biochar 2 at 650 °C (BH2). BH1 elemental composition was: 1.36% N, 74.13% C, 3.03% H, 0.03 % S; Specific Surface Area (BET) 26.03 ± 0.41 m^2 g^{-1}; EPA16-PAHs 0.027 mg kg^{-1} dry matter. BH2 elemental composition: 1.04% N, 83.50% C, 1.81% H, 0.00% S; Specific Surface Area (BET) 6.73 ±

0.19 m^2 g^{-1}; EPA 16-PAHs 0.032 mg kg^{-1} dry matter. Commercial rhamnolipids were purchased from Sigma Aldrich.

4.2.6. Experimental design for the microcosms.

Different soil mixtures were prepared for the different treatments adding 5% (w:w) of biochar (BH1 and BH2) or 1 % (w:w) rhamnolipids, nutrient solution (BHB2), and inoculum and water was added to achieve a moisture content of 40% of the water retention capacity (WRC), using a concrete mixer. On day 43, when the second consortia inoculation was conducted, the WRC was increased to 50%, utilizing this volume to incorporate water, the inoculum, or the nutrients solution. Eight treatments were stablished: control soil (abbreviated as CT), soil with BH1, soil with BH2, soil using RML as carriers, and these same treatments with the addition of the microbial consortium suspended in BHB-solution 2 (BACT, BABH1, BABH2, and BARML). The CT was a control for natural attenuation effects on the bioremediated soil.

Approximately 200 g (dry basis) of soil mixtures were weighed and transferred to 1 L hermetic containers. Sampling was carried out at day 0 (T0), 2 (T1), 15 (T2), 30 (T3), 45 (T4), 60 (T5), and 90 (T6) days of treatment. Three experimental replicates were introduced with a total of 144 soil microcosms, considering all sampling points and treatments. Each microcosm was opened twice a week to check moisture and was manually mixed to improve soil aeration. The containers were incubated in a chamber at 22 °C (± 0.5 °C) in the dark. At each sampling point, one part of the soil was dried in an oven at 30 °C for 48 hours, and the other part was immediately frozen at - 20 °C until microbial analyses were done.

4.2.7. Analytical methods.

4.2.7.1. Soil physical and chemical analysis.

The physicochemical characteristics of the soil were carried out as for the initial soil, using standard analytical methods, whereby all treatments were analysed at the different sampling times to determine the evolution of their properties. A pH

meter (GLP21, Crison) was used to determine the pH of soil through 5 g of sample and 25 mL of water (1:5, w:v) stirred at 60 RPMs for 30 min. Subsequently, the sample was centrifuged at 2,000 g for 20 min, filtered and measured in a conductivity meter (GLP31, Crison) to determine the electrical conductivity (EC). Nutrients were analysed in an autoanalyzer (San++ Skalar, Beda); available P was extracted from 2 g of soil with 40 mL of 0.5 M $NaCO_3$ at pH 8.5 which was shaken at 60 RPMs for 30 min, filtered, analysed and quantified by the molybdenum blue method for orthophosphate; nitrate and soluble ammonium were extracted from 10 g of soil with 40 mL of 0.5 M K_2SO_4 stirred at 60 RPMs for 45 min, filtered, analysed and quantified nitrate by Griess reaction after reduction on Cu-Cd column, and ammonium by the indophenol blue method. This extract was also used to analyse organic C and total N using the TOC-V CSN autoanalyzer (Shimadzu). Using an ICP-OES (Genesis, Spectro), nutrients and trace elements were determined from extracts of 2 g of soil with 20 mL of the extractant solution (0.2 M CH_3COOH, 0.001 M EDTA, 0, 013 M HNO_3, 0.015 M NH_4F, and 0.25 M NH_4NO_3) corresponding to Mehlich method 3, was shaken at 120 RPMs for 5 min, centrifuged at 2,000 g for 10 min and filtered prior to analysis. As the main objective of this manuscript is TPHs degradation, while the HMs were analysed, no significant changes in the levels were observed from the initial to final HMs content in soil, however the information regarding HMs was presented in Supplementary Table 1. Basal soil respiration (BSR) was quantified by 0.1 M HCl using an automatic titrator (718 Stat Titrino, Metrohm). 20 g of frozen soil was tempered for 72 h at 22 ± 0.5 °C, then placed in a 1 litre jar with a tight seal together with a beaker containing 4 mL of 0.5 M NaOH, which acted as a gas trap, and incubated for 24 h. Then 2 mL of 0.5 M $BaCl_2$ was added, causing precipitation of CO_2 adsorbed by barium carbonate, and finally the remaining NaOH was quantified.

4.2.7.2. Scanning Electron Microscopy.

Scanning electron microscopy (SEM) was used to visualize the bacterial adhesion onto the biochar. The consortium growth and subsequent incubation with both forms of biochar were carried out as explained above. After the samples had been

incubated, they were centrifuged at 2,000 *g* for 10 minutes, rinsed in phosphate-buffered saline without calcium and magnesium (DPBS), and fixed for 30 minutes in 4% (v:v) paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. After fixation, samples were washed twice with DPBS, and dehydrated for 10 minutes in 50, 70, 90, and 100% (v:v) ethanol. Finally, the samples were coated with a gold layer and analysed in a scanning electron microscope SEM (FEI-Quanta 200F), in collaboration with "Parque Científico Universidad de Valladolid".

4.2.7.3. Fourier Transform Infrared Spectroscopy - Attenuated Total Reflectance (FTIR-ATR).

The bacterial consortium was concentrated 24 times in BHB medium to expose it to BH1, BH2, and RML at 1% soil concentration for 24 h at 100 rpm room temperature (final volume 2.5 mL). The supernatant was removed and washed three times with phosphate-buffered saline (PBS) after shaking 5 minutes. Before associating bacterial consortium growth to biochar and rhamnolipids by studying their functional groups, the samples were ground and lyophilised. The functional groups present in the samples were studied by Fourier transform infrared spectroscopy - attenuated total reflectance (FTIR-ATR), which can be useful to elucidate the kind and abundance of chemical compounds derived from xylochemical (Groß et al., 2021) in the studied biochars, together with the changes in composition at different pyrolytic temperatures and the microorganism association. The spectra were recorded in the 4000-400 cm^{-1} region, with a spectral resolution of 4 cm^{-1} and 128 scans per spectrum, by a JASCO FT-IR 4200 spectrophotometer with an Attenuated Total Reflection ATR PRO ONE device. Graphics were depicted by Kaleidagraph v4.1.1 Synergy Software, (2010).

4.2.8. Statistical analysis.

For the variables analysed in this study, the mean and standard deviation of at least three separate experimental replicates were calculated. The data of the different treatments and times were analysed by one-way ANOVA with various levels of significance, after checking for normality and homoscedasticity assumptions, using

Prism 8.0 (GraphPad). Further to study and identity the statistically significance for to assess whether the applied variable (BA, BH1, BH2, and RML) have major effect with reference to varying time and is there any interaction among the applied variable, two-way ANOVA was applied. Network analysis was performed in JASP (v 0.18.1.0), where the graphs created positioned nodes (soil characteristics and concentration of TPHs) and edges (interrelationships) to reflect their strength of connection. The explanation for this network analysis was inferred with the findings in two-way ANOVA.

4.3. Results.

4.3.1. Soil and organic amendments characterization.

Basic properties of the soil under study can be seen in Table 9. The main soil properties that influence interactions with organic compounds, such as hydrocarbons, are pH, particle size distribution and organic matter content were analysed. In addition, the total petroleum hydrocarbons (TPHs) were 4,275 mg kg^{-1} for the initial soil used to prepare the microcosms.

4.3.2. Microcosms properties evolution.

Soil pH values increased throughout the incubation for treatments in which the microbial consortium was not added (reaching to 7.84, 7.82, 7.85, 7.76 for CT, BH1, BH2, and RML, respectively), whereas for those in which the consortium was added (BACT, BABH1, BABH2, and BARML) soil pH decreased and remained at 6.56 ± 0.06 (Figure 19.a). This decrease in the bioaugmentation treatments may have been favourable for the solubility of nutrients, such as P and other trace elements. Electrical conductivity (EC) results did not show significant differences throughout the incubation (Figure 19.b). Slight variations appeared for the bioaugmentation treatments, except for BABH1, in which EC values increased with the addition of the microbial consortium and a decrease was observed over time due to higher microbial consumption in the soil.

The extractable organic C (EOC) showed a different behaviour depending on the treatment as shown in Figure 19.c, although the greatest differences were observed in the bioaugmentation treatments. In the BACT and BABH1 treatments, a slow increase was observed, during incubation, and BABH2 reached a significant increase at day 45 probably, due to the second inoculation the previous days. However, in the RML and BARML treatments, a larger decrease was observed in the BARML treatment, compared to RML, during incubation, due to the additional degradation produced by enriching the rhamnolipids with the microbial consortium.

Extractable total N (ETN) were affected in the samples with the bioaugmentation treatment (BA) compared to the controls (CT, BH1, BH2, and RML) without addition of microbial consortium in which there were hardly any variations, as shown in Figure 19.d. For the bioaugmentation treatments, it was higher due to the addition of the nutrient solution, decreasing during the first days of incubation, and increasing later, since microorganisms could not extract it easily at the beginning of the experience, but were more able to assimilate it when changing the soil properties, such as pH. In the evolution of available P levels, an increase was observed after both inoculations, due to the addition of the nutrient solution together with the bioaugmentation, however, decreasing slightly a few days after inoculation, due to nutrient consumption by soil microorganisms and remaining stable, thereafter as shown in Figure 19.e. This consumption is higher for the bioaugmentation treatments because the net nutrient consumption is higher, when the soil microorganisms increase, and in turn the microbial growth of the soil microorganisms increases. It should be noted that in the second inoculation, no significant effect was observed for either the biostimulation treatments or bioaugmentation treatments.

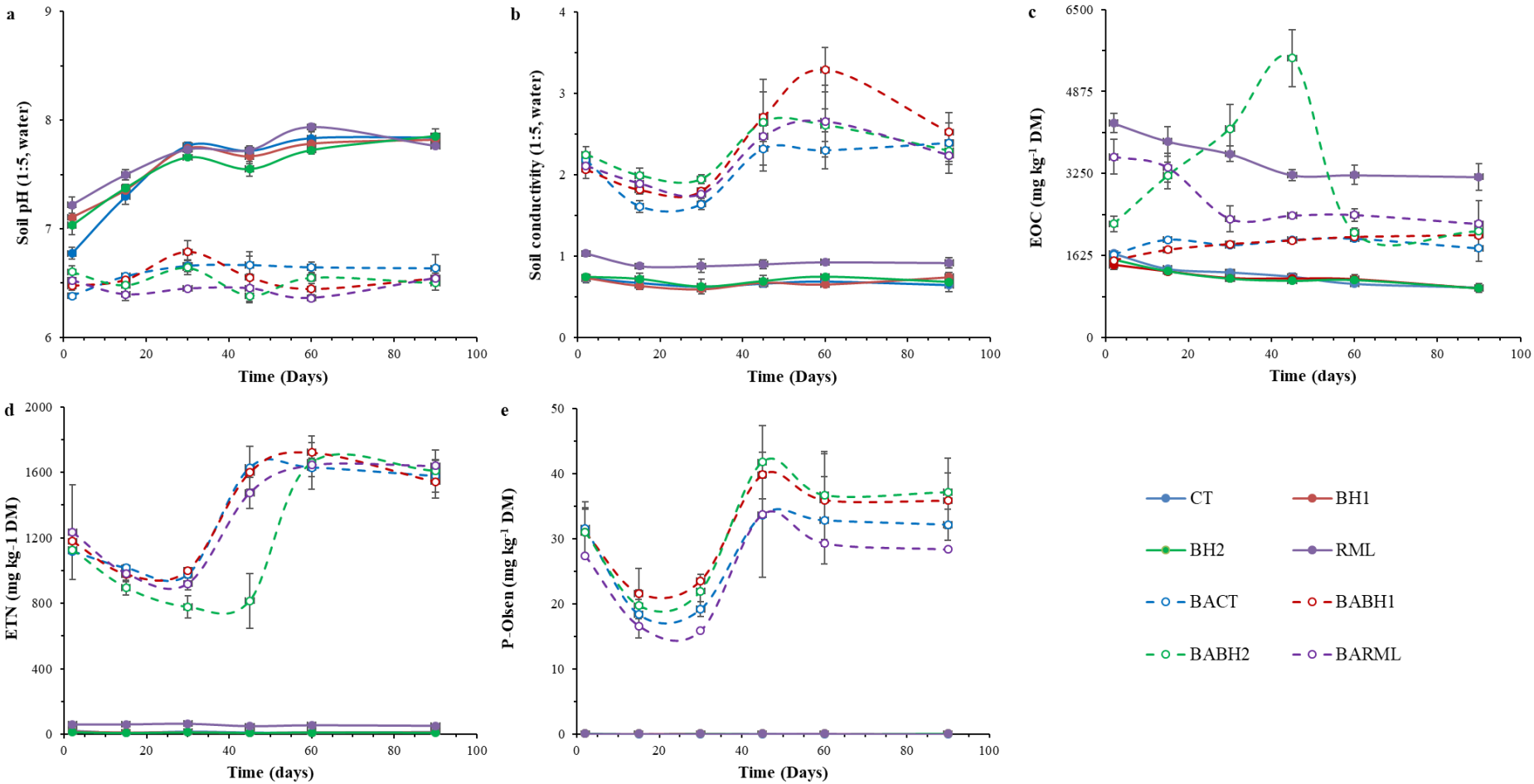


Figure 19. Evolution of chemical soil properties during incubation: (a) pH; (b) Electrical conductivity; (c) Extractable organic carbon; (d) Extractable total nitrogen; and (e) Available P. Treatments: CT, control soil, BH1, soil and 5% biochar 450 °C, BH2, soil and 5% biochar 600 °C; RML, soil and 1% rhamnolipids; BACT, bioaugmented control soil; BABH1, bioaugmented soil and 5% biochar 450 °C; BABH2, bioaugmented soil and 5% biochar 600 °C; BARML, bioaugmented soil and 1% rhamnolipids. Mean values ± standard deviation.

4.3.3. Biological parameters evolution.

Basal soil respiration (BSR) showed a considerable increase during the first 15 days of incubation in the bioaugmentation treatments, due to the increase in both soil and added microbial activity. This increase in basal soil respiration was not the same during the second inoculation even though additional nutrients were also added, perhaps because the assimilable nutrients in the initial soil had already been consumed by the microorganisms, or due to the lack of available hydrocarbons that could be used as a carbon source. These effects are shown in Figure 20. The control samples (CT, BH1, and BH2) did not change throughout the incubation, and a possible initial inhibitory effect could be shown in the BH1 and BH2 treatments. However, in RML the basal soil respiration increased slightly in both inoculations, showing an increase in the following days. This was not a major effect, but it allowed maintaining a constant respiration throughout the whole experiment.

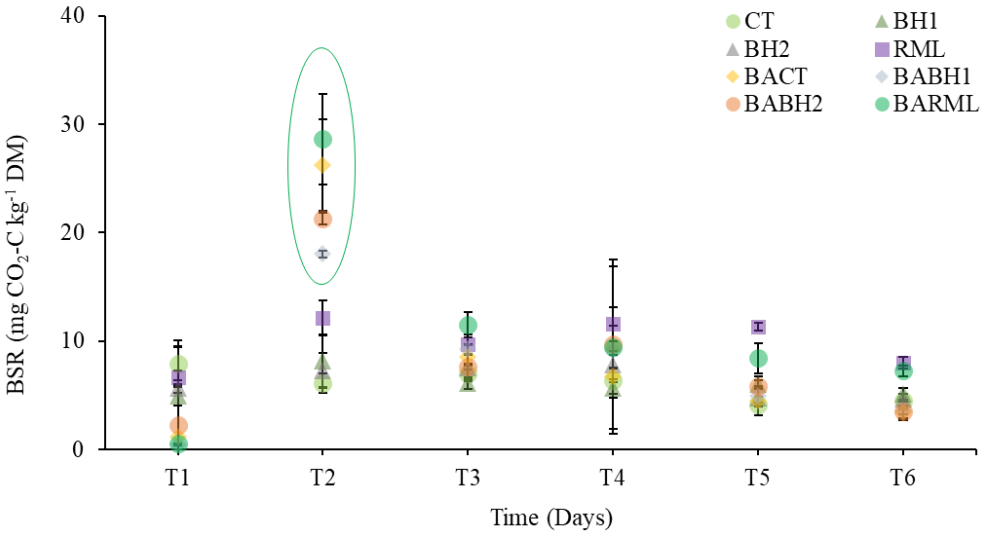


Figure 20. Basal soil respiration of the applied treatments, and evolution along the 90 days. DM: Dry Matter.

4.3.4. Visualization of microbial attachment by Environmental Scanning Electron Microscopy.

The attachment of the microbial consortium to the biochar particles was visualized by Environmental Scanning Electron Microscopy. As it is shown in Figure 21, microorganisms appeared distributed throughout the surface of the BH1 particles. By the same token, BH2 particles displayed a high microbial load in their surface. In both

cases, the irregular topography that the surface of the particles present increases the available surface area, providing thus a scaffold for the microorganisms that facilitates their adhesion and, consequently, increasing the susceptibility of the biochar particles to be colonized. Due to the nature of biochar and its large number of pores, it is very difficult to quantify the bacteria that colonise it, but it can be stated that bacteria are able to use it as a medium for growth and thus improve their survival.

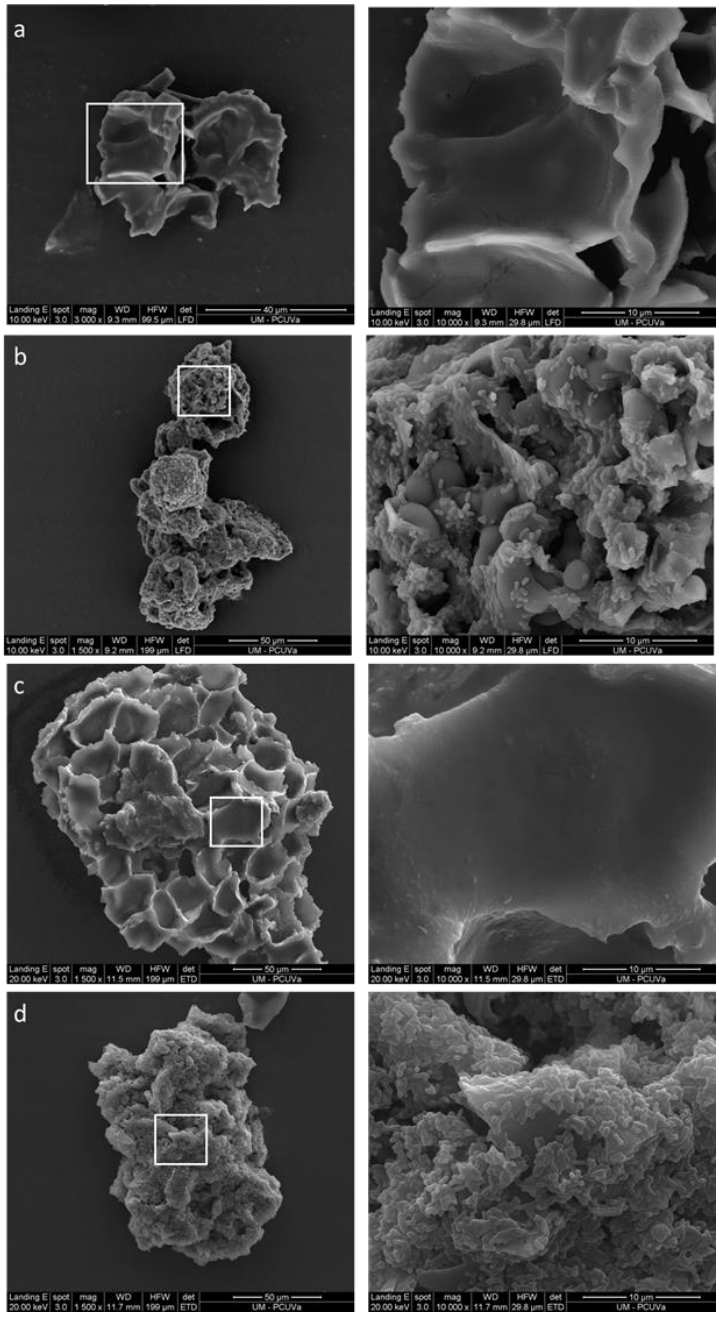


Figure 21. Scanning electron microscopy (SEM) images of growing bacterial cells on biochar. Growing bacterial cells not exposed to biochar, CT BH1 (a) and CT BH2 (c), and growing bacterial cells exposed to BH1 (b) and BH2 (d) at lower magnification in the left panels and at higher magnification in the right panels.

4.3.5. FTIR-ATR results.

The BH1 sample colonized by the microbial consortia (BH1_R) and the milled BH1 sample without bacteria (BH1_T) essentially show the same FTIR spectra (Figure 22.a). The bands and their proposed assignments can be summarized as follows. At higher energies, a broad band around 3332 cm^{-1} (band 13 in Figure 22.a, attributed to stretching OH and, in a lesser extent, to NH vibrations with different origins, probably spread to be involved in hydrogen bonding interactions), a very weak absorption at 3045 cm^{-1} (band 12 in Figure 22.a, stretching vibrations of aromatic C–H bonds), and three bands placed in the 2950 – 2850 cm^{-1} region, characteristic of aliphatic ν(CH) modes (Kim & Ko, 2020). Around 1690 cm^{-1} there is a shoulder attributed to ν(C=O) modes in α,β-substituted carbonyl derivatives and aryl ketones (Kim & Ko, 2020; Pretsch et al., 1976). The band in the 1580 – 1550 cm^{-1} region can be assigned to cooperative contributions of the ν(COO) and ν(C=C) modes, as those in ν(C=C–COO^-) moieties but is more often ascribed to the antisymmetric stretching vibration $\nu_a(COO^-)$ in carboxylate groups (Lago et al., 2021). A weak band at 1429 cm^{-1} can be assigned to δ(CH) vibrations (Li et al., 2007). The weak absorption around 1370 cm^{-1} could be attributed to combinations of δ(COH) and ν(C=C) ring modes from in phenyl-rich regions, or to CH vibrations in lignin and polysaccharides (Kim & Ko, 2020), but also to the carboxylate symmetric $\nu_s(COO^-)$ vibration, as mentioned above. The strong and broad band at 1152 cm^{-1} could be the envelope of absorptions with different origins, as lignin ν(C–O–R) modes (Kiefer et al., 2017; Moradi-Choghamarani et al., 2019). The band around 1034 cm^{-1} with an unresolved shoulder could be attributed to the presence of ash in the samples (Nakamoto, 2008; Sharma et al., 2018), as the in-plane stretching Si –O vibration modes in silicate minerals (Fourdrin et al., 2009; Shahverdi-Shahraki et al., 2015), but organic contributions cannot be discarded. The three band pattern observed in the 870 – 740 cm^{-1} region could be attributed as a whole to H–C–C rocking vibrations in different aromatic environments (Cooke et al., 1986; Odeh, 2015), being those around 864 – 861 cm^{-1} assigned to substituted benzene rings with isolated H atoms (Pretsch et al., 1976), that in the 809 – 787 cm^{-1} region to substituted benzene rings with two adjacent H atoms and/or condensed ring systems (Cooke et al., 1986) and, finally, the band at 751 – 740 cm^{-1} could be related to O-

substituted benzenes and/or monosubstituted benzene rings. A BH2_R colonized biochar and the sample without cell colonization (BH2_R4C) follow the same trend discussed above for the BH1 samples, but the intensity of the bands in these samples is lower than those in the BH1 biochar derivatives due to the lack of functional groups, being hardly distinguished from the baseline (Figure 22.b).

The nearly identic spectra only exhibit very slight differences that fall inside the resolution limits of the technique. The FTIR spectrum of an RML sample colonized by the microbial consortium (RML_R) is provided in Figure 22.c, together with the IR spectra of the microbial consortium without biochar or rhamnolipid scaffolds named as microbial control (Control). Regarding the RML_R spectrum, a medium-strong band at 3275 cm^{-1} is observed, with shoulders about 3390 and 3190 cm^{-1}, which could correspond to stretching ν(NH) and ν(OH) modes from proteins and/or other organic molecules, as the hydroxyl groups in the rhamnose and carboxylic moieties in the rhamnolipids, and water (band 1 in Figure 22.c). Weaks bands around 3070 cm^{-1} can be attributed to aromatic ν(CH) vibrations (band 2 in Figure 22.c). Medium band at 2954 cm^{-1}, sharp and medium-strong at 2924 cm^{-1} and shoulder at 2870 cm^{-1} together with another sharp absorption at 2853 cm^{-1} are characteristic of aliphatic ν(CH) modes (bunch of bands labelled as 3 in Figure 22.c). Concretely, that absorption at 2950 cm^{-1} could be assigned to aliphatic ν(CH_3) groups, and those between 2925 and 2850 cm^{-1} could involve aliphatic ν(CH_3, CH_2, CH) modes rich in ν(CH_2) character (Cooke et al., 1986).

A weak shoulder about 1730 cm^{-1} could be assigned to the carbonyl group in carboxylic acids, esters and/or cyclic ketones (Pretsch et al., 1976; Sharma et al., 2018). A very strong (vs) band with at least two minima can be observed at 1642 and 1625 cm^{-1} which can be attributed to water bending δ(HOH) modes overlapped with δ(NH_2) and amide I vibrations from peptide content (Nakamoto, 2008; Sharma et al., 2018). Strong bands appear at 1535 cm^{-1} and in the 1372 – 1352 cm^{-1} range, which could be due to the antisymmetric and symmetric COO modes of deprotonated carboxylate groups (Kiefer et al., 2017; Li et al., 2007; Li & Chen, 2018; Nakamoto, 2008), but the influence of the amide II contribution in the former absorption cannot

be ruled out. The medium intensity absorption at 1445 cm^{-1} has been attributed to in plane C−O−H bending in the carboxylic group (Li & Chen, 2018; Sharma et al., 2018), but also to bending δ(CH) aliphatic vibrations (Lago et al., 2021; Li et al., 2007). A medium band at 1238 cm^{-1} could be due to combinations of CH_2 wagging and C−O−H bending modes (Lago et al., 2021). Finally, the very strong band at 1052 cm^{-1} could be attributed to antisymmetric stretching C−O−C modes (Pretsch et al., 1976; Trusovas et al., 2016).

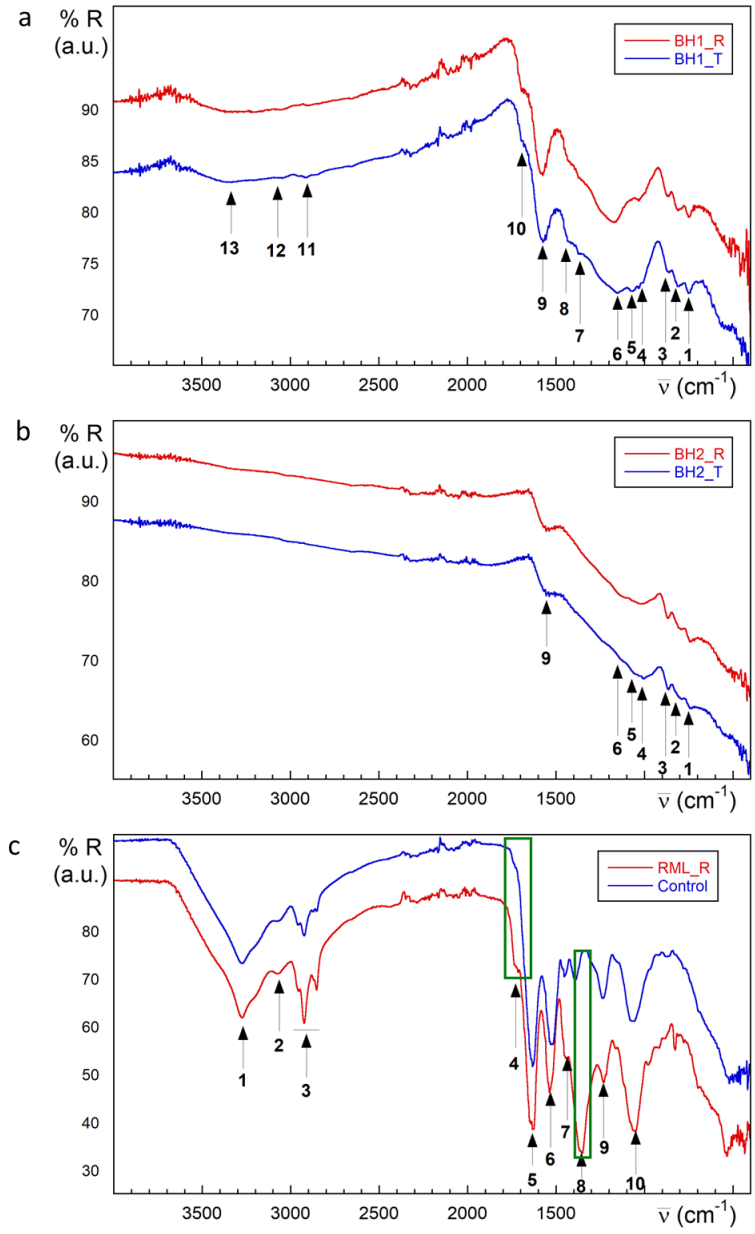


Figure 22. Representative FTIR spectra (a) the BH1_R together with BH1, in the 4000 – 400 cm^{-1} region, (b) the BH2_R together with BH2, in the 4000 – 400 cm^{-1} region, and (c) Control_1, in blue, and RML, in red, in the 4000 – 400 cm^{-1} region, highlighting the main differences between them (green rectangles).

The comparison between Control and RML suggests the former is contained in the latter one. The spectra evidence strong analogies above 1000 cm^{-1}, except for the strong band arising at 1352 cm^{-1} in RML. In addition, the shoulder at 1730 cm^{-1} in RML_R are stronger than that in Control. Unfortunately, the comparison between the spectra of RML, BH1_R and BH2_R is revealed as not very informative (Supplementary Figure 1). This lack of significance is ratified in Supplementary Figure 2, where the subtle differences found in the spectra of BH1_R1 and BH1 cannot be interpreted as due to the influence of RML_1.

4.3.6. Extractable Petroleum Hydrocarbons quantitative determination.

The high molecular weight extractable petroleum hydrocarbons (EPHs) were from the beginning the predominant fractions of the hydrocarbon mixture contained in the soil under study, the average distribution by chain groups being as follows: C10-C12: 8.7 mg kg^{-1}, C12-C16: 213.3 mg kg^{-1}, C16-C21: 183.3 mg kg^{-1}, C21-C30: 2,166.7 mg kg^{-1}, C30-C35: 1,333.3 mg kg^{-1}, and C35-C40: 370.0 mg kg^{-1}. Therefore, it can be stated that most of the hydrocarbons present in the soil were of a recalcitrant nature, and therefore very difficult to reduce, due to their low availability to the microorganisms that are involved in their biodegradation. Figure 23 shows the percentage degradation of EPHs. Increased EPHs degradation in the bioaugmentation treatments (BACT, BABH1, BABH2, and BARML) were noted, compared to the treatment in which no BA was provided (CT, BH1, BH2, and RML). Although most of the degradation occurred during the first 30 days, a trend of hydrocarbon degradation was maintained until the end of the incubation, but in the BH1 treatment, it was observed that degradation was not as fast and increased significantly at 90 days. While in the BH2 treatment degradation did not increase from day 30 onwards.

The high resistance of these EPHs to microbial degradation was demonstrated by the results obtained after 90 days of incubation, under ideal humidity and temperature conditions. Although significant differences were observed in treatments, where BH1 and BH2 were added, probably due to the population of native soil microorganisms in these amendments, a higher significance was shown when all treatments were bioaugmented with the microbial community. Therefore, it was concluded that at 90

days, higher degradation was achieved with the treatments in which the microbial consortium was inoculated, where degradation percentages of 21.6 for BACT, 27.5 for BABH1, and 29.8 for BABH2 and BARML were achieved. In Figure 23, it has been shown that all bioaugmentation treatments have biodegraded in the same way, however, in Table 10 it has been shown that this is not the case, and that for long chain hydrocarbons (C21-C30 and C30-C35) the significance of degradation is higher for those treatments in which amendments have been added (BABH1, BABH2, and BARML).

In addition, from different treatments raw data of soil physicochemical characteristics and total petroleum hydrocarbons, a network analysis was performed to understand the relationships between them (Supplementary Figure 3). The network study showed a maximum number of possible edges of 36, but not all of them were present in the treatment groups applied in the experiment. The treatment groups corresponding to the controls (CT, BH1, BH2, and RML) contained no edges, however, for the bioaugmentation treatments 36 edges were observed, the BACT and BARML groups had 24 edges, the BABH1 group 23 edges, and the BABH2 group 27 edges. The measure of the degree of network dispersion was highest for BABH1 with a dispersion of 0.361, followed by BACT and BARML with a dispersion of 0.333, and lastly BABH2 with 0.250, indicating that the interactions between the different properties in the bioaugmentation treatments were 63.9%, 66.7% and 75% respectively (Supplementary Table 2 and Supplementary Table 3). These high dispersions, the high number of interactions between the different properties, and the fact that the TPHs factor is not shown as a property with the most interconnections, suggest that the degradation of hydrocarbons is not due to the modification of soil properties, as we could observe in BH1, BH2, but is due to the growth of microorganisms that benefit in turn from the application of the different organic amendments (BABH1, BABH2 and BARML). The reason for only performing the network analysis with BA treatments is that the most prominent impact on TPHs degradation and other studied parameters are observed in BA treatments - applied individually and combined (Supplementary Table 4). The analysis and information provided in Supplementary Table 4 complement the Supplementary Figure 1, as the variable that was showing highest

variation in data is BA. The studied parameters that are impacted with individual and combined application of variables (BA, BH1, BH2, and RML) can help understand the most important parameter, and the important of association with other parameters. For instance, even though the sparsity of BABH1, BABH2, and BARML is much higher (supplementary Figure 1), to draw conclusion from network analysis, the best parameters to study varied. As seen in the supplementary Table 4, the individual application of BA resulted in the significant variation in all the treatment, except in the case of BAS which is also influenced by the indigenous microbial population changes. While individual application of BH1 and BH2 showed impacts in the levels of NO_3 and NH_4, while RML individual applications. The co-application has a combined effect on some parameters. For instance, with BABH1 impacts were noted only on NO_3, the BABH2 showed impacts on NO_3 and NH_4, while the BARML combined application showed influence in TC, NO_3, and NH_4.

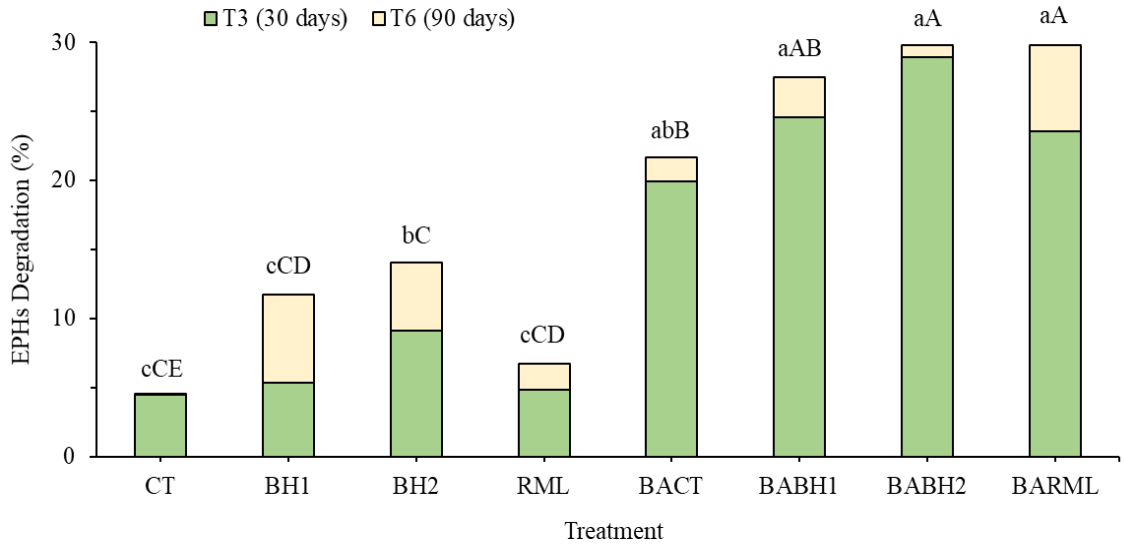


Figure 23. Extractable hydrocarbons degradation percentage along the experiment compared with initial soil (T0). Bars with different letters showed significant statistical differences, with small letter showing differences at T30 days, while capital letters at T90 days ($p < 0.05$).

Table 10. Concentration of the fractions of EPHs from the different treatments at 90 days (T6) compared with initial soil (T0) expressed in mg kg^{-1}.

Technique	Treatment ID	EPH C10-C12	EPH C12-C16	EPH C16-C21	EPH C21-C30	EPH C30-C35	EPH C35-C40
Biostimulation	**CT**	5.17 ± 0.15	156.67 ± 5.77	143.33 ± 11.55	2100.00 ± 0.00	1233.33 ± 115.47	370.00 ± 0.00
	BH1	5.83 ± 0.78	153.33 ± 5.77	96.50 ± 4.95	2133.33 ± 57.74	1133.33 ± 57.74 *	283.33 ± 15.28
	BH2	5.30 ± 0.35	153.33 ± 11.55	133.33 ± 5.77	2066.67 ± 152.75	1166.67 ± 57.74	285.00 ± 7.07
	RML	5.07 ± 0.55	163.33 ± 5.77	136.67 ± 5.77	2233.33 ± 57.74	1166.67 ± 57.74	283.33 ± 11.55
Bioaugmentation	**BA**	5.00 ± 0.60	153.33 ± 5.77	125.00 ± 7.07	1633.33 ± 57.74 ****	1100.00 ± 100.00 **	303.33 ± 35.12
	BABH1	6.00 ± 0.00	155.00 ± 7.07	100.50 ± 13.44	1650.00 ± 70.71 ****	986.67 ± 23.09 ****	276.67 ± 20.82
	BABH2	5.37 ± 0.21	150.00 ± 0.00	29.00 ± 0.00	1566.67 ± 115.47 ****	986.67 ± 105.99 ****	273.33 ± 41.63
	BARML	5.33 ± 0.83	150.00 ± 10.00	110.00 ± 10.00	1500.00 ± 173.21 ****	986.67 ± 32.15 ****	270.00 ± 26.46

Columns with different symbols showed significant statistical differences, * meaning significant difference at $p \leq 0.05$, ** significant difference at $p \leq 0.01$, and **** significant difference at $p \leq 0.0001$).

4.4. Discussion.

The imperative search of mitigating the pollutant soil environments points towards the promising inoculation of microorganism technique, where the microbial consortium approach has an optimal requirement for alleviating the harmful effects of contaminants, for which a specific microbial consortium previously isolated from the contaminated soil under study was prepared, as done by Garrido-Sanz et al., (2019) and Curiel-Alegre et al., (2022). The method used for the microorganisms' application is also a pivotal step for the bioremediation of contaminated site which was already explained in depth by Behera et al., (2021). The addition of the bioaugment in two steps, to increase its growth and thus improve its survival is a key point to achieve efficient degradation, so in relation to this study, the preparation of the pre-inoculum to a final concentration of approximately 10^{11} CFU kg^{-1} of soil was necessary to bioaugment the consortia in the optimal conditions for an efficient treatment. The type of microbial consortium, its growth, and survival are not the only factors that influence hydrocarbon degradation, the degradation process is also affected by environmental and biological factors of the soil, such as pH, temperature, oxygen availability, and nutrient content (Koshlaf & Ball, 2017). Therefore, the study was conducted under controlled temperature and humidity conditions for the microcosms. The soils in which the bioaugmentation treatments were carried out were enriched with a nutrient solution (BHB) to improve the soil characteristics and the survival of the microorganisms. Koshlaf & Ball, (2017) also highlight that the biodegradability of hydrocarbons is closely related to the concentration and bioavailability, something that has been demonstrated in this experiment, with the soil under study being a determining factor due to its high recalcitrance and difficulty for degradation.

The application of amendments to hydrocarbon contaminated soils is a way to improve the different bioremediation techniques commonly used in many studies aimed at the degradation of these pollutants. For example, the addition of biochar acts as a suitable habitat for the growth of microorganisms, both indigenous from the soil and added in the bioaugmentation strategy, thus accelerating the biodegradation of petroleum hydrocarbons (Zhen et al., 2021), enhancing the ability of microorganisms to access and

further degrade short-chain hydrocarbons. Nevertheless, encouraging this growth with organic supports such as a biochar can also be significant for the degradation of hydrocarbons, through enhanced mineralization of recalcitrant components, with mineralization of long-chain hydrocarbons being higher when the pyrolysis temperature of biochar is higher (Ling et al., 2022). Bacterial immobilization on this bio-based material enhances the adsorption, followed by biofilm formation, as shown in Figure 21. In this stage, the biochar became coarse and distorted, and particle adhesion could be observed in contrast to the clean surface of the control, as expected according to (Feng et al., 2020). A deeper study of the interaction between biochar and bacteria was performed with FTIR-ATR analyses, where no microbial colonization was visualized on BH1 and BH2 by FTIR measurements, probably due to the low degree of impregnation that fell below the detection limits of the technique. Although the results achieved by this technique are preliminary, it can be stated that the biochar manufacturing conditions such as pyrolysis temperature and the feedstock used can affect the abundance of functional groups present in them, and therefore, the modification of soil properties; the abundance of functional groups being lower when the pyrolysis temperature increases (Li et al., 2017). Therefore, as already demonstrated by Hoang et al., (2021) it can also affect the material of manufacture of the amendment, and not only the form of production, which significantly affects the remediation efficacy of TPHs. It can also be stated that the faster the pyrolysis process, the more abundant functional groups like carboxylic and hydroxyl groups will be, with CH groups being the most abundant in slow pyrolysis (Zama et al., 2018). On the contrary, the same FTIR analysis was done for the interaction between rhamnolipid and bacteria, where most of the IR bands observed in the colonized rhamnolipid RML samples can be attributed to the microbial consortium. In addition, Zhen et al., (2021) shown how the co-application of a rhamnolipid-modified biochar enhances the degradation of petroleum hydrocarbons, n-alkanes, and PAHs. Although it is an improved biochar, to simplify the technique in this study we have tried to add the amendments separately, achieving similar remediation rates as the previous study, which allows a faster and less economically costly action on the contaminated soil (Hussain et al., 2018). Therefore, environmental impact and economic costs must also be considered in this technology; while BH2 improves bacterial immobilisation and soil remediation compared to BH1, without reaching significant differences between them,

its production results in lower biochar yields when pyrolysis is performed under higher temperatures. In the case of rhamnolipids, they must always be added together with the microbial consortium to influence bioremediation, and their use without bioaugmentation has not led to significant differences with the control in the degradation of hydrocarbons. Numerous studies have demonstrated the capacity of rhamnolipids to help and improve the growth of microorganisms when used in bioaugmentation treatments (Khan et al., 2023, 2017). Rong et al., (2021) showed how better results were achieved with rhamnolipids than even with chemical surfactants, however the form of application of rhamnolipids and the nature of these affect the results obtained. Thus, although in this study good biodegradation percentages were achieved with the use of rhamnolipids, these did not have significant results in comparison with biochars, which are economically more viable, so the search for more specific biosurfactants for this type of soil will continue in future studies.

However, much work remains to be done to remove long-chain hydrocarbons, which, although significantly reduced in this study, are still found in high quantities, as shown in other studies such as that of Zhen et al., (2021), where degradation of long carbon chains has also been slow, due to the recalcitrant nature of these types of compounds. This may be due not only to the sort of duration of this study and the high molecular weight of these compounds, but also to their hydrophobicity, which makes the pollutant less available to both native and bioaugmentation microorganisms. When the expected degradation rates were not achieved (Chen et al., 2017), the consortium previously isolated from the soil was added, but apparently this was not enough to reach complete mineralisation. On the other hand, due to the high concentrations of hydrocarbons, the most recalcitrant fractions, the carbon/nutrient ratios can be affected or even have toxic effects on micro-organisms with biodegradation capacities, thus limiting their growth and degradation activity (Abena et al., 2019). These factors are decisive in understanding how these treatments have achieved significant differences and could lead to even higher degradation rates in the future.

4.5. Conclusions.

This study has shown significant differences between biostimulation treatments and bioaugmentation treatments for hydrocarbon degradation. In addition, smaller significant differences are also shown in the addition of biochar to the soil (BH1 and BH2), which leads us to the conclusion that addition of biochar can be an easy and effective technique for the degradation of hydrocarbons; however, the degradation of each of the fractions, and especially of the long chain fractions, is not satisfying if biochar is not added together with a microbial consortium. Although biochar can serve as a habitat for native soil microorganisms and improve their degradation capabilities, they will not be fully efficient if they are not applied with a bioaugmentation that enhances their potential by increasing the number of microorganisms working for that purpose. In the case of the use of rhamnolipids as an amendment together with bioaugmentation, it has shown significant results as biochar, however its high cost as a raw material raises questions about the economic efficiency. The introduction of an immobilized microbial consortium increased the biological activity in the soil, but only a limited improvement of bioaccessibility to the target pollutants was achieved, due to the fact that the soil, in which the pollutant has been retained for many years, is contaminated by hydrocarbons of a recalcitrant nature, so current research should focus on the use of chemicals, such as more powerful oxidants or surfactants, that improve the mobility of the long-chain fractions of hydrocarbons.

4.6. References.

Abena, M. T., Li, T., Shah, M. N., & Zhong, W. (2019). Biodegradation of total petroleum hydrocarbons (TPH) in highly contaminated soils by natural attenuation and bioaugmentation. *Chemosphere*, 234, 864–874. https://doi.org/10.1016/j.chemosphere.2019.06.111

Behera, I. D., Nayak, M., Biswas, S., Meikap, B. C., & Sen, R. (2021). Enhanced biodegradation of total petroleum hydrocarbons by implementing a novel two-step bioaugmentation strategy using indigenous bacterial consortium. *Journal of Environmental Management*, 292, 112746. https://doi.org/10.1016/j.jenvman.2021.112746

Bera, A., Kumar, S., & Foroozesh, J. (2021). Biosurfactant role in microbial enhanced oil recovery. *Green Sustainable Process for Chemical and Environmental Engineering and Science: Microbially-Derived Biosurfactants for Improving Sustainability in Industry*, 1-33 https://doi.org/10.1016/B978-0-12-823380-1.00011-3

Bolan, S., Hou, D., Wang, L., Hale, L., Egamberdieva, D., Tammeorg, P., Li, R., Wang, B., Xu, J., Wang, T., Sun, H., Padhye, L. P., Wang, H., Siddique, K. H. M., Rinklebe, J., Kirkham, M. B., & Bolan, N. (2023). The potential of biochar as a microbial carrier for agricultural and environmental applications. *Science of The Total Environment*, 886, 163968 https://doi.org/10.1016/j.scitotenv.2023.163968

Chen, W., Li, J., Sun, X., Min, J., & Hu, X. (2017). High efficiency degradation of alkanes and crude oil by a salt-tolerant bacterium *Dietzia* species CN-3. *International Biodeterioration & Biodegradation,* 118, 110-118. https://doi.org/10.1016/j.ibiod.2017.01.029

Chicca, I., Becarelli, S., & Di Gregorio, S. (2022). Microbial Involvement in the Bioremediation of Total Petroleum Hydrocarbon Polluted Soils: Challenges and Perspectives. *Environments*, 9(4), 52. https://doi.org/10.3390/environments9040052

Cooke, N. E., Fuller, O. M., & Gaikwad, R. P. (1986). FT-i.r. spectroscopic analysis of coals and coal extracts. *Fuel*, 65(9), 1254-1260. https://doi.org/10.1016/0016-2361(86)90238-3

Curiel-Alegre, S., Velasco-Arroyo, B., Rumbo, C., Khan, A. H. A., Tamayo-Ramos, J. A., Rad, C., Gallego, J. L. R., & Barros, R. (2022). Evaluation of biostimulation, bioaugmentation, and organic amendments application on the bioremediation of recalcitrant hydrocarbons of soil. *Chemosphere*, 307, 135638. https://doi.org/10.1016/j.chemosphere.2022.135638

Ding, Y., Liu, Y., Liu, S., Huang, X., Li, Z., Tan, X., Zeng, G., & Zhou, L. (2017). Potential Benefits of Biochar in Agricultural Soils: A Review. *Pedosphere*, 27(4), 645–661. https://doi.org/10.1016/S1002-0160(17)60375-8

Ellis, M., Altshuler, I., Schreiber, L., Chen, Y. J., Okshevsky, M., Lee, K., Greer, C. W., & Whyte, L. G. (2022). Hydrocarbon biodegradation potential of microbial communities from high Arctic beaches in Canada's Northwest Passage. *Marine Pollution Bulletin*, 174, 113288. https://doi.org/10.1016/j.marpolbul.2021.113288

Feng, F., Chen, X., Wang, Q., Xu, W., Long, L., Nabil El-Masry, G., Wan, Q., Yan, H., Cheng, J., & Yu, X. (2020). Use of *Bacillus-siamensis*-inoculated biochar to decrease uptake of dibutyl phthalate in leafy vegetables. *Journal of Environmental Management*, 253, 109636. https://doi.org/10.1016/j.jenvman.2019.109636

Fourdrin, C., Balan, E., Allard, T., Boukari, C., & Calas, G. (2009). Induced modifications of kaolinite under ionizing radiation: An infrared spectroscopic study. *Physics and Chemistry of Minerals*, 36, 291-299. https://doi.org/10.1007/s00269-008-0277-8

Garrido-Sanz, D., Redondo-Nieto, M., Guirado, M., Jiménez, O. P., Millán, R., Martin, M., & Rivilla, R. (2019). Metagenomic Insights into the Bacterial Functions of a Diesel-Degrading Consortium for the Rhizoremediation of Diesel-Polluted Soil. *Genes*, 10(6), 456. https://doi.org/10.3390/genes10060456

Groß, J., Grundke, C., Rocker, J., Arduengo, A. J., & Opatz, T. (2021). Xylochemicals and where to find them. *Chemical Communications*, 57(78), 9979-9994. https://doi.org/10.1039/d1cc03512f

Haider, F. U., Ejaz, M., Cheema, S. A., Khan, M. I., Zhao, B., Liqun, C., Salim, M. A., Naveed, M., Khan, N., Nuñez-Delgado, A., & Mustafa, A. (2021). Phytotoxicity of petroleum hydrocarbons: Sources, impacts and remediation strategies. *Environmental Research*, 197, 111031. https://doi.org/10.1016/j.envres.2021.111031

Hoang, S. A., Sarkar, B., Seshadri, B., Lamb, D., Wijesekara, H., Vithanage, M., Liyanage, C., Kolivabandara, P. A., Rinklebe, J., Lam, S. S., Vinu, A., Wang, H., Kirkham, M. B., Bolan, N. S. (2021). Mitigation of petroleum-hydrocarbon-contaminated hazardous soils using organic amendments: A review. *Journal of Hazardous Materials,* 416, 125702. https://doi.org/10.1016/j.jhazmat.2021.125702

Hussain, F., Hussain, I., Khan, A. H. A., Muhammad, Y. S., Iqbal, M., Soja, G., Reichenauer, T. G., Zeshan, & Yousaf, S. (2018). Combined application of biochar, compost, and bacterial consortia with Italian ryegrass enhanced phytoremediation of petroleum hydrocarbon contaminated soil. *Environmental and Experimental Botany*, 153, 80–88. https://doi.org/10.1016/j.envexpbot.2018.05.012

Hussain, F., Khan, A. H. A., Hussain, I., Farooqi, A., Muhammad, Y. S., Iqbal, M., Arslan, M., & Yousaf, S. (2022). Soil conditioners improve rhizodegradation of aged petroleum hydrocarbons and enhance the growth of *Lolium multiflorum*. *Environmental Science and Pollution Research*, 29, 9097–9109. https://doi.org/10.1007/s11356-021-16149-7

Igun, O. T., Meynet, P., Davenport, R. J., & Werner, D. (2019). Impacts of activated carbon amendments, added from the start or after five months, on the microbiology and outcomes of crude oil bioremediation in soil. *International Biodeterioration & Biodegradation*, 142, 1–10. https://doi.org/10.1016/j.ibiod.2019.04.008

Khan, A. H. A., Anees, M., Arshad, M., Muhammad, Y. S., Iqbal, M., & Yousaf, S. (2016a). Effects of illuminance and nutrients on bacterial photo-physiology of hydrocarbon degradation. *Science of The Total Environment*, 557-558, 705–711. https://doi.org/10.1016/j.scitotenv.2016.03.068

Khan, A. H. A., Ayaz, M., Arshad, M., Yousaf, S., Khan, M. A., Anees, M., Sultan, A., Nawaz, I., & Iqbal, M. (2019). Biogeochemical Cycle, Occurrence and Biological Treatments of Polycyclic Aromatic Hydrocarbons (PAHs). *Iranian Journal of Science and Technology, Transactions A: Science*, 43, 1393–1410. https://doi.org/10.1007/S40995-017-0393-8

Khan, A. H. A., Tanveer, S., Alia, S., Anees, M., Sultan, A., Iqbal, M., & Yousaf, S. (2017). Role of nutrients in bacterial biosurfactant production and effect of biosurfactant production on petroleum hydrocarbon biodegradation. *Ecological Engineering*, 104, 158–164. https://doi.org/10.1016/j.ecoleng.2017.04.023

Khan, A. H. A., Tanveer, S., Anees, M., Muhammad Y. S., Iqbal, M., & Yousaf, S., (2016b). Role of nutrients and illuminance in predicting the fate of fungal mediated petroleum hydrocarbon degradation and biomass production. *Journal of Environmental Management*, 176, 54–60. https://doi.org/10.1016/j.jenvman.2016.03.040

Khan, A. H. A., Tanveer, S., Kiyani, A., Barros, R., Iqbal, M., & Yousaf, S. (2023). Biosurfactant-producing *Aspergillus*, *Penicillium*, and *Candida* Performed Higher Biodegradation of Diesel Oil than a Non-producing Fungal Strain. *Applied Biochemistry and Microbiology*, 59, 282–289. https://doi.org/10.1134/S0003683823030109

Khan, M. A. I., Biswas, B., Smith, E., Naidu, R., & Megharaj, M. (2018). Toxicity assessment of fresh and weathered petroleum hydrocarbons in contaminated soil- a review. *Chemosphere*, 212, 755-767. https://doi.org/10.1016/j.chemosphere.2018.08.094

Kiefer, J., Radzuan, M. N., & Winterburn, J. (2017). Infrared spectroscopy for studying structure and aging effects in rhamnolipid biosurfactants. *Applied Sciences*, 7(5), 533. https://doi.org/10.3390/app7050533

Kim, D. G., & Ko, S. O. (2020). Effects of thermal modification of a biochar on persulfate activation and mechanisms of catalytic degradation of a pharmaceutical. *Chemical Engineering Journal*, 399, 125377. https://doi.org/10.1016/j.cej.2020.125377

Koshlaf, E., & Ball, A. S. (2017). Soil bioremediation approaches for petroleum hydrocarbon polluted environments. *AIMS microbiology*, 3(1), 25-49. https://doi.org/10.3934/microbiol.2017.1.25

Lago, B. C., Silva, C. A., Melo, L. C. A., & de Morais, E. G. (2021). Predicting biochar cation exchange capacity using Fourier transform infrared spectroscopy combined with partial least square regression. *Science of The Total Environment*, 794, 148762. https://doi.org/10.1016/j.scitotenv.2021.148762

Li, H., Dong, X., da Silva, E. B., de Oliveira, L. M., Chen, Y., & Ma, L. Q. (2017). Mechanisms of metal sorption by biochars: Biochar characteristics and modifications. *Chemosphere*, 178, 466-478. https://doi.org/10.1016/j.chemosphere.2017.03.072

Li, S., & Chen, G. (2018). Thermogravimetric, thermochemical, and infrared spectral characterization of feedstocks and biochar derived at different pyrolysis temperatures. *Waste Management*, 78, 198-207. https://doi.org/10.1016/j.wasman.2018.05.048

Li, Z., Fredericks, P. M., Rintoul, L., & Ward, C. R. (2007). Application of attenuated total reflectance micro-Fourier transform infrared (ATR-FTIR) spectroscopy to the study of coal macerals: Examples from the Bowen Basin, Australia. *International Journal of Coal Geology*, 70(1-3), 87-94. https://doi.org/10.1016/j.coal.2006.01.006

Ling, L., Luo, Y., Jiang, B., Lv, J., Meng, C., Liao, Y., Reid, B. J., Ding, F., Lu, Z., Kuzyakov, Y., & Xu, J. (2022). Biochar induces mineralization of soil recalcitrant components by activation of biochar responsive bacteria groups. *Soil Biology and Biochemistry*, 172, 108778. https://doi.org/10.1016/j.soilbio.2022.108778

Lu, Y., Zhang, W., Li, Y., Zhang, C., Wang, L., Niu, L., & Zhang, H. (2021). Microbial community shift via black carbon: Insight into biological nitrogen removal from microbial assemblage and functional patterns. *Environmental Research*, 192, 110266. https://doi.org/10.1016/j.envres.2020.110266

Manzoor, M., Khan, A. H. A., Ullah, R., Khan, M., & Ahmad, I. (2016). Environmental Epidemiology of Cancer in South Asian Population: Risk Assessment Against Exposure to Polycyclic Aromatic Hydrocarbons and Volatile Organic Compounds. *Arabian Journal for Science and Engineering*, 41, 2031–2043. https://doi.org/10.1007/s13369-016-2139-x

Moradi-Choghamarani, F., Moosavi, A. A., & Baghernejad, M. (2019). Determining organo-chemical composition of sugarcane bagasse-derived biochar as a function of pyrolysis temperature using proximate and Fourier transform infrared analyses. *Journal of Thermal Analysis and Calorimetry*, 138, 331-342. https://doi.org/10.1007/s10973-019-08186-9

Nakamoto, K. (2008). *Infrared and Raman Spectra of Inorganic and Coordination Compounds: Part B: Applications in Coordination, Organometallic, and Bioinorganic Chemistry*. John Wiley & Sons, Inc. https://doi.org/10.1002/9780470405888

Odeh, A. O. (2015). Qualitative and quantitative ATR-FTIR analysis and its application to coal char of different ranks. *Journal of Fuel Chemistry and Technology*, 43(2), 129-137. https://doi.org/10.1016/s1872-5813(15)30001-3

Pretsch, E., Clerc, T., Seibl, J., & Simon, W. (1976). *Tabellen zur Strukturaufklärung organischer Verbindungen: mit spektroskopischen Methoden*. Springer Verlag.

Rong, L., Zheng, X., Oba, B. T., Shen, C., Wang, X., Wang, H., Luo, Q., & Sun, L. (2021). Activating soil microbial community using bacillus and rhamnolipid to remove TPH contacted soil. *Chemosphere*, 275, 130062. https://doi.org/10.1016/j.chemosphere.2021.130062

Saeed, M., Ilyas, N., Jayachandran, K., Shabir, S., Akhtar, N., Shahzad, A., Sayyed, R. Z., & Bano, A. (2022). Advances in Biochar and PGPR engineering system for hydrocarbon degradation: A promising strategy for environmental remediation. *Environmental Pollution*, 305, 119282. https://doi.org/10.1016/j.envpol.2022.119282

Saravanan, A., Swaminaathan, P., Kumar, P. S., Yaashikaa, P. R., Kamalesh, R., & Rangasamy, G. A. (2023). comprehensive review on immobilized microbes-biochar and their environmental remediation: Mechanism, challenges and future perspectives. *Environmental Research*, 23(1), 116723. https://doi.org/10.1016/j.envres.2023.116723

Shahsavari, E., Schwarz, A., Aburto-Medina, A., & Ball, A. S. (2019). Biological Degradation of Polycyclic Aromatic Compounds (PAHs) in Soil: a Current Perspective. Current *Pollution Reports*, 5, 84–92. https://doi.org/10.1007/s40726-019-00113-8

Shahverdi-Shahraki, K., Ghosh, T., Mahajan, K., Ajji, A., & Carreau, P. J. (2015). Effect of dry grinding on chemically modified kaolin. *Applied Clay Science*, 105-106, 100–106. https://doi.org/10.1016/j.clay.2014.12.026

Sharma, R., Singh, J., & Verma, N. (2018). Optimization of rhamnolipid production from *Pseudomonas aeruginosa* PBS towards application for microbial enhanced oil recovery. *3 Biotech*, 8, 1-15. https://doi.org/10.1007/s13205-017-1022-0

Taccari, M., Milanovic, V., Comitini, F., Casucci, C., & Ciani, M. (2012). Effects of biostimulation and bioaugmentation on diesel removal and bacterial community. *International Biodeterioration & Biodegradation*, 66(1), 39–46. https://doi.org/10.1016/j.ibiod.2011.09.012

Trusovas, R., Račiukaitis, G., Niaura, G., Barkauskas, J., Valušis, G., & Pauliukaite, R. (2016). Recent Advances in Laser Utilization in the Chemical Modification of Graphene Oxide and Its Applications. *Advanced Optical Materials*, 4(1), 37-65. https://doi.org/10.1002/adom.201500469

Udom, G. J., Frazzoli, C., Ekhator, O. C., Onyena, A. P., Bocca, B., & Orisakwe, O. E. (2023). Pervasiveness, bioaccumulation and subduing environmental health challenges posed by polycyclic aromatic hydrocarbons (PAHs): A systematic review to settle a one health strategy in Niger Delta, Nigeria. *Environmental Research*, 226, 115620. https://doi.org/10.1016/j.envres.2023.115620

Yousaf, U., Khan, A. H. A., Farooqi, A., Muhammad, Y. S., Barros, R., Tamayo-Ramos, J. A., Iqbal, M., & Yousaf, S. (2022). Interactive effect of biochar and compost with *Poaceae* and *Fabaceae* plants on remediation of total petroleum hydrocarbons in crude oil contaminated soil. *Chemosphere*, 286(2), 131782. https://doi.org/10.1016/j.chemosphere.2021.131782

Zahed, M. A., Salehi, S., Madadi, R., & Hejabi, F. (2021). Biochar as a sustainable product for remediation of petroleum contaminated soil. *Current Research in Green and Sustainable Chemistry*, 4, 100055. https://doi.org/10.1016/j.crgsc.2021.100055

Zama, E. F., Reid, B. J., Arp, H. P. H., Sun, G. X., Yuan, H. Y., & Zhu, Y. G. (2018). Advances in research on the use of biochar in soil for remediation: a review. *Journal of Soils and Sediments*, 18, 2433-2450. https://doi.org/10.1007/s11368-018-2000-9

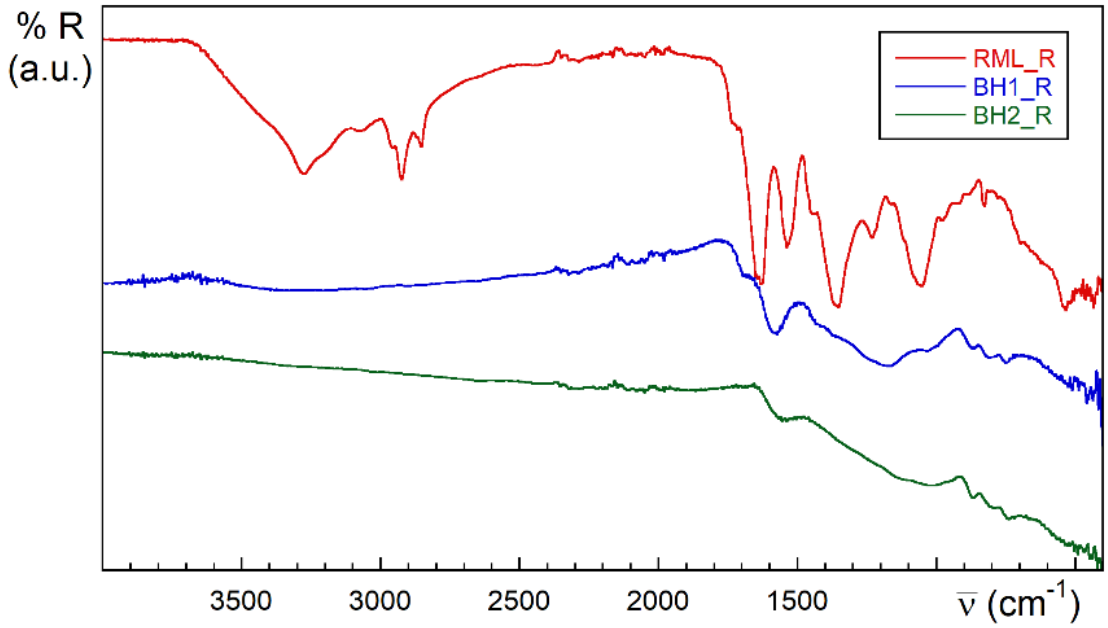
Zhang, B., Zhang, L., & Zhang, X. (2019). Bioremediation of petroleum hydrocarbon-contaminated soil by petroleum-degrading bacteria immobilized on biochar. *RSC Advances*, 9(60), 35304–35311. https://doi.org/10.1039/c9ra06726d

Zhen, M., Tang, J., Li, C., & Sun, H. (2021). Rhamnolipid-modified biochar-enhanced bioremediation of crude oil-contaminated soil and mediated regulation of greenhouse gas emission in soil. *Journal of Soils and Sediments*, 21, 123-133. https://doi.org/10.1007/s11368-020-02746-5

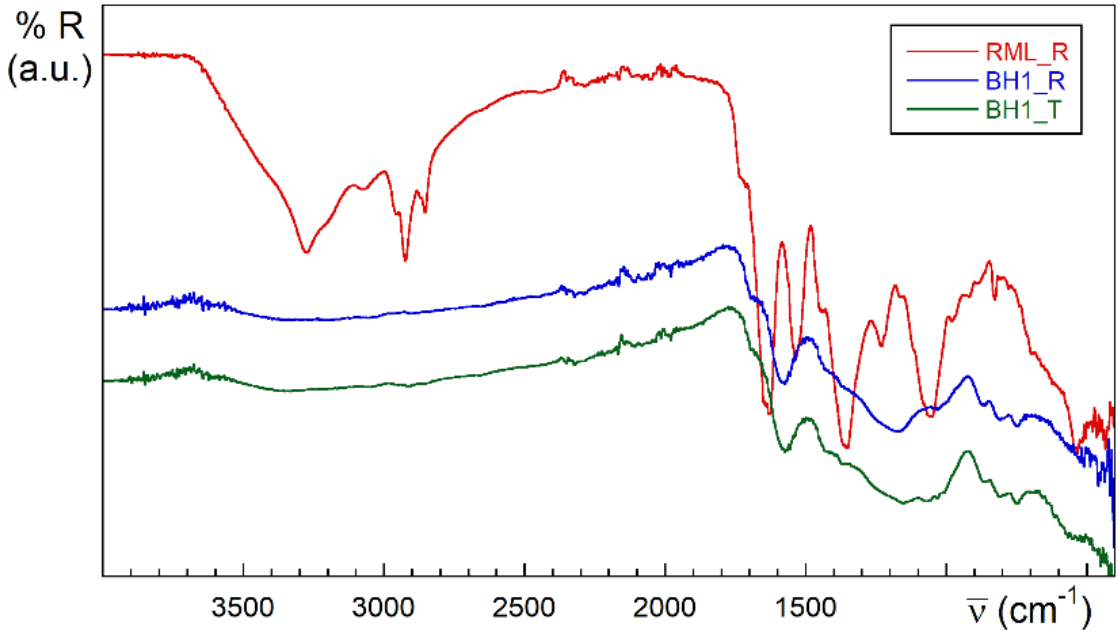
4.7. Supplementary material.

Supplementary Table 1. Metals concentration in soil at the start and end of experiment.

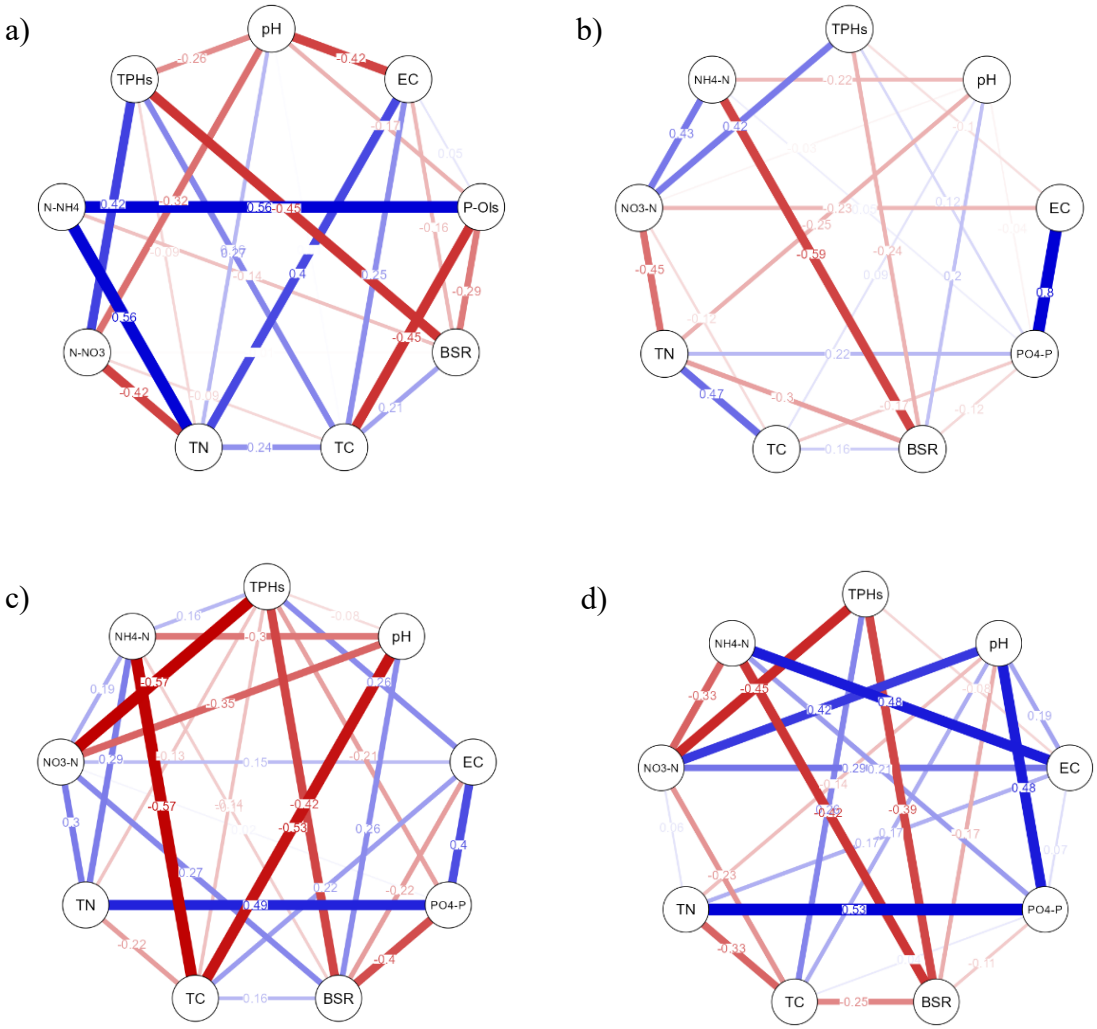
	CT		BH1		BH2		RML		BACT		BABH1		BABH2		BARML	
	T1	T6	T1	T6	T1	T6	T1	T6	T1	T6	T1	T6	T1	T6	T1	T6
Al	16.16 ±2.48	35.48 ±7.87	14.30 ±4.54	14.86 ±3.89	5.53 ±0.22	11.87 ±7.09	29.29 ±6.74	42.53 ±8.06	10.66 ±2.09	9.01 ±1.83	14.84 ±4.82	7.54 ±0.63	6.97 ±3.50	5.03 ±0.98	35.47 ±12.89	21.22 ±1.45
B	2.12 ±0.95	1.10 ±0.03	1.62 ±0.85	1.21 ±0.07	1.67 ±0.89	1.11 ±0.02	1.63 ±0.79	1.14 ±0.04	2.52 ±1.03	1.21 ±0.02	1.83 ±0.76	1.24 ±0.02	1.74 ±0.79	1.08 ±0.03	1.67 ±0.61	1.06 ±0.01
Ca	11687.34 ±786.18	9839.77 ±1076.04	11884.29 ±1286.32	13758.01 ±1249.78	16053.96 ±854.51	11859.18 ±2075.87	9231.62 ±263.83	8844.69 ±550.00	12161.57 ±268.79	13339.84 ±765.11	11940.18 ±407.65	13027.00 ±1136.34	14331.08 ±1392.55	13951.52 ±1079.16	7941.52 ±1062.87	12210.77 ±516.20
Fe	74.81 ±3.51	65.26 ±3.20	71.93 ±2.99	66.61 ±0.78	73.38 ±5.92	68.51 ±2.30	63.02 ±4.69	56.80 ±5.52	82.53 ±1.73	89.09 ±2.74	82.50 ±2.55	93.61 ±1.32	81.80 ±4.20	92.88 ±1.67	61.63 ±5.92	68.10 ±2.75
K	89.55 ±14.61	100.27 ±19.18	121.70 ±16.72	132.71 ±5.24	136.32 ±0.43	131.16 ±4.56	989.82 ±76.03	911.85 ±69.70	364.28 ±11.29	415.24 ±39.30	422.52 ±11.81	570.19 ±37.95	427.75 ±12.59	549.98 ±18.23	1787.31 ±22.00	1398.59 ±53.07
Mg	427.22 ±11.07	424.56 ±11.56	417.71 ±23.63	422.11 ±9.86	423.81 ±13.53	432.45 ±25.19	401.80 ±19.24	415.27 ±18.75	451.70 ±20.79	448.87 ±28.64	422.44 ±8.03	450.47 ±11.59	449.05 ±12.35	447.61 ±8.69	410.88 ±16.43	420.94 ±18.26
Mn	77.55 ±2.68	69.45 ±4.47	72.22 ±8.15	64.81 ±1.45	68.24 ±3.68	67.70 ±4.44	75.05 ±3.51	75.80 ±5.79	86.46 ±1.50	104.15 ±4.03	78.81 ±1.89	102.93 ±2.91	78.97 ±4.40	101.55 ±4.93	83.66 ±1.09	97.78 ±3.22
Na	1116.03 ±76.89	1022.42 ±71.25	1004.32 ±51.21	979.15 ±95.25	1066.71 ±105.93	940.93 ±33.81	913.21 ±59.26	900.07 ±62.67	978.92 ±61.24	1005.76 ±6.66	1052.65 ±75.41	964.69 ±72.46	1271.73 ±115.77	1009.18 ±71.94	1050.36 ±216.74	1058.43 ±122.48
P	2.77 ±0.67	2.71 ±1.06	4.68 ±1.64	5.07 ±1.81	6.41 ±2.63	5.39 ±1.16	7.02 ±0.87	7.37 ±1.86	48.16 ±3.57	57.02 ±8.92	50.91 ±6.64	66.45 ±3.44	44.89 ±11.03	62.26 ±4.01	106.48 ±13.72	66.75 ±9.63
S	251.38 ±9.60	292.98 ±9.23	254.46 ±25.22	284.28 ±17.92	249.09 ±16.93	288.53 ±17.08	265.38 ±14.89	318.82 ±30.75	279.27 ±12.75	298.65 ±23.86	265.84 ±7.13	299.43 ±18.02	302.09 ±13.66	312.33 ±15.36	335.29 ±36.52	320.12 ±10.32
Cu	1.51 ±0.17	1.31 ±0.07	1.31 ±0.30	1.52 ±0.19	2.06 ±0.99	1.42 ±0.23	1.41 ±0.13	1.19 ±0.06	1.24 ±0.21	1.04 ±0.05	1.28 ±0.17	1.00 ±0.08	1.32 ±0.22	1.17 ±0.12	1.37 ±0.15	0.90 ±0.08
Zn	72.92 ±4.27	69.25 ±3.09	60.03 ±5.04	66.13 ±3.76	61.97 ±6.82	67.19 ±3.09	71.70 ±4.52	67.76 ±4.97	75.93 ±2.94	82.17 ±3.59	67.45 ±0.58	83.27 ±2.06	68.69 ±4.87	82.42 ±3.26	74.44 ±1.09	70.93 ±0.57



Supplementary Figure 1. IR spectra of RML_R1 (green), BH1_R1 (blue) and BH2_R1 (red) in the 4000 – 400 cm^{-1} region.



Supplementary Figure 2. IR spectra of RML_R (red), BH1_R (blue) and BH1 (green) in the 4000 – 400 cm^{-1} region.



Supplementary Figure 3. Network analysis to determine the correlation pattern in reference to different bioaugmentation treatments. a) BACT, b) BABH1, c) BABH2, and d) BARML.

Supplementary Table 2 Centrality measures per property for different treatments.

BACT

Property	Betweenness	Closeness	Strength	Expected influence
pH	-0.808	-1.289	-0.440	-1.397
EC	0.162	-0.452	-0.662	0.236
PO_4-P	-0.323	0.333	0.538	-0.391
BSR	-0.808	-1.439	-0.815	-1.164
TC	-1.294	0.048	0.484	0.670
TN	2.102	1.842	2.186	1.269
NO_3-N	0.162	0.667	-0.819	-0.555
NH_4-N	0.162	0.333	-0.792	1.450
TPHs	0.647	-0.042	0.321	-0.118

BABH1

Property	Betweenness	Closeness	Strength	Expected influence
pH	-0.745	-0.477	-1.174	0.330
EC	-0.745	-1.071	-1.348	-0.516
PO_4-P	-0.051	-0.861	-0.461	0.834
BSR	-0.051	-0.813	0.660	1.593
TC	-0.745	0.184	0.919	-1.722
TN	-0.745	-0.381	-0.854	0.776
NO_3-N	1.568	1.621	1.160	-0.625
NH_4-N	1.799	1.531	1.113	-0.009
TPHs	-0.283	0.268	-0.016	-0.661

BABH2

Property	Betweenness	Closeness	Strength	Expected influence
pH	-0.578	0.187	1.382	-1.186
EC	1.374	-0.021	-0.547	-1.012
PO_4-P	-1.229	-1.270	-1.674	1.395
BSR	0.072	-0.008	-0.532	0.722
TC	-0.578	0.593	0.781	-0.293
TN	0.072	-0.422	0.780	-1.110
NO_3-N	0.723	0.943	-0.899	1.288
NH_4-N	1.374	1.570	0.817	0.332
TPHs	-1.229	-1.572	-0.108	-0.136

BARML

Property	Betweenness	Closeness	Strength	Expected influence
pH	-0.825	-0.009	-1.136	-0.885
EC	0.525	0.624	0.933	0.973
PO_4-P	-1.500	-0.999	-0.578	1.161
BSR	0.525	-0.339	0.288	1.288
TC	-0.150	-0.171	-0.261	-1.666
TN	-0.150	-0.707	-0.605	-0.501
NO_3-N	-0.825	-1.080	-0.921	0.216
NH_4-N	1.875	2.118	2.017	-0.396
TPHs	0.525	0.565	0.263	-0.189

Supplementary Table 3. Weight matric used for the preparation of network plots for variables (extractable metals) for different treatments.

BACT

Property	pH	EC	PO_4-P	BSR	TC	TN	NO_3-N	NH_4-N	TPHs
pH	0.000	-0.416	-0.173	0.000	0.006	0.160	-0.317	0.000	-0.258
EC	-0.416	0.000	0.055	-0.158	0.252	0.404	0.000	0.000	0.000
PO_4-P	-0.173	0.055	0.000	-0.290	-0.453	0.000	0.000	0.562	0.000
BSR	0.000	-0.158	-0.290	0.000	0.208	0.000	-0.009	-0.139	-0.449
TC	0.006	0.252	-0.453	0.208	0.000	0.244	-0.089	0.000	0.270
TN	0.160	0.404	0.000	0.000	0.244	0.000	-0.418	0.556	-0.091
NO_3-N	-0.317	0.000	0.000	-0.009	-0.089	-0.418	0.000	0.000	0.419
NH_4-N	0.000	0.000	0.562	-0.139	0.000	0.556	0.000	0.000	0.000
TPHs	-0.258	0.000	0.000	-0.449	0.270	-0.091	0.419	0.000	0.000

BABH1

Property	pH	EC	PO_4-P	BSR	TC	TN	NO_3-N	NH_4-N	TPHs
pH	0.000	0.000	-0.039	0.199	0.089	-0.249	-0.033	-0.220	0.000
EC	0.000	0.000	0.804	0.000	0.000	0.000	-0.231	0.000	-0.102
PO_4-P	-0.039	0.804	0.000	-0.121	-0.166	0.224	0.000	0.053	0.122
BSR	0.199	0.000	-0.121	0.000	0.160	-0.304	0.000	-0.592	-0.242
TC	0.089	0.000	-0.166	0.160	0.000	0.471	-0.115	0.000	0.000
TN	-0.249	0.000	0.224	-0.304	0.471	0.000	-0.454	0.000	0.000
NO_3-N	-0.033	-0.231	0.000	0.000	-0.115	-0.454	0.000	0.428	0.424
NH_4-N	-0.220	0.000	0.053	-0.592	0.000	0.000	0.428	0.000	0.000
TPHs	0.000	-0.102	0.122	-0.242	0.000	0.000	0.424	0.000	0.000

BABH2

Property	pH	EC	PO_4-P	BSR	TC	TN	NO_3-N	NH_4-N	TPHs
pH	0.000	0.000	0.000	0.259	-0.530	0.000	-0.347	-0.305	-0.081
EC	0.000	0.000	0.398	-0.220	0.221	0.000	0.151	0.000	0.262
PO_4-P	0.000	0.398	0.000	-0.398	0.000	0.494	0.022	0.000	-0.212
BSR	0.259	-0.220	-0.398	0.000	0.159	0.000	0.270	-0.112	-0.419
TC	-0.530	0.221	0.000	0.159	0.000	-0.219	0.000	-0.567	-0.142
TN	0.000	0.000	0.494	0.000	-0.219	0.000	0.297	0.293	-0.134
NO_3-N	-0.347	0.151	0.022	0.270	0.000	0.297	0.000	0.188	-0.571
NH_4-N	-0.305	0.000	0.000	-0.112	-0.567	0.293	0.188	0.000	0.160
TPHs	-0.081	0.262	-0.212	-0.419	-0.142	-0.134	-0.571	0.160	0.000

BARML

Property	pH	EC	PO_4-P	BSR	TC	TN	NO_3-N	NH_4-N	TPHs
pH	0.000	0.192	0.480	-0.171	0.173	-0.137	0.417	0.000	0.000
EC	0.192	0.000	0.069	0.000	0.000	0.167	0.292	0.481	0.000
PO_4-P	0.480	0.069	0.000	-0.111	0.044	0.533	0.000	0.211	-0.085
BSR	-0.171	0.000	-0.111	0.000	-0.253	0.000	0.000	-0.424	0.000
TC	0.173	0.000	0.044	-0.253	0.000	-0.327	-0.227	0.000	-0.388
TN	-0.137	0.167	0.533	0.000	-0.327	0.000	0.057	0.000	0.258
NO_3-N	0.417	0.292	0.000	0.000	-0.227	0.057	0.000	-0.328	0.000
NH_4-N	0.000	0.481	0.211	-0.424	0.000	0.000	-0.328	0.000	-0.452
TPHs	0.000	-0.085	0.000	-0.388	0.258	0.000	-0.452	0.000	0.000

Supplementary Table 4. Two-way ANOVA analysis for variables and their impacts on parameters.

Source		Sum of Squares[a]	df[b]	Mean Square	F	Significance
Bioaugmentation (BA)	TPHs	3843201	1	3843201	19.035	0
	pH	13.044	1	13.044	159.723	0
	EC	24074725	1	24074725	551.868	0
	PO_4	10554.06	1	10554.06	541.154	0
	BAS	25.664	1	25.664	3.226	0.077
	TC	2312833	1	2312833	8.859	0.004
	TN	19369205	1	19369205	394.457	0
	NO_3	10540.92	1	10540.92	1649.861	0
	NH_4	311382.9	1	311382.9	60532.85	0
Biochar 1 (BH1)	TPHs	113350.1	1	113350.1	0.561	0.456
	pH	0.042	1	0.042	0.515	0.476
	EC	18090.25	1	18090.25	0.415	0.522
	PO_4	14.113	1	14.113	0.724	0.398
	BAS	2.373	1	2.373	0.298	0.587
	TC	8877.754	1	8877.754	0.034	0.854
	TN	558.007	1	558.007	0.011	0.915
	NO_3	289.113	1	289.113	45.252	0
	NH_4	115.133	1	115.133	22.382	0
Biochar 2 (BH2)	TPHs	370566.4	1	370566.4	1.835	0.18
	pH	0.014	1	0.014	0.167	0.684
	EC	29013.44	1	29013.44	0.665	0.418
	PO_4	13.201	1	13.201	0.677	0.414
	BAS	0.383	1	0.383	0.048	0.827
	TC	2265313	1	2265313	8.677	0.004
	TN	7225.283	1	7225.283	0.147	0.703
	NO_3	563.825	1	563.825	88.25	0
	NH_4	313.054	1	313.054	60.858	0
Biosurfactant (RML)	TPHs	60618.54	1	60618.54	0.3	0.586
	pH	0.008	1	0.008	0.099	0.754
	EC	137146.8	1	137146.8	3.144	0.081
	PO_4	31.397	1	31.397	1.61	0.209
	BAS	30.764	1	30.764	3.867	0.054
	TC	25309328	1	25309328	96.945	0
	TN	15627.29	1	15627.29	0.318	0.575
	NO_3	169	1	169	26.452	0
	NH_4	588.143	1	588.143	114.335	0

BA * BH1	TPHs	178	1	178	0.001	0.976
	pH	0.007	1	0.007	0.082	0.776
	EC	2320.028	1	2320.028	0.053	0.818
	PO_4	13.371	1	13.371	0.686	0.411
	BAS	3.105	1	3.105	0.39	0.534
	TC	66823.2	1	66823.2	0.256	0.615
	TN	794.347	1	794.347	0.016	0.899
	NO_3	271.92	1	271.92	42.561	0
	NH_4	12.816	1	12.816	2.492	0.119
BA * BH2	TPHs	0.825	1	0.825	0	0.998
	pH	0.003	1	0.003	0.031	0.862
	EC	12173.44	1	12173.44	0.279	0.599
	PO_4	12.484	1	12.484	0.64	0.427
	BAS	0.965	1	0.965	0.121	0.729
	TC	3063792	1	3063792	11.736	0.001
	TN	5306.103	1	5306.103	0.108	0.743
	NO_3	531.687	1	531.687	83.219	0
	NH_4	91.585	1	91.585	17.804	0
BA * RML	TPHs	17669.06	1	17669.06	0.088	0.768
	pH	0.061	1	0.061	0.745	0.391
	EC	219648.4	1	219648.4	5.035	0.028
	PO_4	32.528	1	32.528	1.668	0.201
	BAS	0.446	1	0.446	0.056	0.814
	TC	4393201	1	4393201	16.828	0
	TN	12.46	1	12.46	0	0.987
	NO_3	151.618	1	151.618	23.731	0
	NH_4	251.275	1	251.275	48.848	0
Error	TPHs	12921913	64	201904.9		
	pH	5.227	64	0.082		
	EC	2791938	64	43624.04		
	PO_4	1248.185	64	19.503		
	BAS	509.097	64	7.955		
	TC	16708467	64	261069.8		
	TN	3142625	64	49103.52		
	NO_3	408.895	64	6.389		
	NH_4	329.218	64	5.144		

A *p-value* lower than 0.05 in sig. the column is considered statistically significant. [a]Type I sum of square, and [b]df = Degree of freedom.

CHAPTER 5

Hydrocarbon Bioremediation: Scaling Up from Lab to Field for Petroleum-Contaminated Soils[3]

[3] This chapter has been submitted for publication to a special publication issue corresponding to the 7th International Symposium on Environmental Biotechnology and Engineering (7ISEBE) held in Marseille (France), for the journal Environmental Science and Pollution Research.

5.1. Introduction.

Soil contamination with hydrocarbons, due both to various human activities and natural processes, is an increasingly common environmental problem. The major contribution to this environmental catastrophe was by the anthropogenic activities. Industrial facilities, oil and gas exploitation, improper waste disposal, and some agricultural practices are examples of human activities that significantly contribute to hydrocarbon contamination (Abdullah et al., 2020). Among these activities, oil refineries, chemical manufacturing plants, and gas stations handle and store large quantities of oil, that are some main culprits of soil pollution, and if improperly managed can lead to crude oil spills and natural gas leaks (Adipah, 2019; Truskewycz et al., 2019). In addition, inadequate waste management procedures, and unregulated dumping of liquid or solid wastes in an open environment, further exacerbate the problem of total petroleum hydrocarbon (TPHs) causing environmental contamination (Gautam et al., 2023). From unbranched alkanes (C10–C25), to branched alkanes, low molecular weight aromatics, high molecular weight hydrocarbons, and asphaltenes, the biodegradation of TPHs proceeds in an increasing sequence (Imam et al., 2019; Kebede et al., 2021). The TPHs polluted soils are known to induce human and environmental health emergencies, however the intensity varies with reference to the exposure routes (Khan et al., 2023). Direct and prolong exposure to soil TPHs, can pose several health risks to humans, including cancer, headache, nausea, fatigue, eye irritation, and skin rash (Yousaf et al., 2022). Also, there exist the food chain contamination risks by the residual TPHs in soils associated to grazing livestock, wildlife, and plant-eating insects (Grifoni et al., 2020). Direct effects of soil TPHs has been determined on crops with consequences such are the discoloration and a poor vegetative growth of plants due to a reduction in nutrients acquisition (Truskewycz et al., 2019). Finally, the effect in the loss on soil quality is reflected by a reduction in the presence and activity of different soil microorganisms (Curiel-Alegre et al., 2022b).

Bioelectrochemical systems (BES) is among one of the latest technologies that combines biological and electrochemical activities of electrogenic bacterial processes used for the treatment of pollution and its management. These techniques contribute to the

remediation of TPH and metal-associated pollution, where the syntrophic and cooperative interactions of the microbial populations involved modify their effectiveness (Ambaye et al., 2023). The most basic type of electrobioremediation technique is almost certainly the microbial electrochemical snorkel (MES). A MES is a non-polarized, electrically conductive object that is applied to short-circuit redox gradients, such as the oxic soil environment outside and the anoxic contaminated soil or sediment. Through the use of snorkels, electrons can migrate to severely reduced to oxidized zones more easily, where they can combine with O_2 to produce water. (Cruz-Viggi et al., 2022). The primary approach in this recently developed technique to provide the oxygen needed for the biodegradation of hydrocarbons in soil is oxygen diffusion (Jin & Fallgren, 2022). This electron transfer can span centimetres, giving microbes in the soil or sediment remote access to O_2 that would otherwise be inaccessible. This leads to increased anaerobic oxidation rates over time (Aulenta et al., 2021; Hoareau et al., 2019). The combined application of BES along with other treatment method can enhance the remediation TPHs-contaminated soil by providing an electron acceptor to the microbial community (Wang et al., 2021).

One of the most prominent challenges in the TPHs bioremediation, is that the results at the lab scale studies are not translated to in the field scale, however many methods are adopted at lab and field scale (Khan et al., 2016; Yousaf et al., 2022). This is since it is very difficult to maintain the optimal environmental conditions and availability of essential nutrients at field scale. Soil composition, water retention capacity, particle size and enzyme activity are some of the soil factors that influence microbial biodegradation (Martínez-Álvarez et al., 2020). It is not wrong to conclude that many such minor details are pivotal for the application of such biotechnological interventions to perform efficient removal and mineralization of the organic pollutants. Chen et al., (2019) advocate for a systematic approach to hydrocarbon bioremediation, encompassing thorough assessment, targeted planning, site preparation, bioaugmentation, nutrient supplementation, ongoing monitoring, and final compliance checks. This holistic process aims to efficiently restore contaminated sites and environmental health while adhering to regulatory standards. Hence, once good results have been obtained at a laboratory scale, it must be upscaled pilot tests, so that it can be studied whether, under usual

ambient environmental conditions, same results are achievable as those obtained at lab scale. Further research is necessary to fully comprehend the consequences of these microorganisms on the ecosystem during bioremediation, since specific organisms introduced to the environment may subsequently form biological pollution via recombination or other mechanisms (Ayilara & Babalola, 2023). Studies conducted on a pilot size have demonstrated that bioremediation can be successful in decreasing TPH pollution and hastening soil recovery (Akbari & Ghoshal, 2014; Nobili et al., 2022). Previous works has been focused on the optimization and validation of different soil bioremediation technologies (biostimulation/bioaugmentation) and organic amendments at laboratory scale (Curiel-Alegre et al., 2022a,b). Hence, to validate the capacity of bioaugmentation coupled with organic amendment, and co-application with MES, the main objective of the present research, is to study the scaling up of these biotechnologies, at their optimal operative conditions, at a scale of 500 kg mesocosms, and identify the impact on chemical, physical, biological (including at soil metagenomics and enzymatic assays) for an effective TPH bioremediation option.

5.2. Materials and methods.

5.2.1. Soil site properties.

The historically TPHs polluted soil was collected from the machinery park in Noblejas, Toledo, Spain. The source of contamination were different fuel spills, engine motor oils, and lubricants leakages from parked vehicles and heavy machinery. After excavation, the soil placed in three separated containers where the respective remediation activities were carried out.

5.2.2. Chemicals reagents.

Standards for hydrocarbons (HC) were purchased from LGC Standards Ltd (Teddington, UK): Alkanes-Mix 12, 100 µg mL^{-1} in toluene, covering the range C8–C40 (total 14 HCs) and PAHs-Mix 9 with 19 PAHs according to EPA Method 610 (acenaphthene, acenaphthylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(g,h,i)perylene, chrysene, dibenzo(a,h)anthracene, fluoranthene, fluorine, indeno(1,2,3-c,d)pyrene,

naphthalene, phenanthrene and pyrene) at 100 µg mL^{-1} in acetonitrile. Certified soil reference materials CRM-357 (sandy loam soil) and CRM-359 (clay soil) were purchased form Sigma-Aldrich. Chromatographic separation was performed using Isolute Sorbent EPH extraction cartridges (25 mL 5 g^{-1}).

5.2.3. Production of inoculum and nutrient solutions.

Microbial consortium was previously isolated from the contaminated soil under study according to the procedure described by Garrido-Sanz et al., (2019) and Curiel-Alegre, et al., (2022b). The microbial consortium was pre-cultured in 1 L of sterile liquid minimum salt medium (MM) (Brazil et al., 1995), supplemented with 1 mL L^{-1} of phosphate-buffered mineral medium salts (PAS) (Bedard et al., 1986), 0.005% (w:v) yeast extract, and 1 mL L^{-1} of diesel oil, added as a unique carbon and energy source. The consortium was incubated for five days at 28 °C, at 200 rpm, before it was scaled up to 20 L, in separate four 25 L containers. These cultures were incubated for 2 weeks at 28 °C, with periodic shaking. Once the inoculant was grown, a representative sample was extracted from it and its optical density and colony-forming units (CFUs mL^{-1}) on Plate Count Agar (PCA) were determined.

5.2.4. Experimental setup.

The experiment was carried out at a pilot scale in 500 kg mesocosms for 90 days. Three different experimental conditions were assayed: control treatment to test the natural attenuation of polluted soil (CT) in which only water was added to reach 40% soil water retention capacity (WRC), a bioaugmentation treatment with inoculation and nutrient incorporation to a 2% (w:w) vermicompost-soil mixture (BAVC), and the same bioaugmentation treatment but with the introduction of a passive MES (BESBAVC) in the form of six 50 cm length and 19 mm diameter graphite rods (https://www.graphitestore.com/, USA) inserted in the soil at 30 cm distance between them. Nutrients were added as salts to reach de following concentrations (g kg^{-1} dry soil): KH_2PO_4 0.6116; NH_4NO_3 3.0454; 0.0153 $FeCl_3$. The amount of N supplemented in the addition of vermicompost was considered by subtracting it from the nutrient solution. For the mesocosms, three different mixtures were prepared by

blending soil, vermicompost, and nutrients salts with a shovel, that were irrigated with water or microbial suspensions with the help of a phytosanitary backpack and stored in 1 m^3 containers in a shed located outside the building. In the case of the bioaugmentation treatments (BAVC and BESBAVC), the consortium was inoculated by spraying at a rate of 40 L of culture in each mesocosms (Supplementary Figure 1). The control (CT) treatment was not inoculated. Water was added in all treatments, including the CT to achieve 40% of soil water retention capacity (WRC). After one month, mesocosms were re-inoculated with an equal amount of inoculant and the water content of all the mesocosms were increased to reach 50% WRC. During the experiment, the mesocosms were irrigated once or twice a week with water to maintain optimum humidity. Sensors were installed to control the temperature and humidity from soil (GS1 Probes by ProCheck, Decagon, USA) and environmental conditions (Tinytag Plus 2, GEMINI Data loggers, UK) in each treatment throughout the experiment (Supplementary Figure 2).

5.2.5. Soil sample collection and physicochemical parameters and metal(loid)s content analysis.

The experiment was conducted for 90 days (Supplementary Figure 3), taking triplicate soil samples with a 7 cm diameter soil auger (Eijkelkamp, The Netherlands) at T0 (before and after inoculation), T1 (7 days after the inoculation), T2 (30 days after inoculation and 2nd inoculation), T3 (60 days after the inoculation), and T4 (90 days after the inoculation). Fresh soil samples were maintained in refrigerated conditions in the lab, after being sieved using at 2 mm mesh and used for microbiological determination: presence of culturable bacteria and extraction of metagenomic DNA. The rest of soil sample was divided in two, one is frozen at -20°C and the other is air-dried at ambient temperature for biochemical and physicochemical analyses, respectively. Briefly, physicochemical analysis, including soil pH and electrical conductivity, were determined in a soil water suspension (1:5 w:v), total C and N by dry combustion in a TruSpec (LECO) autoanalyzer, organic C by wet oxidation using $K_2Cr_2O_7$, lime content by volumetry after acidic attack, soluble N-NO_3 and exchangeable N-NH_4 and available PO_4 were extracted with 1 M KCl and 0.5 M

$NaHCO_3$, respectively, and analysed colorimetrically, using a segmented flow analyser San+ (SKALAR, The Netherlands). All chemical measurements were made in triplicate. To analyse the changes in available metals, Mehlich extraction was performed using Mehlich 3 soil extractant, a modification of Mehlich 2. The protocol adopted was presented by Mehlich (1984). The extraction was then analysed using ICP-OES (Genesis Spectro, AMETEK, Germany), and the raw average results are presented in supplementary material (Supplementary Table 1). The raw data was used for network analysis, which is detailed in section 2.9.

5.2.6. Microbial consortia biodiversity sequencing and analysis.

Bacterial biodiversity was studied by analysis of the 16S RNA gene. For this purpose, DNA was isolated in triplicate from soil samples. Total DNA was extracted from one gram of homogenized sample soil using the FastDNA Spin Kit for Soil (MP Biomedicals, USA) according to manufacturer indications. The isolated DNA was quantified using Qubit 4 fluorometer (Invitrogen). Genomic DNA 16S rDNA region was amplified by PCR using the primers 27F (5'- AGAGTTTGATCMTGGCTCAG -3') and 1492R (5'- CGGTTACCTTGTTACGACTT -3'). Each sample was amplified in triplicate and multiplexed by using a set of 27F and 1492R primers with a specific barcode tail for each sample. After barcoding PCR, the amplicons were pooled, and 100 femtomoles was utilized for library preparation using Oxford Nanopore Ligation Sequencing Kit (SQK-LSK114) and NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (cat #E7180S) according to their specifications. 12 femtomoles of the multiplexed libraries were loaded and subsequently sequenced on Flongle Flow Cel (R10.4.1, ONT) using the MinION Mk1B device. Data acquisition, basecalling, and quality filtering was performed using MinKNOW version 22.03.4 (ONT).

5.2.7. EPH quantification.

EPHs were extracted as described by Curiel-Alegre et al., (2022b). Soil extraction was performed from 1 g of dried soil sample and 20 mL of a mixture acetone-hexane (1:1, v:v) in a microwave oven (Ethos X, Milestone, Italy) at 150 °C for 20 min. After centrifugation (30 min at 2,500 *g*), the supernatant was filtered (0.22 µm) and

evaporated to a volume of 1 mL on a rotary evaporator (SAVANT SPD111V, Thermo). Extracted EPHs were fractionated using Isolute EPH cartridges (25 mL/5 g) previously conditioned with 30 mL hexane, at ambient pressure, and eluted with 12 and 20 mL of hexane and DCM (dichloromethane) at a flow rate of 2–3 mL min^{-1}, for the elution of aliphatic and aromatic hydrocarbons, respectively. The fractions obtained were evaporated in a stream of N_2 to above 1 mL and injected on a Varian 3900 Gas Chromatograph (GC) equipped with a Flame Ionization Detector (FID) device and a Varian CP8907 capillary column (25 m, 0.25 mm inner diameter, with a film thickness of 0.25 mm). The splitless injection was carried out with a temperature of 250 °C and a volume of 3 µL. The initial temperature of the oven was 80 °C, rising to 200 °C at 7 °C min^{-1}, then reaching 300 °C at 11 °C min^{-1}, which was maintained for 17 min. Helium was the carrier gas (74 kPa). The FID operated at 325 °C and 20 Hz. The hydrocarbon decontamination process was evaluated by determining the degradation yield for each soil sample, through the following equation (Micle & Sur, 2021):

$$\eta = \frac{C_i - C_f}{C_i} 100\ [\%]$$

Where:

η is the yield, in %;

C_f is EPHs concentration in the soil at the end of the treatment time, in mg kg^{-1};

C_i is initial EPHs concentration in the soil, in mg kg^{-1}.

5.2.8. Soil enzyme activities.

The studied activities were analysed with fluorogenic 4-methylumbelliferone (MUF) or 7-amino-4-methylcoumarin (AMC) substrates in 96-microtiter plates using a fluorometric plate-reader (GENios, TECAN) with 360 and 450 nm excitation and emission filters, (Marx et al. 2001). These measured hydrolytic activities were acid phosphatases (EC 3.1.3.2 – AcPA), alkaline phosphatase (EC 3.1.3.3 – AlkPA), α-glucosidases (EC 3.2.1.20 – aGA), β-glucosidases (EC 3.2.1.21 – bGA), N-acetyl-β-

glucosaminidase (EC 3.2.1.30 – bNAG), β-xylosidase (EC 3.2.2.27 – bXyl), leucine-aminopeptidase (EC 3.4.11.1 – LeuAMP) and sulfatase (EC 3.1.6.1 – AS).

5.2.9. Statistical analysis.

For statistically treated data, the mean and standard error of three separate experimental replicates were calculated. The normality and homogeneity of variances were then assessed using Kolmogorov's and Levene's tests. The data were analysed with a unidirectional ANOVA (meaning significant difference at $p < 0.05$ for treatment versus control for each time) and double ANOVA (showing significate difference at $p < 0.05$ for time zero versus other sampling time points) depending on the data. Significant differences between means were obtained using Tukey's test (HSD). The program Prism 8.0 (GraphPad) was also used for statistical analyses. For soil metagenomic analysis Demultiplexing and a finest basecalling was carried out by SUP algorithm of Guppy 6.0.7. Operational Taxonomic Units (OTUs) were obtained by clustering raw reads at an 80% similarity threshold using VSEARCH 2.22.1 (Rognes et al., 2016). OTU taxonomic assignment was conducted using DADA2 assign Taxonomy function (Callahan et al., 2016) and the SILVA SSU 138 database (Quast et al., 2013). Network analysis was conducted using the Extended Bayesian Information Criterion Graphical Least Absolute Shrinkage and Selection Operator (EBICglasso) method. The resulting network was visualized using the Fruchterman-Reingold algorithm, which positioned nodes (metals and metalloids) and edges (interrelationships) to reflect their strength of connection. The network analysis was performed on JASP (v 0.18.1.0), while the details. Statistical analysis of genomic data, including diversity metrics and community structure assessments, was performed using R packages Phyloseq, (McMurdie & Holmes, 2013), Microbiome (v 0.99.41), and Vegan: Community Ecology Package (v 2.6-4).

5.3. Results.

5.3.1. Characterization of properties of soil and organic amendment.

Polluted soil of the machinery park has been excavated from different areas with oil spills, mixed, divided in three mesocosms and analysed. A commercial vermicompost

(Vermicultura Duque S.L., Madrid, Spain) was chosen as organic amendment for the BAVC and BESBAVC treatments, at a rate of 2% (w:w). The physicochemical properties of the vermicompost used are as follows: organic matter 40% (w:w), pH 7.2, organic N 1.5%, soluble P_2O_5 1.5%, exchangeable K_2O 1%, Mg 1%, Fe 1.5%, Mn 356 mg kg^{-1}, Cu 100 mg kg^{-1}, total humic acids 20%, and bacterial load 10^{10} g kg^{-1}. The analytical properties of the three mesocosms are summarized in Table 11.

Table 11. Basic properties and hydrocarbon concentration of soil mesocosms at the beginning of the experience.

Basic properties	CT	BAVC	BESBAVC
Humidity (%)	0.364	4.279	10.740
Field capacity (%)	28.22	24.80	24.80
Total carbon (%)	6.21	5.75	5.98
Total nitrogen (%)	0.037	0.025	0.028
Electrical conductivity (dS m^{-1})	0.490	0.405	0.394
pH (1:5 H_2O)	8.35	8.61	8.35
Organic matter (%)	10.38	8.51	7.34
$CaCO_3$ (%)	68.13	75.27	69.31
Soluble N-NO_3 (mg Kg^{-1})	1.320	3.878	2.722
Extractable N-NH_4 (mg Kg^{-1})	18.636	22.746	17.450
Available P-PO_4 (mg Kg^{-1})	1.750	0.560	0.801
EPHs (mg Kg^{-1})	40,000	34,000	31,000
PAHs (mg Kg^{-1})	0.18	0.26	<0.16

5.3.2. Evolution of soil properties.

During the 90 days of the study, humidity and temperature data were recorded, which are shown in Supplementary Figure 2. Temperature and atmospheric humidity have been monitored with sensors placed in the air environment and under the ground at 20 cm depth, and results were similar, with minimum temperatures of 15.8 °C and a maximum of 40.4 °C for the CT and BAVC treatments, which in the case of the BESBAVC treatment reached 50.8 °C, it could be due graphite rods added to this respective treatment, and as the graphite is good conductor of heat and electricity

this could be the possible reason. Regarding the humidity data for the CT, BAVC and BESBAVC treatments, the minimum recorded were 30.5% RH (relative humidity), 0% RH and 12.1% RH, respectively, with a maximum of 100 % RH. The soil properties evolution was studied through the results obtained from the soils at different sampling times. There are no significant differences in soil pH values (Figure 24.A) in the CT throughout the experiment. In the treatments with bioaugmentation (BAVC and BESBAVC) a small rise in pH is shown at the beginning of the experiment, followed by an acidification that will be related to the microbial respiration due to the addition of the microbial consortia, vermicompost or nutrients. The electrical conductivity results (Figure 24.B) of CT and BESBAVC soil treatment showed a sightly variation during the 90 days of the experiment. BAVC treatment had significant variations in the first days before stabilizing on day 30. It was observed that the CT treatment has a much lower electrical conductivity than the two bioaugmentation treatments (BAVC and BESBAVC) because of the effect of organic nutrients does not appear in the absence of organic amendment. Both bioaugmentation treatments have shown similar values in the evolution of available orthophosphate, ammonium, and nitrate levels compared to the CT treatment, as shown in Figure 25. In Figure 25.B shows the most significant difference, a large decrease in ammonium values is observed, probably due to ammonium consumption by the microbial community. The rest of the physicochemical analyses showed no significant differences to be considered in future real scale-ups.

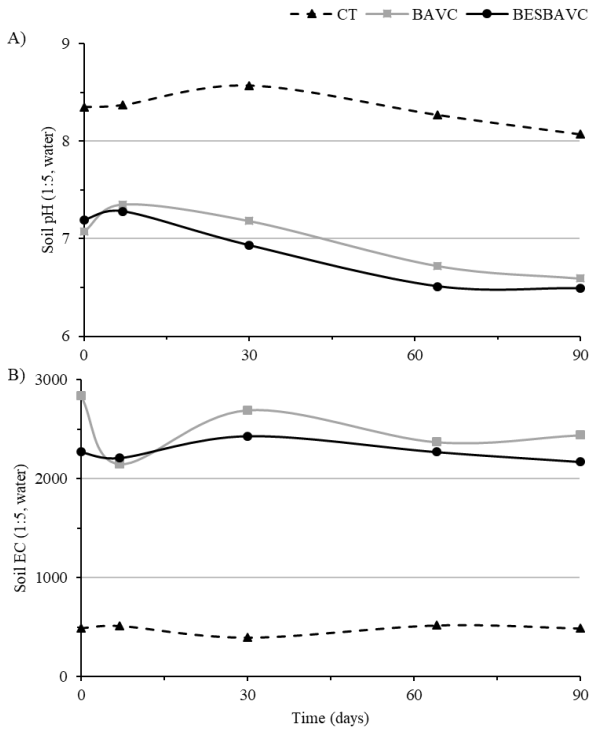


Figure 24. Soil physiochemical parameters. A) pH and B) Electrical conductivity during the pilot scale experience.

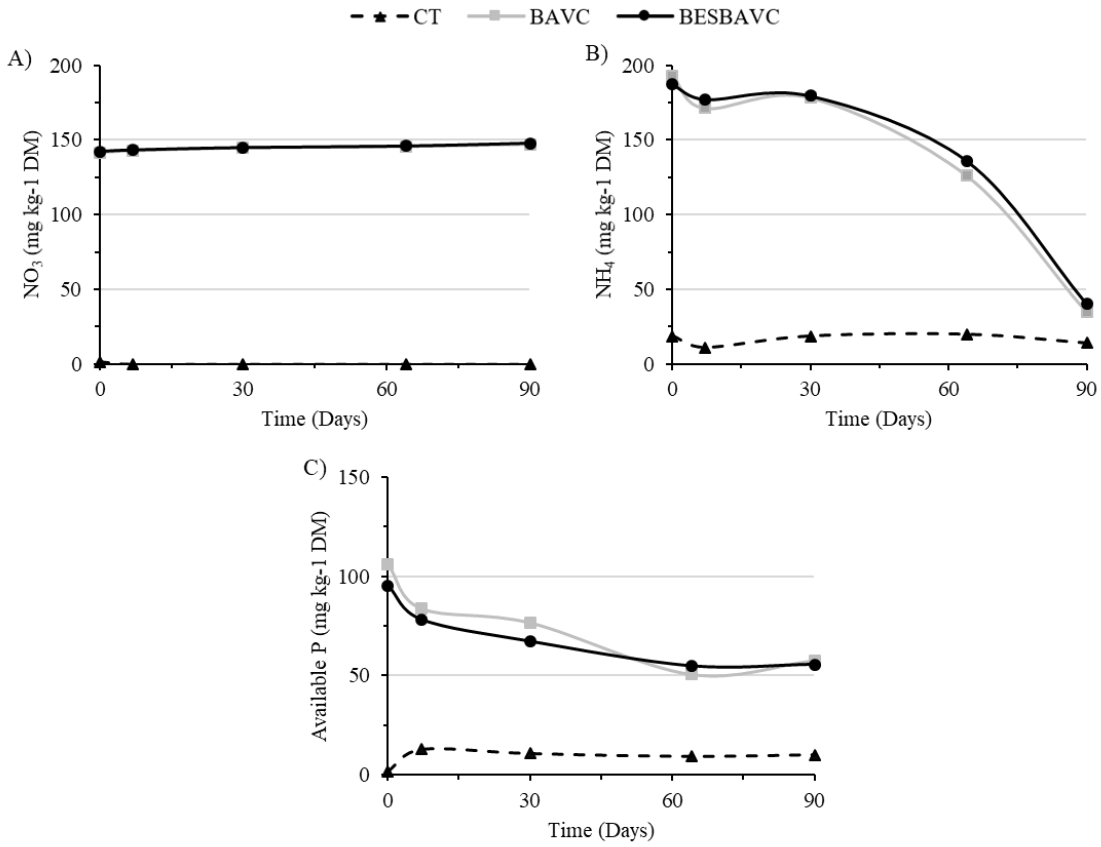


Figure 25. Nutrients evolution (mg kg^{-1} DW) at the pilot scale experiment: A) Nitrates, B) Ammonium, and C) Orthophosphate.

5.3.3. Evolution of biological parameters.

Complementary to the physicochemical analyses, hydrolytic enzyme activities were evaluated to understand the soil microbial activity evolution, as shown in Figure 26. For further information, the enzyme activity profile of the samples was analysed for five intervals (T0, T1, T2, T3 and T4), the values are provided in the Supplementary Table 2. After 7 days of incubation (T1), an increase in microbial activity began, which continued until day 60 (T3), where it stabilised and stopped moderately, with a further increase at 90 days (T4). For the TC treatment, a significant growth of phosphatases (AlkPA) was observed, which is not common in treatments without the addition of microbial consortia, because this enzyme activity is related to bacterial growth. In the case of treatment with bioaugmentation (BAVC and BESBAVC), however, microbial growth is observed in all genera that form the synthetic community, with proteases (LeuAMP) being predominant for both treatments. These results show an increase in the catabolic capacities of the microbial community.

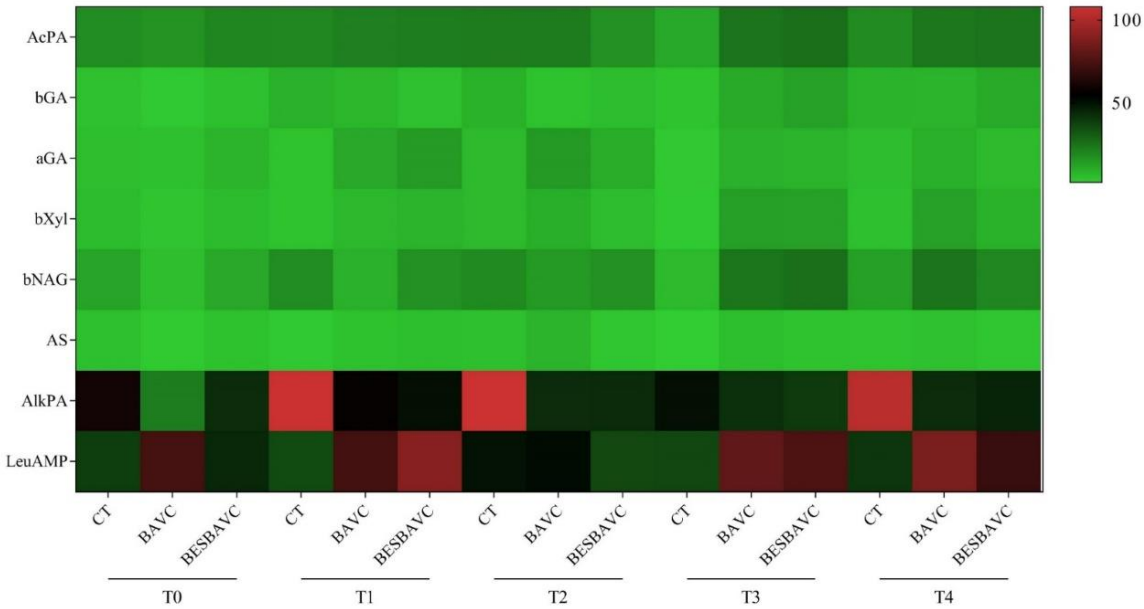


Figure 26. Heat map of the enzyme activities of the three treatments (CT, BAVC, and BESBAVC) throughout the different sampling points (T0: 0 days; T1: 7 days; T2: 30 days; T3: 60 days; and T4: 90 days). Green indicates low values and red indicates high values.

5.3.4. EPHs and PAHs biodegradation.

EPHs were analysed from the initial time to 90 days of experience, also including analyses for days 30 (T2) and 60 (T3). These analyses were carried out to know the hydrocarbon degradation efficiency of the tested treatments. Evaluation of the effectiveness of the bioremediation treatments, through the degradation percentages for EPHs were obtained at the end of the experience, after 90 days (T4). In addition, the variation of the EPHs throughout the experience can be observed in Figure 27, and details are provided in Supplementary Table 3. For CT treatment, the EPHs content (mg kg^{-1}) at 90 days (T4) was 34,000, which was initially at 40,000. While for BAVC is reduced to 3,300 mg kg^{-1}, from initial levels of 34,000, and in the case BESBAVC treatment, and from 31,000 mg kg^{-1} to 4,100 mg kg^{-1}, that represents a degradation yield (%) of 15.0, 90.3, and 86.8 for each treatment respectively, compared with the initial content. The three study strategies have shown a decrease in EPHs levels over time, for the bioaugmentation treatments (BAVC and BESBAVC) significant differences have been obtained with respect to the control (CT) at each sampling point. The BESBAVC treatment showed a more progressive and continuous decrease in EPHs degradation during the experiment, compared with the BAVC treatment, whose decrease was less abrupt and stabilized over time. In addition, significant differences were observed at 90 days of the experiment, if the 0-day value is compared with the other sampling points. The 16 EPA (Environmental Protection Agency) PAHs for each sampling period, from the start of the experiment to the end at 90 days, including 30 and 60 days, were analysed and shown in Table 12. All the treatments studied showed a decreasing trend, and at the end of the experiment the values were undetectable. Phenanthrene with a concentration of 32 µg kg^{-1}, fluoranthene with 32 µg kg^{-1}, and pyrene with 120 µg kg^{-1} were found in the highest amounts at time 0, also becoming undetectable at the last sampling point.

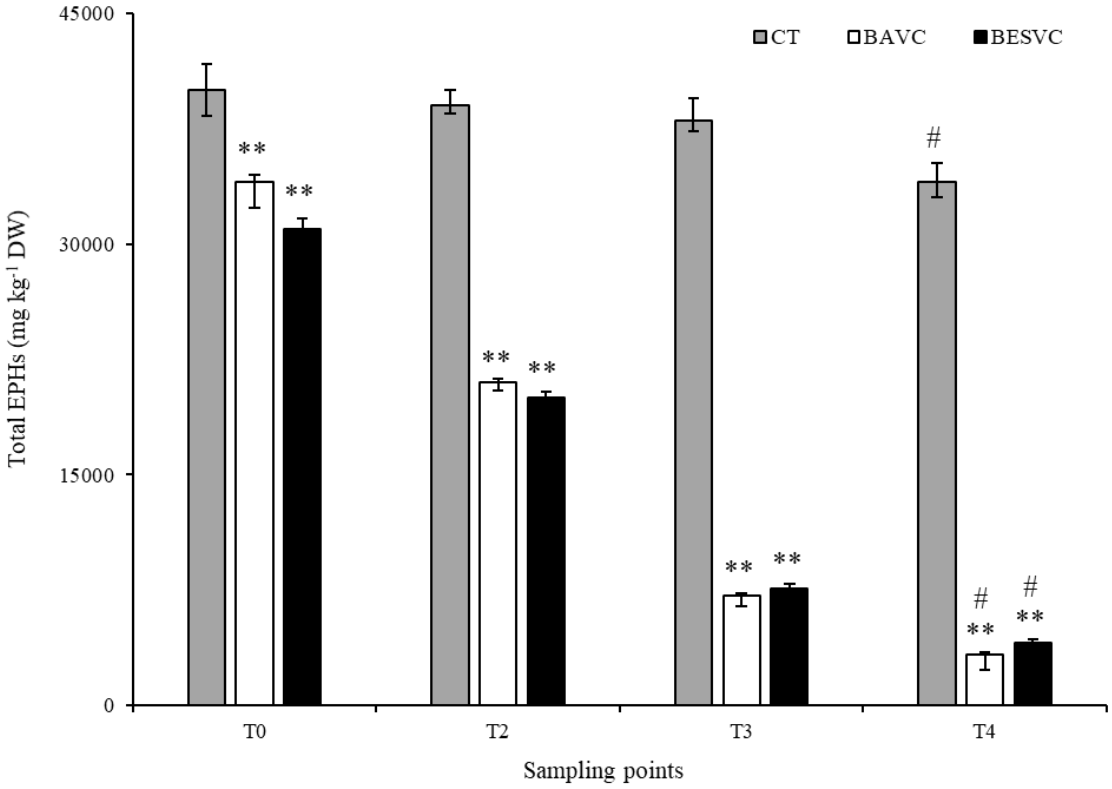


Figure 27. Content of EPHs during the pilot scale experience. Columns with different symbols showed significant statistical differences, ** meaning significant difference at $p < 0.05$ for treatment versus control for each time, while # showing significate difference at $p < 0.05$ for time zero versus other sampling time points.

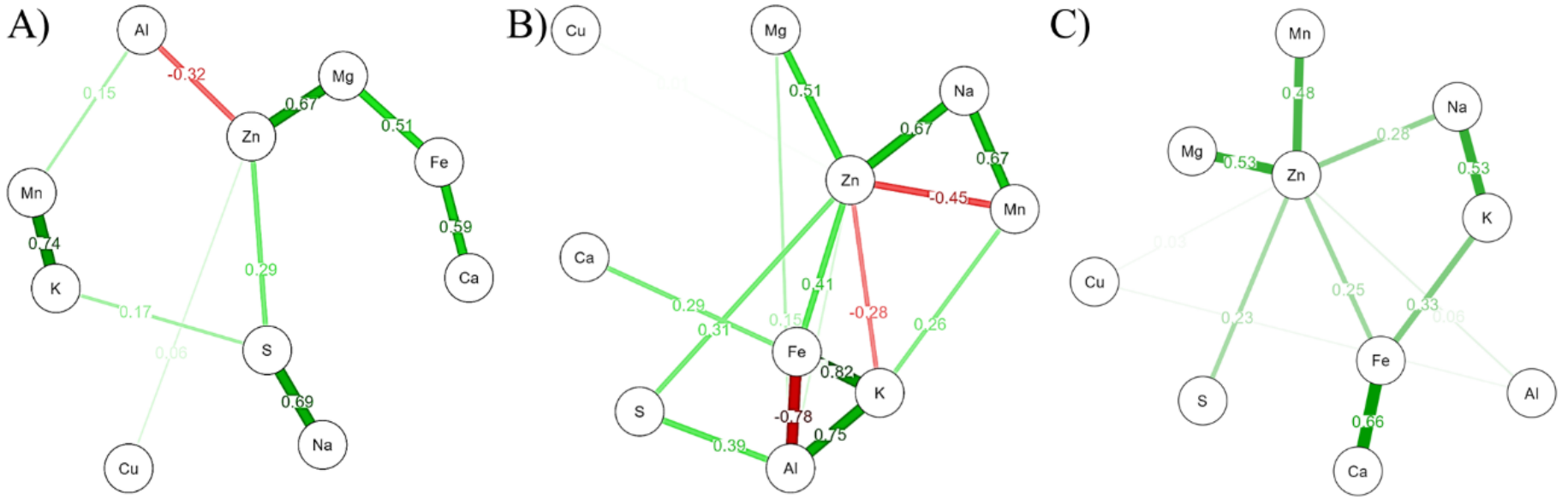
Table 12. Concentration of 16 EPA PAHs from the initial contaminated soil (T0) to the different sampling points (T2, T3, and T4) expressed in mg kg^{-1}.

Treatment ID	CT	BAVC	BESBAVC	CT	BAVC	BESBAVC	CT	BAVC	BESBAVC	CT	BAVC	BESBAVC
Sampling Point	T0			T2			T3			T4		
Naphthalene	<0.010	<0.010	<0.010	<0,010	*	<0,010	*	*	<0,010	*	*	*
Acenaphthylene	<0.010	<0.010	<0.010	<0,010	*	0,014	*	*	<0,010	*	*	*
Acenaphthene	<0.010	<0.010	<0.010	<0,010	*	<0,010	*	*	<0,010	*	*	*
Fluorine	<0.010	<0.010	<0.010	<0,010	*	<0,010	*	*	<0,010	*	*	*
Phenanthrene	0,032	<0.010	<0.010	<0,010	*	0,013	*	*	<0,010	*	*	*
Anthracene	<0.010	<0.010	<0.010	<0,010	*	<0,010	*	*	<0,010	*	*	*
Fluoranthene	0,036	<0.010	<0.010	<0,010	*	0,03	*	*	<0,010	*	*	*
Pyrene	0,12	0,078	0,076	<0,010	*	0,051	*	*	0,014	*	*	*
Benzo(a)anthracene	<0.010	<0.010	<0.010	<0,010	*	<0,010	*	*	<0,010	*	*	*
Chrysene	<0.010	<0.010	<0.010	<0,010	*	<0,010	*	*	<0,010	*	*	*
Benzo(b)fluoranthene	<0.010	<0.010	<0.010	<0,010	*	<0,010	*	*	<0,010	*	*	*
Benzo(k)fluoranthene	<0.010	<0.010	<0.010	<0,010	*	<0,010	*	*	<0,010	*	*	*
Benzo(a)pyrene	<0.010	0,18	<0.010	<0,010	*	<0,010	*	*	<0,010	*	*	*
Dibenzo(a,h)anthracene	<0.010	<0.010	<0.010	<0,010	*	<0,010	*	*	<0,010	*	*	*
Benzo(g,h,i)perylene	<0.010	<0.010	<0.010	<0,010	*	<0,010	*	*	<0,010	*	*	*
Indeno(1,2,3-c,d)pyrene	<0.010	<0.010	<0.010	<0,010	*	<0,010	*	*	<0,010	*	*	*
HAP 16 EPA	0,18	0,26	<0.16	<0,16	*	<0,16	*	*	<0,16	*	*	*

*Non-detectable fraction

5.3.5. Metals interaction network.

The results of Mehlich-3 extractable metal(loid)s are shown in Supplementary Table 1. Using raw data, a network analysis was done to understand relations between available metal and metalloids (Figure 28), while the details of the weight matrix used for the preparation of these networks is presented in the Supplementary Table 4 and Supplementary Table 5. Specifically, the study found that the maximum number of possible edges between the metals was 45. However, the network analysis showed that only a subset of these edges was present in each of the treatment groups. The CT treatment group had 10 edges, the BAVC treatment group had 16 edges, and the BESBAVC treatment group had 11 edges. The sparsity of the network, which is a measure of how spread out the edges are, was also found to be different for each treatment group. The CT treatment group had the highest sparsity (0.778), followed by the BESBAVC treatment group (0.756) and the BAVC treatment group (0.644). It indicates that only 22 to 35% interaction between the metals were observed. Zn in all treatment was showing the highest number of edges/interactions, with 4 in CT (positive interaction with Mg, S, and Cu, while negative with Al), 8 in BAVC (positive interaction with Fe, Mg, NA, S, and Cu, while negative with K and Mn), and 7 in BESBAVC (all positive with Al, Fe, Mg, Mn, Na, S, and Cu). While most interactions were positive, few negative interactions were also noted, including between Zn and Al (-0.32) in CT, Zn and K (-0.45), Zn and Mn (-0.45), and Fe with Al (-0.78). In the case of BESBAVC no negative interaction was noted, suggesting that in this treatment now antagonist behaviour was observed in the metal with increment in time. Based on the analysis, it can also be concluded that there were no significant differences between CT, BAVC, and BESBAVC, based on the sparsity (around 0.6 to 0.7 in all cases).



Network summary for CT		
Total nodes	Total non-zero edges	Sparsity
10	10 / 45	0.778

Network summary for BAVC		
Total nodes	Total non-zero edges	Sparsity
10	16 / 45	0.644

Network summary for BESBAVC		
Total nodes	Total non-zero edges	Sparsity
10	11 / 45	0.756

Figure 28. Network analysis performed using EBICglasso estimator based on Fruchterman-Reingold algorithm to assess the correlation pattern in reference to different applied treatments. A) Control, B) Bioaugmentation and Vermicompost (BAVC), and C) passive snorkel bioelectrochemical system + BAVC.

5.3.6. Evolution of the bacterial community during bioremediation.

Before inoculation, the inoculant and the vermicompost used were tested for bacterial density by determining CFU mL^{-1} or CFU g^{-1}. The inoculant that showed an OD_{600} of 1.2, contained over 10^8 CFU mL^{-1} while the vermicompost showed ca. 10^4 CFU g^{-1}, indicating that contribution of vermicompost to the bacterial community was negligible. Soil samples were also tested for bacterial abundance and the values were typically between 10^7-10^8 CFU g^{-1}, indicating a suitable bacterial community in the soil for biodegradation. Furthermore, the inoculated mesocosms (BAVC and BESBAVC) had a similar, although slightly higher, number of CFU g^{-1} than the uninoculated control treatment.

Because no statistically significant variations in biodegradation were found between treatments BAVC and BESBAVC, the evolution of the microbial community throughout the bioremediation process was only studied and compared between the treatments of CT and BAVC. This evolution study was constructed using the amplicon sequencing of 16S rRNA, in three replicates for the control and BAVC samples, in the five timepoints (T0 to T4). As shown in Figure 29.A. The taxonomy distribution according to the relative bacterial OTUs abundance of the twenty-three major bacterial families identified, indicates clear variation between the families presents in the control and the BAVC sample (Figure 29.A) at times T1 to T4. At T4 more than 50 percent of the OTUs identified in the BAVC treatment belonged to families *Alcaligenaceae* and *Nocardiaceae*. These families were less predominant in CT sample. It is also evident that the bacterial composition of the vermicompost bacterial population is completely different than that of the soil and due to the limited number of bacteria, it is not expected to have a major influence in the microbial processes in biopiles. As shown in Figure 29.B, alpha-diversity, presented as Shannon index revealed than the control and the BAVC soil possessed a similar value at T0. However, after the inoculation, Shannon index dropped in all BAVC samples, indicating that the addition of the consortium produced a big impact of inoculation. Furthermore, analysis of beta-diversity (Figure 30), showed that the communities of the control treatment clustered together and with the T_0 sample of the treated soil, indicating that at the

experiment beginning both soils harboured similar bacterial populations, and that this CT community did not have significant changes during the treatment. Conversely, the community in the BAVC treated, contaminated soil, strongly changed after inoculation and this change was maintained through the bioremediation process. The community in the vermicompost was very different from the other two communities.

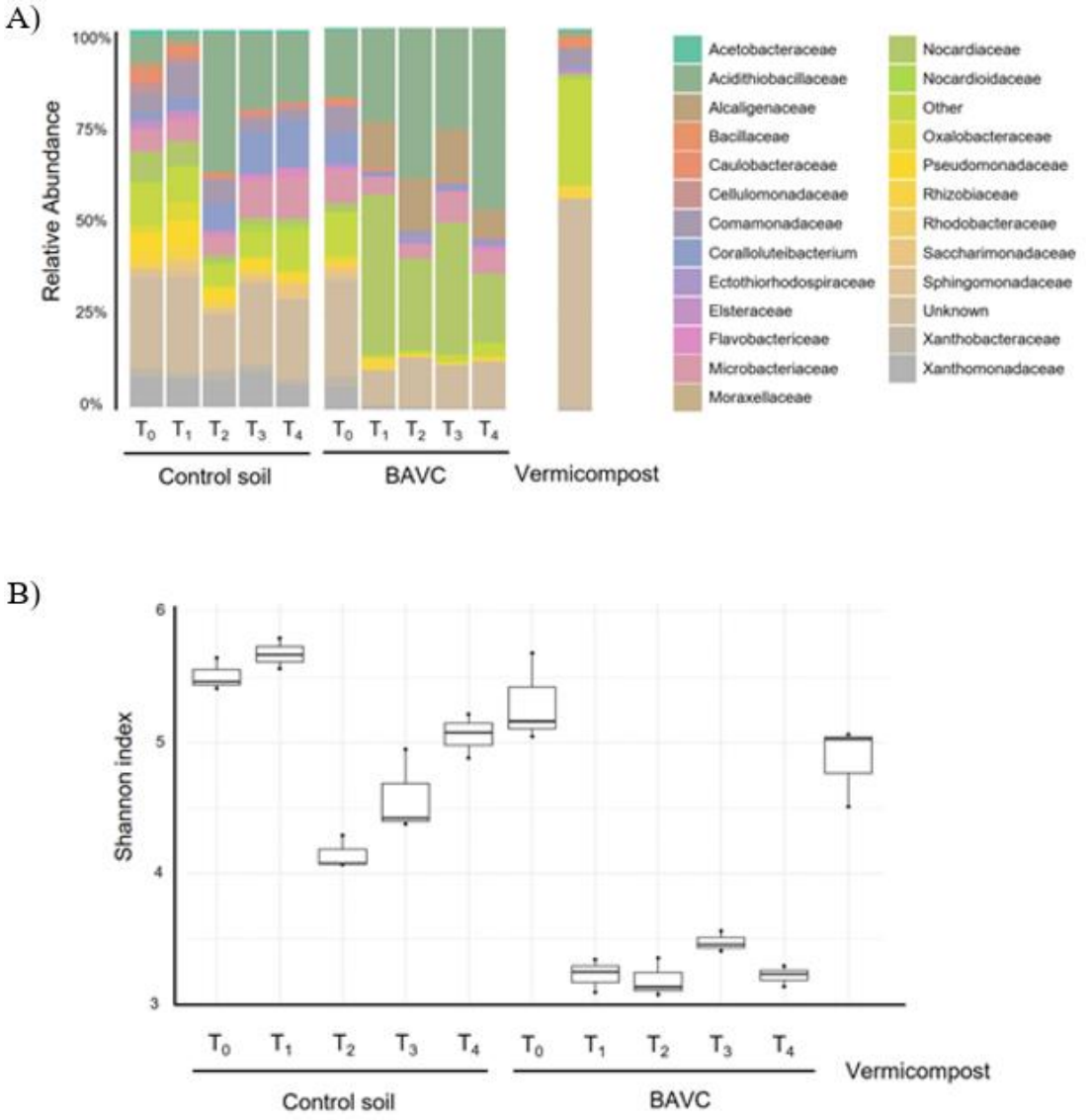


Figure 29. Compositional analysis of the control and treated soil communities, A) Bacterial relative abundances at the level of family for the different samples at the different sampling times. Relative bacterial OTUs abundance at the level of family of the mesocosms at each of the timepoints for the 23 most abundant families. The remaining classes were grouped under the category Other. The bar-plots represent the sum of OTUs from the three replicates. B) Boxplot representing the bacterial Shannon index values for each timepoint and sample. Differences between Shannon index values at the different timepoints were assessed with the Kruskal-Wallis test. Three independent biological replicates of each sample were analysed.

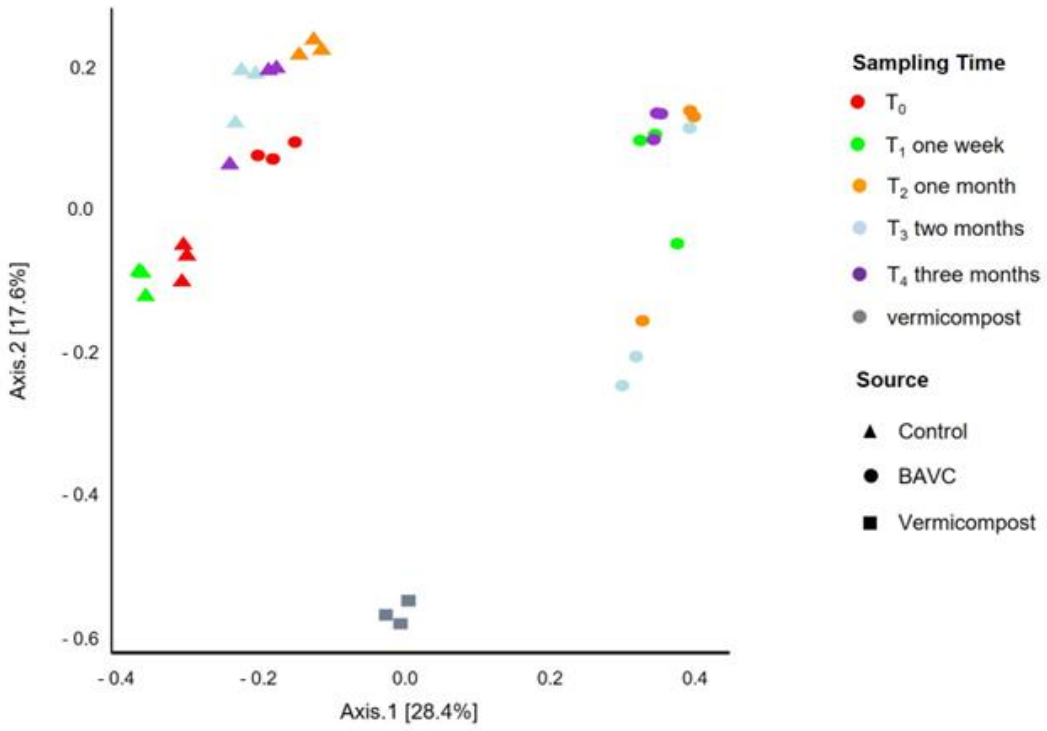


Figure 30. Clustering analysis of the bacterial communities within CT and BAVC at the different timepoints. Principal coordinate analysis (PCoA) of mesocosms using Bray-Curtis distances. Colours according to mesocosm, shapes according to timepoint and size according to Shannon index. Averages for each mesocosms and sampling time are represented.

5.4. Discussion.

Bioaugmentation has been widely recognized as an effective procedure used for soil decontamination that are TPHs contaminated (Khan et al., 2017). In these studies, bioaugmentation processes using a microbial inoculum, which was isolated from the TPHs polluted soil, was effective for contaminated soil treatment. Bakina et al., (2021) also analysed effectiveness of the bioaugmentation to enhance hydrocarbon remediation and removal from soils that were oil-contaminated, emphasizing the environmentally friendly nature of bioaugmentation as a method for remediation of TPHs pollution. Nwankwegu et al., (2022) provided an overview of the advantages of bioaugmentation technologies for soil remediation, as they can be cost effective treatments, performed in situ, without the need of applying hazardous chemical inputs, and able to achieve high biodegradation levels of organic pollutants if favourable

environmental conditions are maintained. Martínez-Rivera & Cardona-Gallo, (2021), studying the degradation of oil spills in tropical soils, demonstrate the successfulness of bioaugmentation and biostimulation methods combining inoculation with compost, nutrients, surfactants and other organic amendments, such is leonardite, as promising strategies for the treatment of oil spills. The results presented here show that soil humectation has a positive impact on bacterial diversity after 7 days of incubation (T2) but this diversity decreased at T3 when humectation was incremented at 50% WRC, possibly by a decrease in aeration. The inoculation with the consortium had a profound impact on the bacterial community of polluted soil, resulting in changes both in alpha and beta diversity; alpha diversity decreased drastically in the inoculated soil because of the predominance of Nocardiaceae and Alcaligenaceae families that was maintained during the 90 days of incubation. These results indicate that after inoculation large changes in bacterial populations arose. Martínez-Rivera & Cardona-Gallo, (2021) also found that their biostimulation treatments decreased alpha diversity of the polluted soil, due to the increase of Actinobacteria filum in treated soils. Our results also agreed with the effect of bioaugmentation in Ecopiles shown by Martínez-Cuesta et al., (2023), besides in this case the predominance between the different families of bacteria changes during a year of monitoring obeying to environmental changes These studies provide consistent testimonies that are advocating the potential of bioaugmentation application, as a promising technique capable to perform biological treatment of hydrocarbon contaminated soil and other matrices. All these studies also highlight the environmentally friendly nature, efficacy, and potential scalability of bioaugmentation for environmentally friendly, sustainable, efficient and low-cost solutions.

Furthermore, the results of our study further support previous studies, demonstrating the effectiveness of the combination of bioaugmentation techniques with organic amendments and MES for soil bioremediation. Our results also highlight the importance of selecting an adequate bioremediation strategy depending on the type of oil contamination. In this case, the incidence of soil pollution with motor oils, along with lubricants, was associated with a higher concentration of long-chain (C21-C35) petroleum hydrocarbon contaminants (Supplementary Table 3), which are more difficult to degrade. Zhang et al., (2019) studied the feasibility of a biological degradation-based

remediation process applied to contaminated soils from a decommissioned refinery and demonstrated the effectiveness of combined bioremediation strategies, including bioaugmentation-assisted landfarming and BES, for the TPHs contaminated soil treatment. The study concluded that the co-application of bioremediation strategies was the most beneficial choice, to perform TPHs remediation. However, considering the results obtained in the current investigation, finding no statistically momentous alterations were observed between the two bioaugmentation treatments (BAVC and BESBAVC), it is not advisable to use the MES technique, since the economic cost will be higher, and the results were similar.

The addition of compost has been recognized to be an advantageous approach for the recovery and remediation of contaminated soils, affected with a wide number of pollutants (Hussain et al., 2018). These organic pollutants include TPHs, aromatic compounds (including the notorious contaminants like BTEX and PAHs), and halogenated hydrocarbons, including chlorophenols, explosives, and pesticides (Khan et al., 2018; Hussain et al., 2018, 2022). An organic amendment for soil such as compost can stabilize the soil structure and promote the activity of degrading microorganisms by improving oxygen diffusion, water, and nutrient availability. It is also very inexpensive because it is derived from biodegradable trash, therefore it can help to the environmental sustainability of remediation procedures. The use of compost amendments positively affects the activity, size, and composition of the soil microbial community, although, their effects were mainly due to the physicochemical characteristics of compost matrix rather than to compost-borne microorganisms (Saison et al., 2006). The metagenome analysis of the bacterial community, in the present work, suggested that the bacterial population naturally present in vermicompost are not expected to have a major effect on biodegradation, since the bacterial levels are very low in comparison with those already present in soil or with the bioaugmentation consortium and the main role of vermicompost was to provide nutrients and stimulate microbial activities. In a previous experiment at a lab scale (Curiel-Alegre et al., 2022b), the introduction of 2% (w:w) of vermicompost stimulated hydrocarbon removal in this TPHs' contaminated soil with a clear increase in basal soil respiration, bacterial biomass, and enzyme activities; in this work, the same results were obtained at a pilot scale, increasing TPHs degradation and

stimulating other soil biochemical processes such are nitrification and AlkPA and LeuAMP. Koolivand et al., (2020) confirmed the effectiveness of enriching soil and vermicompost mixtures with native bacterial consortia, that was isolated from oily petroleum sludge, increased the degradation rates form 31-49% in vermicompost to 85-91% for the bioaugmented vermicompost.

The bioelectrochemical systems (BES) usage is also reported to have significant implications for the field of bioremediation of TPHs contaminated soils and other environmental matrices. MES is a passive BES that uses microbes to generate an electric current field, enhancing the contaminant biodegradation, removal, and reduction of pollutant mobility due to an efficient electron transfer, generated during growth. Several research studies have highlighted the advantages and effectiveness of BES in soil bioremediation. Studies such as Lan et al. (2023) show BES-based systems as an efficient and biologically responsive method towards the bioremediation of polluted soil. Similarly, they proposed that BES energized and enhanced the anaerobic oxidation of various organic wastes to minimize soil and groundwater contaminants such as petroleum hydrocarbons and halogenated chemicals. The use of BES in the present study improved the biodegradation rates of TPH, in line with previous studies. The aim of this research investigation was to establish the most appropriate techniques to carry out on a real scale, so after the results were obtained it is seen that they can be scaled, as well Al-Mailem et al., (2019) could be observed in a similar study achieving effective treatments to be tested on a real scale. According to prior research, microbial consortia immobilized on plant-based residues increase hydrocarbon breakdown efficiency after 30 days, demonstrating relevance for beneficial impact owing to growth-promoting transporter and extra supply of C and N (Tao et al., 2019). However, this result is associated with soil respiration (Pacwa-Płociniczak et al., 2019) and the accumulation of metabolites from enzymatic action (Meyer et al., 2018). For the first 30 days, the crude oil (TPHs) is under degradation by microbial activity, and the activation of alkane-degrading enzymes is linked to an increase of amino compounds (Curiel-Alegre et al., 2022b). Longer incubation time removal PAHs and the role of biostimulation gain importance, while bioaugmentation did not contribute significantly to the process according to Haleyur et al., (2019) study. It might be in concordance with the quantitative

reduction of nutrients and the increase of metabolites in the edaphic medium that affect ecotoxicity, translating into reduced viability in eukaryotic organisms as shown in the results. While *Pseudomonas sp.* enhance TPH decontamination of the soil mediated by rhamnolipid production under bioremediation treatments (Ángeles & Refugio, 2013; Ramadass et al., 2018).

5.5. Conclusions.

The results obtained for the optimized bioaugmentation treatments with the application of an organic amendment (BAVC) and the use of a MES (BESBAVC) after 90 days of experience were satisfactory to achieve green, sustainable, efficient, and low-cost solutions for soil bioremediation. The degradation of soil EPHs has reached very promising results with significance between the bioaugmentation treatments (BAVC and BESBAVC) and the control treatment. No significant differences were observed between the BAVC and BESBAVC treatments, achieving similar biodegradation values. Therefore, it is expected that the BAVC technique will be used to bring this experience to full scale, since it has achieved very promising results and with lower economic costs than BESBAVC. After 90 days of experience, a higher concentration of long-chain pollutant petroleum hydrocarbons such as C21-C30, followed by the C30-C35 fraction, was observed, as shown in Supplementary Table 3. This has been associated with the fact that contamination by heavy hydrocarbon fractions is related to the presence in the treated soil of motor oils and lubricants, which are much more difficult to degrade than the hydrocarbons present in gasoline or diesel. To achieve significant biodegradation of TPHs in the bioaugmentation treatments by the microbial community previously isolated from the soil, temperature, humidity, and aeration were key factors. The soil under study contaminated with TPHs from motor oils and other types of hydrocarbons contains a significant microbial population capable of degrading hydrocarbons and which is metabolically very active. The bioaugmentation inoculum, obtained from the isolation of this microbial community and its subsequent growth to treat the soil, the addition of vermicompost and the use of BES favour the biodegradation rates of TPHs.

5.6. References.

Abdullah, S. R. S., Al-Baldawi, I. A., Almansoory, A. F., Purwanti, I. F., Al-Sbani, N. H., & Sharuddin, S. S. N. (2020). Plant-assisted remediation of hydrocarbons in water and soil: Application, mechanisms, challenges and opportunities. *Chemosphere*, 247, 125932. https://doi.org/10.1016/j.chemosphere.2020.125932

Adipah, S. (2019). Introduction of petroleum hydrocarbons contaminants and its human effects. *Journal of Environmental Science and Public Health,* 3, 1-9. https://doi.org/10.26502/jesph.96120043

Akbari, A., & Ghoshal, S. (2014). Pilot-scale bioremediation of a petroleum hydrocarbon-contaminated clayey soil from a sub-Arctic site. *Journal of Hazardous Materials*, 280, 595–602. https://doi.org/10.1016/j.jhazmat.2014.08.016

Al-Mailem, D. M., Kansour, M. K., Radwan, S. S. (2019). Cross-bioaugmentation among four remote soil samples contaminated with oil exerted just inconsistent effects on oil-bioremediation. *Frontiers in Microbiology*, 10, 2827. https://doi.org/10.3389/fmicb.2019.02827

Ambaye, T. G., Vaccari, M., Franzetti, A., Prasad, S., Formicola, F., Rosatelli, A., Hassani, A., Aminabhavi, T. M., & Rtimi, S. (2023). Microbial electrochemical bioremediation of petroleum hydrocarbons (PHCs) pollution: Recent advances and outlook. *Chemical Engineering Journal*, 452, 139372. https://doi.org/10.1016/j.cej.2022.139372

Ángeles, M. T., Refugio, R. V. (2013). In situ biosurfactant production and hydrocarbon removal by *Pseudomonas putida* CB-100 in bioaugmented and biostimulated oil-contaminated soil. *Brazilian Journal of Microbiology*, 44, 595-605. https://doi.org/10.1590/S1517-83822013000200040

Aulenta, F., Palma, E., Marzocchi, U., Cruz Viggi, C., Rossetti, S., & Scoma, A. (2021). Enhanced hydrocarbons biodegradation at deep-sea hydrostatic pressure with microbial electrochemical snorkels. *Catalysts*, 11(2), 263. https://doi.org/10.3390/catal11020263

Ayilara, M. S., & Babalola, O. O. (2023). Bioremediation of environmental wastes: the role of microorganisms. *Frontiers in Agronomy*, 5, 1183691. https://doi.org/10.3389/fagro.2023.1183691

Bakina, L. G., Chugunova, M. V., Polyak, Y. M., Mayachkina, N. V., & Gerasimov, A. O. (2021). Bioaugmentation: possible scenarios due to application of bacterial preparations for remediation of oil-contaminated soil. *Environmental Geochemistry and Health,* 43(6), 2347–2356. https://doi.org/10.1007/s10653-020-00755-4

Bedard, D. L., Unterman, R., Bopp, L. H., Brennan, M. J., Haberl, M. L., & Johnson, C. (1986). Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. *Applied and environmental microbiology*, 51(4), 761-768. https://doi.org/10.1128/aem.51.4.761-768.1986

Brazil, G. M., Kenefick, L., Callanan, M., Haro, A., De Lorenzo, V., Dowling, D. N., & O'gara, F. (1995). Construction of a rhizosphere pseudomonad with potential to degrade polychlorinated biphenyls and detection of bph gene expression in the rhizosphere. *Applied and Environmental Microbiology*, 61(5), 1946-1952. https://doi.org/10.1128/aem.61.5.1946-1952.1995

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, 13, 581-583. https://doi.org/10.1038/nmeth.3869

Chen, F., Li, X., Zhu, Q., Ma, J., Hou, H., & Zhang, S. (2019). Bioremediation of petroleum-contaminated soil enhanced by aged refuse. *Chemosphere*, 222, 98-105. https://doi.org/10.1016/j.chemosphere.2019.01.122

Viggi, C. C., Tucci, M., Resitano, M., Matturro, B., Crognale, S., Feigl, V., Molnár, M., Rossetti, S., & Aulenta, F. (2022). Passive electrobioremediation approaches for enhancing hydrocarbons biodegradation in contaminated soils. *Science of The Total Environment*, 845, 157325. https://doi.org/10.1016/j.scitotenv.2022.157325

Curiel-Alegre, S., Velasco-Arroyo, B., Martínez, A., Rumbo, C., Tamayo-Ramos, J. A., Khan, A. A., Rad, C., & Barros, R. (2022a). Application of a microbial consortium immobilized onto a biochar for the remediation of a polluted soil with hydrocarbons. *Revista de Ciências Agrárias*, 45(4), 295–299. https://doi.org/https://doi.org/10.19084/rca.28440

Curiel-Alegre, S., Velasco-Arroyo, B., Rumbo, C., Khan, A. H. A., Tamayo-Ramos, J. A., Rad, C., Gallego, J.L.R., & Barros, R. (2022b). Evaluation of biostimulation, bioaugmentation, and organic amendments application on the bioremediation of recalcitrant hydrocarbons of soil. *Chemosphere*, 307, 135638. https://doi.org/10.1016/j.chemosphere.2022.135638

Garrido-Sanz, D., Redondo-Nieto, M., Guirado, M., Pindado Jiménez, O., Millán, R., Martin, M., & Rivilla, R. (2019). Metagenomic Insights into the Bacterial Functions of a Diesel-Degrading Consortium for the Rhizoremediation of Diesel-Polluted Soil. *Genes*, 10(6), 456. https://doi.org/10.3390/genes10060456

Gautam, K., Sharma, P., Dwivedi, S., Singh, A., Gaur, V. K., Varjani, S., Srivastava, J.K., Pandey, A., Chang, J.S. & Ngo, H. H. (2023). A review on control and abatement of soil pollution by heavy metals: Emphasis on artificial intelligence in recovery of contaminated soil. *Environmental Research*, 225, 115592. https://doi.org/10.1016/j.envres.2023.115592

Grifoni, M., Rosellini, I., Angelini, P., Petruzzelli, G., & Pezzarossa, B. (2020). The effect of residual hydrocarbons in soil following oil spillages on the growth of *Zea mays* plants. *Environmental Pollution*, 265, 114950. https://doi.org/10.1016/j.envpol.2020.114950

Haleyur, N., Shahsavari, E., Jain, S. S., Koshlaf, E., Ravindran, V. B., Morrison, P. D., Osborn, A.M., & Ball, A. S. (2019). Influence of bioaugmentation and biostimulation on PAH degradation in aged contaminated soils: Response and dynamics of the bacterial community. *Journal of environmental management*, 238, 49-58. https://doi.org/10.1016/j.jenvman.2019.02.115

Hoareau, M., Erable, B., & Bergel, A. (2019). Microbial electrochemical snorkels (MESs): A budding technology for multiple applications. A mini review. *Electrochemistry Communications*, 104, 106473. https://doi.org/10.1016/j.elecom.2019.05.022

Hussain, F., Hussain, I., Khan, A. H. A., Muhammad, Y. S., Iqbal, M., Soja, G., Reichenauer, T. G., Zeshan, & Yousaf, S. (2018). Combined application of biochar, compost, and bacterial consortia with Italian ryegrass enhanced phytoremediation of petroleum hydrocarbon contaminated soil. *Environmental and Experimental Botany,* 153, 80–88. https://doi.org/10.1016/j.envexpbot.2018.05.012

Hussain, F., Khan, A. H. A., Hussain, I., Farooqi, A., Muhammad, Y. S., Iqbal, M., Arslan, M., & Yousaf, S. (2022). Soil conditioners improve rhizodegradation of aged petroleum hydrocarbons and enhance the growth of *Lolium multiflorum*. *Environmental Science and Pollution Research*, 29, 9097–9109. https://doi.org/10.1007/s11356-021-16149-7

Imam, A., Suman, S. K., Ghosh, D., & Kanaujia, P. K. (2019). Analytical approaches used in monitoring the bioremediation of hydrocarbons in petroleum-contaminated soil and

sludge. *TrAC Trends in Analytical Chemistry*, 118, 50-64. https://doi.org/10.1016/j.trac.2019.05.023

Jin, S., & Fallgren, P. H. (2022). Feasibility of using bioelectrochemical systems for bioremediation. *Microbial Biodegradation and Bioremediation. Techniques and Case Studies for Environmental Pollution*. 493–507. https://doi.org/10.1016/b978-0-323-85455-9.00026-6

Kebede, G., Tafese, T., Abda, E. M., Kamaraj, M., & Assefa, F. (2021). Factors Influencing the Bacterial Bioremediation of Hydrocarbon Contaminants in the Soil: Mechanisms and Impacts. *Journal of Chemistry*, 2021, 1-17. https://doi.org/10.1155/2021/9823362

Khan, A. H. A., Ayaz, M., Arshad, M., Yousaf, S., Khan, M. A., Anees, M., Sultan, A., Nawaz, I., & Iqbal, M. (2019). Biogeochemical cycle, occurrence and biological treatments of polycyclic aromatic hydrocarbons (PAHs). *Iranian Journal of Science and Technology, Transactions A: Science*, 43, 1393-1410. https://doi.org/10.1007/s40995-017-0393-8

Khan, A. H. A., Tanveer, S., Alia, S., Anees, M., Sultan, A., Iqbal, M., & Yousaf, S. (2017). Role of nutrients in bacterial biosurfactant production and effect of biosurfactant production on petroleum hydrocarbon biodegradation. *Ecological Engineering*, 104, 158–164. https://doi.org/10.1016/j.ecoleng.2017.04.023

Khan, A. H. A., Tanveer, S., Anees, M., Muhammad Y. S., Iqbal, M., & Yousaf, S., (2016). Role of nutrients and illuminance in predicting the fate of fungal mediated petroleum hydrocarbon degradation and biomass production. *Journal of Environmental Management*, 176, 54–60. https://doi.org/10.1016/j.jenvman.2016.03.040

Khan, A. H. A., Tanveer, S., Kiyani, A., Barros, R., Iqbal, M., & Yousaf, S. (2023). Biosurfactant-producing *Aspergillus*, *Penicillium*, and *Candida* Performed Higher Biodegradation of Diesel Oil than a Non-producing Fungal Strain. *Applied Biochemistry and Microbiology,* 59, 282–289. https://doi.org/10.1134/S0003683823030109

Koolivand, A., Saeedi, R., Coulon, F., Kumar, V., Villaseñor, J., Asghari, F., & Hesampoor, F. (2020). Bioremediation of petroleum hydrocarbons by vermicomposting process bioaugmentated with indigenous bacterial consortium isolated from petroleum oily sludge. *Ecotoxicology and Environmental Safety,* 198, 110645. https://doi.org/10.1016/j.ecoenv.2020.110645

Lan, J., Wen, F., Ren, Y., Liu, G., Jiang, Y., Wang, Z., & Zhu, X. (2023). An overview of bioelectrokinetic and bioelectrochemical remediation of petroleum-contaminated soils. *Environmental Science and Ecotechnology*, 16, 100278. https://doi.org/10.1016/j.ese.2023.100278

Martínez-Álvarez, L. M., Ruberto, L. A. M., Gurevich, J. M., Mac Cormack, W. P. (2020). Environmental factors affecting reproducibility of bioremediation field assays in Antarctica. *Cold Regions Science and Technology*, 169, 102915. https://doi.org/10.1016/j.coldregions.2019.102915

Martínez-Cuesta, R., Conlon, R., Wang, M., Blanco-Romero, E., Durán, D., Redondo-Nieto, M., Dowling, D., Garrido-Sanz, D., Martín, M., Germaine, K., & Rivilla, R. (2023). Field scale biodegradation of total petroleum hydrocarbons and soil restoration by Ecopiles: microbiological analysis of the process. *Frontiers in Microbiology*, 14, 1158130.https://doi.org/10.3389/fmicb.2023.1158130

Martínez-Rivera, A., & Cardona-Gallo, S. A. (2021). Oil bioremediation in soils contaminated with oil spills in tropical environments. *Anais da Academia Brasileira de Ciências*, 93(4), e20201102. https://doi.org/10.1590/0001-3765202120201102

Marx, M. C., Wood, M., & Jarvis, S. C. (2001). A microplate fluorometric assay for the study of enzyme diversity in soils. *Soil Biology and Biochemistry*, 33(12-13), 1633-1640. https://doi.org/10.1016/S0038-0717(01)00079-7

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS One*, 8(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Mehlich, A. (1984) Mehlich 3 soil test extractant: A modification of Mehlich 2 extractant. *Communications in Soil Science and Plant Analysis*, 15(12), 1409-1416. https://doi.org/10.1080/00103628409367568

Meyer, D. D., Beker, S. A., Heck, K., Peralba, M. D. C. R., & Bento, F. M. (2018). Simulation of a surface spill of different diesel/biodiesel mixtures in an ultisol, using natural attenuation and bioaugmentation/biostimulation. *Anais da Academia Brasileira de Ciências*, 90, 2741-2752. https://doi.org/10.1590/0001-3765201820170268

Micle, V., & Sur, I. M. (2021). Experimental investigation of a pilot-scale concerning ex-situ bioremediation of petroleum hydrocarbons contaminated soils. *Sustainability*, 13(15), 8165. https://doi.org/10.3390/su13158165

Nobili, S., Masin, C. E., Zalazar, C. S., & Lescano, M. R. (2022). Bioremediation of hydrocarbon contaminated soil using local organic materials and earthworms. *Environmental Pollution*, 314, 120169. https://doi.org/10.1016/j.envpol.2022.120169

Nwankwegu, A. S., Zhang, L., Xie, D., Onwosi, C. O., Muhammad, W. I., Odoh, C. K., Sam, K., & Idenyi, J. N. (2022). Bioaugmentation as a green technology for hydrocarbon pollution remediation. Problems and prospects. *Journal of Environmental Management,* 304, 114313. https://doi.org/10.1016/j.jenvman.2021.114313

Pacwa-Płociniczak, M., Czapla, J., Płociniczak, T., & Piotrowska-Seget, Z. (2019). The effect of bioaugmentation of petroleum-contaminated soil with *Rhodococcus erythropolis* strains on removal of petroleum from soil. *Ecotoxicology and Environmental Safety*, 169, 615-622. https://doi.org/10.1016/j.ecoenv.2018.11.081

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, 41(D1), D590-D596. https://doi.org/10.1093/nar/gks1219

Ramadass, K., Megharaj, M., Venkateswarlu, K., & Naidu, R. (2018). Bioavailability of weathered hydrocarbons in engine oil-contaminated soil: Impact of bioaugmentation mediated by *Pseudomonas* spp. on bioremediation. *Science of The Total Environment*, 636, 968-974. https://doi.org/10.1016/j.scitotenv.2018.04.379

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: A versatile open source tool for metagenomics. *PeerJ*, 4, e2584. https://doi.org/10.7717/peerj.2584

Saison, C., Degrange, V., Oliver, R., Millard, P., Commeaux, C., Montange, D., & Le Roux, X. (2006). Alteration and resilience of the soil microbial community following compost amendment: effects of compost level and compost-borne microbial community. *Environmental microbiology*, 8(2), 247-257. https://doi.org/10.1111/j.1462-2920.2005.00892.x

Tao, K., Zhang, X., Chen, X., Liu, X., Hu, X., & Yuan, X. (2019). Response of soil bacterial community to bioaugmentation with a plant residue-immobilized bacterial consortium for crude oil removal. *Chemosphere*, 222, 831-838. https://doi.org/10.1016/j.chemosphere.2019.01.133

Truskewycz, A., Gundry, T. D., Khudur, L. S., Kolobaric, A., Taha, M., Aburto-Medina, A., Ball, A. S., & Shahsavari, E. (2019). Petroleum hydrocarbon contamination in terrestrial ecosystems—fate and microbial responses. *Molecules*, 24(18), 3400. https://doi.org/10.3390/molecules24183400

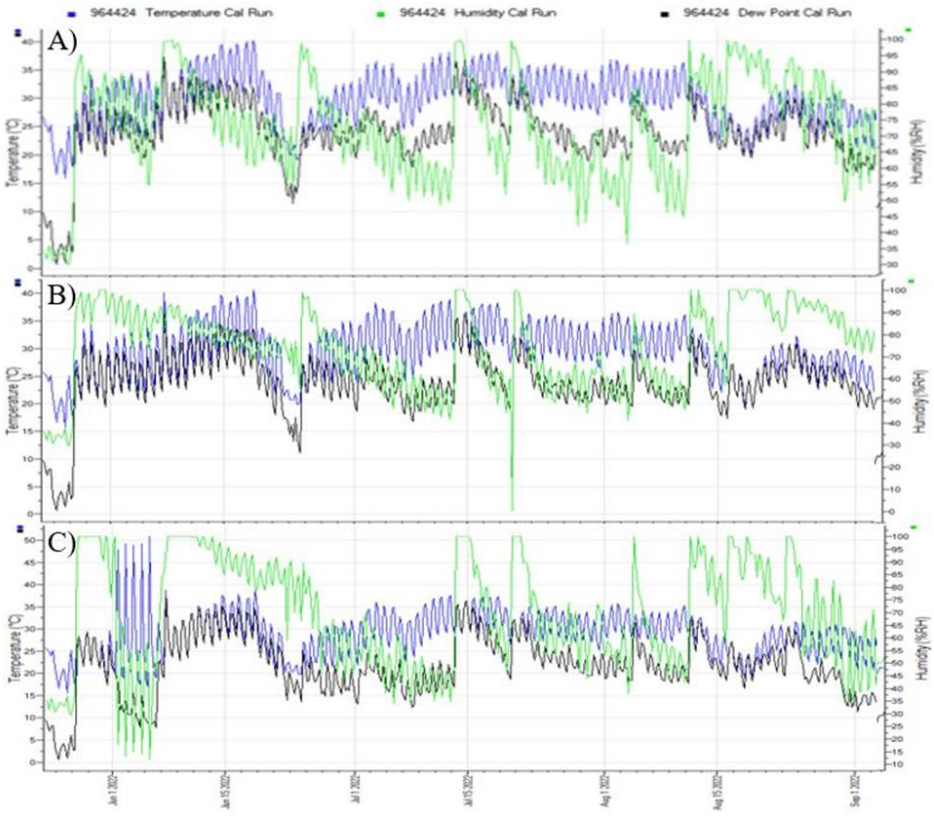
Wang, H., Xing, L., Zhang, H., Gui, C., Jin, S., Lin, H., Li, Q., & Cheng, C. (2021). Key factors to enhance soil remediation by bioelectrochemical systems (BESs): A review. *Chemical Engineering Journal*, 419, 129600. https://doi.org/10.1016/j.cej.2021.129600

Yousaf, U., Khan, A. H. A., Farooqi, A., Muhammad, Y. S., Barros, R., Tamayo-Ramos, J. A., Iqbal, M., & Yousaf, S. (2022). Interactive effect of biochar and compost with *Poaceae* and *Fabaceae* plants on remediation of total petroleum hydrocarbons in crude oil contaminated soil. *Chemosphere*, 286, 131782. https://doi.org/10.1016/j.chemosphere.2021.131782

Zhang, B., Zhang, L., & Zhang, X. (2019). Bioremediation of petroleum hydrocarbon-contaminated soil by petroleum-degrading bacteria immobilized on biochar. *RSC Advances*, 9, 35304–35311. https://doi.org/10.1039/c9ra06726d

5.7. Supplementary material.

Supplementary Figure 1. The application of nutrients and bacterial suspension at the start of experiment during first addition of bacterial inoculants spray.



Supplementary Figure 2. Results of monitorization for temperature and moisture of: A) CT treatment, B) BAVC treatment, and C) BESBAVC treatment.

Supplementary Figure 3. Pilot scale in ACCIONA´s Facilities - off site (Alcobendas, Madrid): A) three containers for treatments under study, B) Visualization of soils under study, and C) sample collected using auger.

Supplementary Table 1. The evolution of extractable metals in the soil of applied treatment with reference to time (0, 7, 30, 60, and 90 days).

CT	Al	Ca	Fe	K	Mg	Mn	Na	S	Cu	Zn
T0	0.299 ± 0.110	3183 ± 12	12.45 ± 0.57	10.06 ± 0.75	110.98 ± 0.14	7.58 ± 0.23	98.49 ± 5.80	11.87 ± 0.78	0.232 ± 0.041	5.211 ± 0.031
T7	0.267 ± 0.005	2577 ± 62	11.47 ± 0.43	7.53 ± 0.27	102.19 ± 2.17	11.53 ± 0.31	106.86 ± 5.41	17.11 ± 0.56	0.308 ± 0.021	5.242 ± 0.190
T30	1.359 ± 0.015	2862 ± 151	14.29 ± 1.02	16.82 ± 0.34	109.75 ± 10.48	21.50 ± 3.15	105.28 ± 12.24	16.03 ± 1.54	0.291 ± 0.082	4.228 ± 0.301
T60	0.236 ± 0.064	2373 ± 289	11.17 ± 0.84	8.209 ± 0.616	98.88 ± 0.11	12.81 ± 1.70	103.50 ± 7.43	14.50 ± 1.93	0.315 ± 0.020	4.401 ± 0.219
T90	2.837 ± 0.095	2998 ± 129	9.06 ± 0.90	13.920 ± 0.895	104.19 ± 9.59	17.95 ± 6.24	104.01 ± 8.67	19.82 ± 2.37	0.340 ± 0.015	4.349 ± 0.211
BAVC	**Al**	**Ca**	**Fe**	**K**	**Mg**	**Mn**	**Na**	**S**	**Cu**	**Zn**
T0	0.130 ± 0.025	3647 ± 73	12.17 ± 0.94	40.14 ± 2.89	111.6 ± 0.7	9.85 ± 0.24	95.8 ± 2.1	118.2 ± 6.1	0.180 ± 0.005	3.58 ± 0.09
T7	0.158 ± 0.017	3478 ± 36	12.54 ± 0.38	33.67 ± 0.49	109.8 ± 1.0	12.19 ± 0.20	101.6 ± 10.4	122.4 ± 3.9	0.197 ± 0.021	3.55 ± 0.06
T30	2.515 ± 0.017	3674 ± 128	9.42 ± 1.75	14.21 ± 1.44	112.6 ± 5.7	11.54 ± 2.57	90.9 ± 5.4	123.3 ± 7.2	0.294 ±0.016	3.60 ± 0.47
T60	0.239 ± 0.068	3247 ± 129	10.15 ± 0.51	37.65 ± 2.26	97.4 ± 3.1	12.46 ± 0.65	94.2 ± 8.9	129.7 ± 12.3	0.214 ± 0.018	3.46 ± 0.19
T90	0.701 ± 0.214	3100 ± 191	7.28 ± 0.85	12.80 ± 0.96	105.2 ± 4.9	10.66 ± 1.50	100.5 ± 8.8	103.9 ± 4.9	0.345 ± 0.007	3.19 ± 0.95
BESBAVC	**Al**	**Ca**	**Fe**	**K**	**Mg**	**Mn**	**Na**	**S**	**Cu**	**Zn**
T0	0.229 ± 0.052	3404 ± 77	13.06 ± 0.28	43.35 ± 0.33	109.5 ± 0.0	11.99 ± 0.17	110.1 ± 8.2	78.8 ± 2.2	0.248 ± 0.044	4.71 ± 0.10
T7	0.310 ± 0.080	3238 ± 57	13.57 ± 0.82	38.74 ± 0.05	109.1 ± 1.6	14.36 ± 0.10	95.4 ± 4.2	108.6 ± 1.6	0.236 ± 0.008	4.84 ± 0.09
T30	0.406 ± 0.084	3236 ± 32	12.73 ± 0.30	40.99 ± 1.55	100.8 ± 0.3	15.14 ± 0.34	99.2 ± 14.8	105.5 ± 0.7	0.242 ± 0.033	4.70 ± 0.09
T60	0.176 ± 0.021	2929 ± 29	13.72 ± 0.38	35.90 ± 0.38	101.7 ± 1.4	14.07 ± 0.36	99.2 ± 4.6	97.4 ± 7.0	0.221 ± 0.011	4.48 ± 0.09
T90	0.515 ± 0.080	3237 ± 15	12.41 ± 0.54	34.12 ± 1.13	99.3 ± 3.6	15.76 ± 0.54	102.6 ± 7.2	117.2 ± 4.9	0.273 ± 0.016	5.02 ± 0.12

Supplementary Table 2. Enzymatic evolution from the initial contaminated soil (T0) to the different sampling points (T0, T1, T2, T3, and T4), expressed in nmol g^{-1} min^{-1}.

Treatment	Soil enzyme	T0	T1	T2	T3	T4
CT	AcPA	18.55 ± 1.80	19.92 ± 1.88	23.07 ± 1.89	11.26 ± 1.21	18.96 ± 0.88
	bGA	5.31 ± 0.96	9.32 ± 1.31	9.15 ± 0.95	4.39 ± 0.34	8.89 ± 1.64
	aGA	5.98 ± 0.25	4.47 ± 0.28	7.11 ± 0.51	3.27 ± 0.19	6.16 ± 0.47
	bXyl	6.48 ± 0.52	4.45 ± 0.30	7.01 ± 0.52	3.06 ± 0.21	5.43 ± 0.26
	bNAG	12.59 ± 0.57	18.52 ± 1.17	19.35 ± 0.71	6.83 ± 0.64	13.60 ± 1.65
	AS	5.42 ± 0.18	3.16 ± 0.25	5.54 ± 0.35	2.23 ± 0.14	4.09 ± 0.16
	AlkPA	59.94 ± 1.90	107.00 ± 1.76	107.94 ± 3.09	50.76 ± 2.53	102.92 ± 2.51
	LeuAMP	39.28 ± 2.22	35.72 ± 1.22	50.29 ± 2.27	36.37 ± 2.90	40.95 ± 1.90
BAVC	AcPA	16.99 ± 1.07	21.97 ± 1.91	22.97 ± 0.62	24.92 ± 1.81	24.38 ± 1.76
	bGA	3.05 ± 0.74	7.71 ± 1.50	4.65 ± 0.44	10.95 ± 0.57	8.52 ± 0.43
	aGA	5.40 ± 0.23	11.65 ± 0.54	15.30 ± 0.46	9.38 ± 0.46	9.64 ± 0.39
	bXyl	4.33 ± 0.17	7.25 ± 0.21	9.74 ± 0.41	13.62 ± 0.62	13.41 ± 0.77
	bNAG	5.97 ± 0.59	9.24 ± 1.47	15.30 ± 0.93	24.62 ± 1.88	24.82 ± 1.31
	AS	2.84 ± 0.26	4.86 ± 0.23	8.19 ± 0.29	5.65 ± 0.29	5.09 ± 0.15
	AlkPA	22.60 ± 3.45	56.81 ± 1.76	43.27 ± 1.79	42.61 ± 1.54	43.35 ± 2.86
	LeuAMP	72.65 ± 3.46	72.58 ± 2.00	52.11 ± 1.75	79.91 ± 1.99	86.50 ± 2.00
BESBAVC	AcPA	20.40 ± 1.58	22.73 ± 1.02	17.51 ± 3.44	26.44 ± 1.59	24.97 ± 1.19
	bGA	5.51 ± 0.43	5.08 ± 0.45	6.32 ± 0.95	13.41 ± 1.65	11.04 ± 0.53
	aGA	8.49 ± 0.28	14.91 ± 0.61	10.15 ± 0.63	8.40 ± 0.48	6.62 ± 0.21
	bXyl	6.50 ± 0.30	8.36 ± 0.39	6.24 ± 0.90	13.06 ± 0.46	9.10 ± 0.45
	bNAG	11.53 ± 0.89	17.67 ± 0.87	17.46 ± 1.41	26.39 ± 1.60	20.05 ± 1.02
	AS	4.98 ± 0.38	5.68 ± 0.14	3.86 ± 0.51	4.45 ± 0.21	4.02 ± 0.09
	AlkPA	43.36 ± 2.97	50.76 ± 1.66	43.66 ± 2.90	39.91 ± 3.00	45.77 ± 2.36
	LeuAMP	44.68 ± 1.50	89.66 ± 2.45	36.12 ± 2.29	74.76 ± 2.84	69.73 ± 2.91

Supplementary Table 3. Concentration of TPHs from the initial contaminated soil (T0) to the different sampling points (T2, T3, and T4) expressed in mg kg^{-1}.

Treatment ID	Sampling Point	EPH C10-C12	EPH C12-C16	EPH C16-C21	EPH C21-C30	EPH C30-C35	EPH C35-C40	Total EPH
CT	**T0**	<30	<50	430	22000	15000	2000	40000
BAVC		<30	<50	410	19000	13000	200	34000
BESBAVC		<30	<50	390	17000	12000	190	31000
CT	**T2**	<30	54	410	21000	16000	2100	39000
BAVC		<30	<50	280	12000	7700	1200	21000
BESBAVC		<30	<50	300	11000	7300	1100	20000
CT	**T3**	<30	<50	410	21000	14000	2000	38000
BAVC		9	52	230	3300	2700	530	7100
BESBAVC		8	45	210	3900	2900	430	7600
CT	**T4**	<30	<50	380	19000	13000	1900	34000
BAVC		4,7	30	120	1700	1200	190	3300
BESBAVC		5,6	36	140	2100	1500	250	4100

Supplementary Table 4. Centrality measures per variable (extractable metals) for different treatments.

For Control

Variable	Betweenness	Closeness	Strength	Expected influence
Al	-0.204	0.677	-0.113	-1.315
Ca	-0.842	-0.443	-0.952	-0.575
Fe	0.179	0.123	0.446	0.684
K	-0.587	-0.681	0.109	0.412
Mg	0.945	0.803	0.824	1.078
Mn	-0.715	-0.795	-0.084	0.233
Na	-0.842	0.197	-0.509	-0.163
S	0.817	0.901	1.342	1.56
Cu	-0.842	-2.039	-2.041	-1.588
Zn	2.093	1.258	0.979	-0.324

For BAVC

Variable	Betweenness	Closeness	Strength	Expected influence
Al	0.297	0.581	0.853	-0.328
Ca	-0.752	-0.719	-1.194	-0.916
Fe	-0.227	0.726	0.699	-0.59
K	0.822	0.619	1.107	1.968
Mg	-0.752	-0.142	-0.79	-0.227
Mn	-0.752	0.113	4.784×10^{-6}	-0.54
Na	-0.752	0.176	-0.039	1.055
S	0.647	0.327	-0.695	-0.064
Cu	-0.752	-2.521	-1.388	-1.253
Zn	2.222	0.841	1.448	0.896

For BESBAVC

Variable	Betweenness	Closeness	Strength	Expected influence
Al	-0.588	-1.105	-0.915	-1.142
Ca	-0.588	0.052	-0.193	-0.124
Fe	0.334	0.502	1.184	1.116
K	-0.357	0.166	0.224	0.211
Mg	-0.588	0.502	-0.204	-0.281
Mn	0.219	0.605	-0.353	-0.285
Na	0.104	0.454	0.092	0.154
S	-0.588	-0.192	-0.643	-0.563
Cu	-0.588	-2.246	-1.292	-1.197
Zn	2.641	1.262	2.101	2.111

Supplementary Table 5. Weight matric used for the preparation of network plots for variables (extractable metals) for different treatments.

For Control

Variable	Al	Ca	Fe	K	Mg	Mn	Na	S	Cu	Zn
Al	0	0	0	0	0	0.148	0	0	0	-0.322
Ca	0	0	0.586	0	0	0	0	0	0	0
Fe	0	0.586	0	0	0.514	0	0	0	0	0
K	0	0	0	0	0	0.741	0	0.17	0	0
Mg	0	0	0.514	0	0	0	0	0	0	0.671
Mn	0.148	0	0	0.741	0	0	0	0	0	0
Na	0	0	0	0	0	0	0	0.69	0	0
S	0	0	0	0.17	0	0	0.69	0	0	0.287
Cu	0	0	0	0	0	0	0	0	0	0.063
Zn	-0.322	0	0	0	0.671	0	0	0.287	0.063	0

For BAVC

Variable	Al	Ca	Fe	K	Mg	Mn	Na	S	Cu	Zn
Al	0	0	-0.78	0.75	0.15	0	0	0.39	0	0.083
Ca	0	0	0	0.291	0	0	0	0	0	0
Fe	-0.78	0	0	0.817	0	0	0	0	0	0.405
K	0.75	0.291	0.817	0	0	0.261	0	0	0	-0.278
Mg	0.15	0	0	0	0	0	0	0	0	0.508
Mn	0	0	0	0.261	0	0	0.67	0	0	-0.446
Na	0	0	0	0	0	0.67	0	0	0	0.668
S	0.39	0	0	0	0	0	0	0	0	0.312
Cu	0	0	0	0	0	0	0	0	0	0.014
Zn	0.083	0	0.405	-0.278	0.508	-0.446	0.668	0.312	0.014	0

For BESBAVC

Variable	Al	Ca	Fe	K	Mg	Mn	Na	S	Cu	Zn
Al	0	0	0	0	0	0	0	0	0.036	0.065
Ca	0	0	0.661	0	0	0	0	0	0	0
Fe	0	0.661	0	0.329	0	0	0	0	0	0.249
K	0	0	0.329	0	0	0	0.528	0	0	0
Mg	0	0	0	0	0	0	0	0	0	0.534
Mn	0	0	0	0	0	0	0	0	0	0.476
Na	0	0	0	0.528	0	0	0	0	0	0.28
S	0	0	0	0	0	0	0	0	0	0.228
Cu	0.036	0	0	0	0	0	0	0	0	0.029
Zn	0.065	0	0.249	0	0.534	0.476	0.28	0.228	0.029	0

CHAPTER 6

Conclusions, Final Remarks and, Future Perspectives

6.1. General conclusions and final remarks.

The evaluation of bioremediation techniques for the retention and degradation of hydrocarbons and other xenobiotic compounds in soils developed throughout this doctoral thesis has revealed important knowledge about the efficiency of the techniques used, the environmental sustainability and the specific optimisation for the type of soil under study.

The soils under which this doctoral thesis has been developed are soils that have shown a large difficulty for their treatment with biodegradation techniques for the hydrocarbons contained in them, this is due to their recalcitrant nature in which most of the hydrocarbons present are of long chain with more than 22 carbon atoms. Furthermore, this is not the only difficulty present in these soils, in addition to petroleum hydrocarbons, the soils also contained other contaminants such as heavy metals, oils and lubricants because they were taken from a machinery park, making them more difficult to depollute.

The combination of bioremediation technologies has shown synergistic effects on hydrocarbon degradation. It has been observed that with bioaugmentation technologies, where a microbial consortium has been added, together with biostimulation technologies, where a nutrient solution has been added to improve the growth of both the indigenous microorganisms and those of the bioaugmentation, higher degradation percentages have been obtained than in the rest of the treatments. Moreover, if the technology has been enriched with an organic amendment or additive, this percentage is even higher. The evaluation of biostimulation technologies, bioaugmentation and organic amendments and additives has demonstrated their effectiveness in enhancing hydrocarbon degradation. These strategies have shown promising results in the degradation of total aliphatic and aromatic fractions of petroleum hydrocarbons, indicating their potential to address a wide range of hydrocarbon contaminants. One of the organic amendments, vermicompost, has also been tested at laboratory, pilot and real scale, with very promising results due not only to the use of the amendment, but also to the nutrient supply provide to the bacterial consortium added to the soil in the form of bioaugmentation. The optimisation of soil

characteristics with external inputs through both amendments and nutrient solutions is also a key factor in achieving better results, with soil pH, temperature and nutrient availability being some of the limiting factors in optimising the techniques and minimising the ecological effects derived from this type of contamination.

The results obtained by the biostimulation and bioaugmentation techniques tested are closely related to the microbial communities involved in hydrocarbon degradation, and it was observed that the contaminated soil contained a significant microbial population that possessed this biodegradation capacity and was also metabolically very active. Furthermore, it was shown that degradation is in turn influenced by the composition and diversity of the microbial communities, as well as by the number of microorganisms present in them.

All these results give us an interesting insight into how microorganisms work and live within hydrocarbon contaminated soils. Giving us the knowledge of how difficult it is to degrade the high carbon fractions, and how microorganisms can degrade the lower ones. The most important thing is to improve the bioavailability of the recalcitrant part, as it is the most difficult to degrade, but at the same time as it is not bioavailable, it is less toxic for microorganisms and for humans, so it is less harmful because of this fact. Thus, further research is needed to optimise the application of bioremediation strategies for different types of soils and contaminants. Therefore, efficiency, environmental sustainability, and optimisation of these strategies according to the type of soil to be treated are essential to maximise the metabolism of organic pollutants and minimise the ecological effects of soil contamination. It can be concluded that bioremediation represents an environmentally sustainable and economically viable technology for the decontamination of soils from areas with both acute and diffuse petroleum hydrocarbon contamination, even when other contaminants are present that hinder the performance of the technologies.

6.2. Future perspectives.

As a final step to end the European GREENER project under which this doctoral thesis has been developed, together with other partners such as ACCIONA and the Universidad Autónoma de Madrid (UAM), a real scale bioremediation experiment was carried out. This work is not shown as part of any chapter of this thesis because the experiment is still in progress, since the degradation of hydrocarbons will be studied in two biopiles of 10 tonnes each (Figure 31). In this experiment, the aim is to test the degradation of TPHs and PAHs through two different treatments on a real scale, under optimal operational conditions at laboratory and pilot scale (1 tonne): natural attenuation (control), and bioaugmentation with vermicompost, this time using the synthetic community composed of *Pseudomonas putida*, *Rhodococcus jialingiae*, *Rhodococcus WAY-2*, *Achromobacter aegrifaciens*, *Delftia acidovorans*, and *Novosphingobium silvae*, designed in order to enhance the degradative capacities of the microorganisms and improve the efficiency of the bioaugmentation biodegradation treatment. So far, all the samples taken periodically have been analysed and the degradation of the compounds in the bioaugmentation biopile has been compared with the control pile with promising results, which has conditioned the initial duration of the experiment of three months, and finally the biopiles will be kept for a year for a more exhaustive study. Once the experiment is over, the aim is to carry out all the physicochemical, biological, and genomic analyses of the soil in order to determine both the degradation achieved and the evolution of all the soil characteristics.

Figure 31. Preparation of the real scale experiment at ACCIONA facilities in Alcobendas, Madrid (Spain).

APPENDIX

Congress Publications

AquaConSoil 2021 – Online Congress, 15/17 June 2021

Improvement of bioaugmentation and biostimulation techniques for the biodegradation of hydrocarbons in contaminated soils

Sandra Curiel Alegre[1,2], Carlos Rad[1], Carlos Rumbo Lorenzo[2], Blanca Velasco Arroyo[2], Juan Antonio Tamayo Ramos[2], Sonia Martel Martín[2], Rocío Barros García[2,*].

[1] UBUCOMP, Universidad de Burgos, Facultad de Ciencias, Pl. Misael Bañuelos s/n. 09001 Burgos (Spain).

[2] ICCRAM, Universidad de Burgos (Spain). Plaza Misael Bañuelos, s/n. Edificio I+D+i. 09001, Burgos (Spain).

Session: 4a1 - Enhanced bioremediation

Topic: "5. Sustainable remediation technologies in context of the EGD and energy transition. 5a. New low-carbon solutions for conventional and emerging contamination."

Congreso Ibérico de Suelos y Desarrollo Sostenible (CISDS 2021) – Online Congress, 17/18 June 2021

Bioremediation of soils contaminated with hydrocarbons through the use of edaphic microbial consortia

Sandra Curiel Alegre[1,2], Andrea Martínez Santamaría[1], Blanca Velasco Arroyo[2], Carlos Rumbo Lorenzo[2], Juan Antonio Tamayo Ramos[2], Carlos Rad[1], Rocío Barros García[2,*].

[1] UBUCOMP, Universidad de Burgos, Facultad de Ciencias, Pl. Misael Bañuelos s/n. 09001 Burgos (Spain).

[2] ICCRAM, Universidad de Burgos (Spain). Plaza Misael Bañuelos, s/n. Edificio I+D+i. 09001, Burgos (Spain).

EUROSOIL 2021 – Geneva Virtual Congress (Switzerland), 23/27 August 2021

New opportunities for bioremediation technologies combining the use of local microbial consortia and organic amendments

Carlos Rad[1,*], Sandra Curiel[1,2], Blanca Velasco[2], Carlos Rumbo[2], Juan Antonio Tamayo[2], Rocío Barros[2].

[1] UBUCOMP, Universidad de Burgos, Facultad de Ciencias, Pl. Misael Bañuelos s/n. 09001 Burgos (Spain).

[2] ICCRAM, Universidad de Burgos (Spain). Plaza Misael Bañuelos, s/n. Edificio I+D+i. 09001, Burgos (Spain).

Poster number: PO138

8th European Bioremediation Conference (EBC-VIII) – Chania (Greece), 12/17 June 2022

Vermicompost improves the bioremediation efficiency in an aged contaminated soil with recalcitrant hydrocarbons

Sandra Curiel-Alegre[1,2], Blanca Velasco-Arroyo[1], Andrea Martínez[2], Carlos Rumbo[1], Aqib Hassan-Ali Khan[1], Juan Antonio Tamayo-Ramos[1], José Luis R. Gallego[3], Carlos Rad[2] and Rocío Barros[1,*].

[1] ICCRAM, Universidad de Burgos, Edificio I+D+i, Pl. Misael Bañuelos s/n. 09001 Burgos (Spain)

[2] UBUCOMP, Universidad de Burgos, Facultad de Ciencias, Pl. Misael Bañuelos s/n. 09001 Burgos (Spain)

[3] Environmental Biogeochemistry & Raw Materials Group and INDUROT. Campus de Mieres, University of Oviedo, C/ Gonzalo Gutiérrez Quirós s/n, 33600 Mieres (Spain)

Poster number: ID106

Congreso Ibérico de Ciencias del Suelo (CICS 2022) – Oeiras, Lisboa (Portugal), 22/24 June 2022

Application of a microbial consortium immobilized onto a biochar for the remediation of a polluted soil with hydrocarbons

Sandra Curiel Alegre[1,2], Blanca Velasco-Arroyo[2], Andrea Martínez[1,2], Carlos Rumbo[2], Juan Antonio Tamayo-Ramos[2], Aqib Hassan Ali Khan[2], Carlos Rad[1,*], Rocío Barros[2].

[1] Grupo de Investigación en Compostaje (UBUCOMP). Facultad de Ciencias. Pl. Misael Bañuelos s/n. 09001 Burgos (Spain). *crad@ubu.es

[2] Grupo de Investigación Medioambiente y Sostenibilidad (ICCRAM-Ma-Sos). Pl. Misael Bañuelos s/n. 09001 Burgos (Spain).

22nd World Congress of Soil Science (WCSS 2022) – Glasgow (United Kingdom), 31 July / 5 August 2022

Use of biochar and rhamnolipids as enhancers of TPHs' bioremediation processes

Carlos Rad[1,*], Sandra Curiel-Alegre[1,2], Blanca Velasco-Arroyo[2], Carlos Rumbo[2], Aqib Hassan Ali Khan[2], Juan Antonio Tamayo-Ramos[2], Rocío Barros[2].

[1] Grupo de Investigación en Compostaje (UBUCOMP). Facultad de Ciencias. Pl. Misael Bañuelos s/n. 09001 Burgos (Spain).

[2] Grupo de Investigación Medioambiente y Sostenibilidad (ICCRAM-Ma-Sos). Pl. Misael Bañuelos s/n. 09001 Burgos (Spain).

7th International Symposium on Environmental Biotechnology and Engineering – Marseille (France), 22-26 May 2023

Bioremediation of a TPHs' polluted soil using vermicompost and a microbial consortium at a pilot scale

Sandra Curiel-Alegre[1,5], Rafael Rivilla[2], Marta Martín[2], David Durán[2], Eduard Borràs[3], Soledad Martín-Castellote[4], Blanca Juez[4], Aqib H.A Khan[5], Blanca Velasco-Arroyo[5], Carlos Rad[1], Rocío Barros[5].

[1] Research Group in Composting UBUCOMP, University of Burgos, Faculty of Sciences, Misael Bañuelos. 09001 Burgos (Spain).

[2] Dpt. Biology, Universidad Autónoma de Madrid, Faculty of Sciences, Darwin 2. 28049 Madrid (Spain).

[3] LEITAT, Carrer de la Innovació, 2. 08225 Terrassa, Barcelona (Spain).

[4] ACCIONA, C/ Valportillo II, 8. 28108 Alcobendas, Madrid (Spain).

[5] ICCRAM, University of Burgos, Misael Bañuelos. 09001 Burgos (Spain).

3rd International Meeting on New Strategies in Bioremediation/Restoration Processes (BIOREMID 2023) – Muttenz (Switzerland), 29/30 June 2023

Soil TPHs bioremediation using bacterial consortia, vermicompost, and passive bioelectrochemical system: a pilot scale study

Sandra Curiel-Alegre1,5, Rafael Rivilla2, Marta Martín2, David Durán2, Eduard Borràs3, Soledad Martín-Castellote4, Blanca Juez4, Aqib Hassan Ali Khan5, Blanca Velasco-Arroyo5,6, Carlos Rad1, Rocío Barros5

[1] Research Group in Composting UBUCOMP, University of Burgos, Faculty of Sciences, Misael Bañuelos. 09001 Burgos (Spain)

[2] Dpt. Biology, Universidad Autónoma de Madrid, Faculty of Sciences, Darwin 2. 28049 Madrid (Spain).

[3] LEITAT, Carrer de la Innovació, 2. 08225 Terrassa, Barcelona (Spain).

[4] ACCIONA, C/ Valportillo II, 8. 28108 Alcobendas, Madrid (Spain).

[5] ICCRAM, University of Burgos, Misael Bañuelos. 09001 Burgos (Spain).

[6] Dpt. Biotechnology and Food Science,University of Burgos, Faculty of Sciences. Plaza Misael Bañuelos s/n. 09001 Burgos (Spain).