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BIOREMEDIATION OF SOIL CONTAMINATED WITH PETROLEUM HYDROCARBONS: PROCESS EVALUATION THROUGH BIOSLURRY REACTOR AND BIOELECTROCHEMICAL SYSTEM APPROACHES

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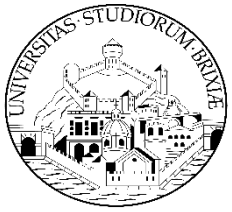

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I dedicate this work to my father, to the soul of my mother, and my brother Tesfay. You are my strength.

This work is also dedicated to the Tigray people, who live in the most testing time of their history, and to those who lost their beloved ones in a brutal way that left the world in complete amazement and shock. This, too, shall pass.

Abstract

Excessive use of petroleum hydrocarbons (PHCs) leads to high pollution of water and soil environments. Spillage of crude oil contamination is common during offshore oil drilling, transport, and transfer to onshore in many parts of the world. PHCs in the soil can also change the physical and chemical properties of the topsoil and subsoil. They also have a phytotoxic effect on seed germination, crop growth, and crop yields. Therefore, to lower the danger of exposure, restore soil function, and provide ecosystem services, remediation of the contamination is required for the restoration and reclamation of affected soil for various uses, including agriculture. For this reason, bioremediation and, particularly, the use of bioslurry and microbial electrochemical treatment (MET) approaches has emerged as an effective and feasible technique with a high remediation potential, especially for soils polluted with petroleum hydrocarbons, which often contain the highest pollution levels and are hard to treat with conventional approaches. However, the mechanisms involved in how adding an external stimulant enhances PHC biodegradation in the bioreactor and those involved in the extracellular transfer of electrons in the MET system are still not completely understood. Therefore, the main objective of this thesis was to develop a sustainable approach to bioslurry and a MET system for the effective and efficient treatment of PHCs from the soil.

The biodegradation of PHCs in soil was investigated in six bioslurry reactors using different external stimulants such as nutrients, activated sludge, synthetic surfactants, and long-chain rhamnolipids. The comparative analysis revealed that the combination of activated sludge, nutrients, and biosurfactant showed a 79.4% reduction in PHCs after ten days in the mesocosm experiment compared to the control treatment.

A reactor system that includes boron-doped diamond as an electro-fenton-based anode and biological processes driven by long-chain rhamnolipids was developed to treat soil contaminated with petroleum hydrocarbons. In addition, the parameters that affect the optimum conditions, such as changes in intermediate generation, pH, hydroxide concentration, and soil organic matter composition, were also studied. The study showed that the combination of systems led to a PHC degradation rate of 93.6% in 72 hours, which is 40% higher as compared to a single system due to the excellent synergistic effects.

To enhance the degradation of PHCs and electron transfer, 10 mg/L, 50 mg/L, and 100 mg/L of rhamnolipid biosurfactants were added to the anolyte of MET. The results showed that rhamnolipids at a dose of 100 mg/L facilitated the degradation of PHCs (72 %) and promoted the highest bioelectricity generation (9.5 Am^{-2}) in 20 days. Furthermore, the biosurfactants induced a special microbial enrichment associated with electrochemically active bacteria like *Geobacter* and *Desulfovibrio* and oil-degrading bacteria like *Pseudomonas* and *Acinetobacter*. With MET as a tool, this study confirmed for the first time that novel long-chain rhamnolipid produced from non-Pseudomonas bacteria could remarkably facilitate the degradation of PHCs via extracellular electron transfer, which provides novel insights into understanding the mechanisms of rhamnolipid regulating petroleum hydrocarbon degradation.

The treatment of soil polluted with PHCs using a double chamber MET designed for treating soil polluted with PHCs fed with biochar ex-situ applications in aqueous systems was investigated for 20 days. The study has shown a significant improvement in the biodegradation efficiency and electricity generation of PHCs from polluted soil by adding rice straw biochar. This work demonstrated that by adding an optimal dose of biochar (8% w/w), the maximum current density was 3.5 A/m^{-2}, and the highest PHC reduction rate was achieved at 87.8 % in 20 days. However, excessive biochar use (12% w/w) caused adverse effects, with extracellular electron transfer efficiency diminished by 87.5% compared to the biochar dose (8%). Furthermore, microbial community analysis confirmed that biochar enrichment could form electroactive biofilms of gram-negative sulfur-reducing bacteria, *Desulfuromonas*.

Finally, a life cycle assessment was also conducted using SimaPro 9.1 software to compare the environmental performance of bioslurry and MET treatment using primary data from the experiment, identify the significant environmental hotspots of the system, and assess its environmental impact category with the ReCiPe 2016 characterization method. The results showed that the bioelectrochemical systems of MET treatment options present the most environmentally favorable option compared to the bioslurry treatment process due to the offset of the environmental loads from the avoided impacts contributed by their value-added by-product, which is the generation of electricity, which leads to outweighs a factor of 1.6 for the Global warming potential (GWP) offset of the environmental loads incurred by the system. The overall findings of this study suggest that MET technology could be used to treat petroleum hydrocarbon-contaminated soil systems while also recovering energy effectively.

Sintesi

L'uso eccessivo di idrocarburi petroliferi (PHC) porta a un elevato inquinamento dell'acqua e dell'ambiente del suolo. La fuoriuscita di contaminazione da petrolio greggio è ora comune durante le trivellazioni petrolifere offshore, il trasporto e il trasferimento a terra in molte parti del mondo. I PHC nel suolo possono anche modificare le proprietà fisiche e chimiche del suolo e del sottosuolo. Hanno un effetto fitotossico sulla germinazione dei semi, sulla crescita delle colture e sui raccolti. Pertanto, per ridurre il pericolo di esposizione, ripristinare la funzione del suolo e fornire servizi ecosistemici, è necessaria la bonifica della contaminazione per il ripristino e il risanamento del suolo colpito per vari usi, compresa l'agricoltura. Per questo motivo, l'uso di bioslurry e trattamento elettrochimico microbico (MET) è emerso come una tecnica efficace e fattibile con un elevato potenziale di bonifica, soprattutto per i suoli inquinati da idrocarburi petroliferi, che spesso contengono i livelli di inquinamento più elevati e sono difficili da trattare con approcci convenzionali. Tuttavia, i meccanismi coinvolti nel modo in cui l'aggiunta di uno stimolante esterno migliora la biodegradazione del PHC nel bioreattore e quelli coinvolti nel trasferimento extracellulare di elettroni nel sistema MET non sono ancora completamente compresi. Pertanto, l'obiettivo principale di questa tesi è stato quello di sviluppare un approccio sostenibile al bioslurry e un sistema MET per il trattamento efficace ed efficiente dei PHC dal suolo.

La biodegradazione del suolo inquinato da PHC è stata studiata in un mesocosmo costituito da sei reattori bioslurry che utilizzano diversi stimolanti esterni quali nutrienti, fanghi attivi, tensioattivi sintetici e ramnolipidi a catena lunga. L'analisi comparativa ha rivelato che la combinazione di fanghi attivi, nutrienti e biotensioattivo ha mostrato una riduzione del 79,4% dei PHC dopo dieci giorni nell'esperimento del mesocosmo rispetto al trattamento di controllo.

È stato sviluppato un sistema di reattori che include diamante drogato di boro come anodo basato sull'elettro-fentone e processi biologici guidati da ramnolipidi a catena lunga per trattare il suolo contaminato da idrocarburi di petrolio. Inoltre, sono stati studiati anche i parametri che influenzano le condizioni ottimali, come i cambiamenti nella generazione intermedia, il pH, la concentrazione di idrossido e la composizione della sostanza organica del suolo. Lo studio ha mostrato che la combinazione dei sistemi ha portato a un tasso di degradazione del PHC del 93,6% in 72 ore, il 40% in più rispetto a un singolo sistema grazie agli eccellenti effetti sinergici.

Per migliorare la degradazione dei PHC e il trasferimento di elettroni, all'anolita del processo MET sono stati aggiunti 10 mg/L, 50 mg/L e 100 mg/L di biotensioattivi ramnolipidici. I risultati hanno mostrato che i ramnolipidi, a una dose di 100 mg/L, facilitavano la degradazione dei PHC (72%) e promuovevano la più alta generazione di bioelettricità (9,5 Am-2) in 20 giorni. Inoltre, i biotensioattivi hanno indotto uno speciale arricchimento microbico associato a batteri elettrochimicamente attivi come Geobacter e Desulfovibrio e batteri che degradano il petrolio come Pseudomonas e Acinetobacter. Con la MET, questo studio ha confermato per la prima volta che i nuovi ramnolipidi a catena lunga prodotti da batteri non Pseudomonas potrebbero facilitare notevolmente la degradazione dei PHC tramite il trasferimento di elettroni extracellulari, il che fornisce nuove informazioni sulla comprensione dei meccanismi dei ramnolipidi che regolano l'idrocarburo del petrolio degradazione.

Per 20 giorni è stato studiato il trattamento del suolo inquinato con PHC mediante un MET a doppia camera alimentato con biochar. Lo studio ha mostrato un miglioramento significativo nell'efficienza della biodegradazione e nella generazione di elettricità dei PHC da terreni inquinati aggiungendo biochar prodotto a partire da pula di riso. Questo lavoro ha dimostrato che aggiungendo una dose ottimale di biochar (8% p/p), la densità di corrente massima era di 3,5 A/m-2 e il più alto tasso di riduzione del PHC (87,8%) è stato raggiunto in 20 giorni. Tuttavia, l'uso eccessivo di biochar (12% p/p) ha causato effetti negativi, con l'efficienza del trasferimento di elettroni extracellulare diminuita dell'87,5% rispetto alla dose di biochar (8%). Inoltre, l'analisi della comunità microbica ha confermato che l'arricchimento del biochar potrebbe formare biofilm elettroattivi di batteri gram-negativi zolfo riducenti, Desulfuromonas.

Infine, è stata condotta anche una valutazione del ciclo di vita utilizzando il software SimaPro 9.1 per confrontare le prestazioni ambientali del processo bioslurry e del trattamento MET utilizzando i dati primari dell'esperimento, identificare gli hotspot ambientali significativi del sistema e valutarne la categoria di impatto ambientale con la caratterizzazione ReCiPe 2016 metodo. I risultati hanno mostrato che i sistemi bioelettrochimici delle opzioni di trattamento MET presentano l'opzione più favorevole dal punto di vista ambientale rispetto al processo di trattamento del bioslurry a causa della compensazione dei carichi ambientali dagli impatti evitati forniti dal loro sottoprodotto a valore aggiunto, che è la generazione di elettricità, che porta a un fattore di 1,6 per la compensazione del potenziale di riscaldamento globale (GWP) dei carichi ambientali sostenuti dal sistema. I risultati complessivi di questo studio

suggeriscono che la tecnologia MET potrebbe essere utilizzata per trattare suoli contaminati da idrocarburi petroliferi, recuperando allo stesso tempo energia in modo efficace.

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List of abbreviations

AOPs	Advanced oxidation processes
BC	Biochar
BTEX	Benzene, toluene, ethyl benzene and xylene
BES	Bio-electrochemical systems
BDD	Boron-doped diamond
C6–C35	Alkane mix
C_6H_{14}	Hexane
$C_7H_6O_3$	Salicylic acid
CV	Cyclic voltammograms
CEC	Soil cation exchange capacity
DNA	Deoxyribonucleic acid
EDS	Energy dispersive X-ray spectroscopy
EDTA	Ethylenediaminetetraacetic acid
EEC	electron exchange capacity
EC	Electrical conductivity
EB	Electro-bioremediation
EF	Electro-Fenton
FA	fulvic acid
G	Germinated seeds
G_C	Germinated seeds control
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GC-FID	Gas Chromatography with flame-ionization detection
GI	Germination index
HA	humic acid
H_2SO_4	Sulfuric acid
HPLC	high-performance liquid chromatography
K	kinetic biodegradation rates
KH_2PO_4	Potassium dihydrogen phosphate
$K_2Cr_2O_7$	Potassium dichromate
KWH	Kilowatt-Hours
KNO_3	Potassium nitrate

lcRLs	Long-chain rhamnolipids
L	Root elongation
LCA	Life cycle assessment
L_C	Root elongation control
LTTD	low-temperature thermal desorption
MET	Microbial electrochemical technologies
MTBE	Methyl tert butyl ether
MMT	Million metric tons
NGS	Next-generation sequencing
Na_2SO_4	Sodium sulfate
$Na_2H_2P_2O_7$	Sodium pyrophosphate
OH	Hydroxyl radicals
OTU	Operation Taxonomic Units
PHCs	Petroleum hydrocarbons
PAHs	Polycyclic aromatic hydrocarbons
PCR	Polymerase chain reaction
R	External resistance
Rha	Rhamnose;
RL	Rhamnolipid
rDNA	Ribosomal DNA
SA	Salicylic acid
SEM	Scanning electron microscopy
SHE	Standard Hydrogen Electrode
SOM	soil organic matter
SOD	superoxide dismutase
T	Treatment
TOC	Total organic carbon
TPH	Total petroleum hydrocarbons
TW80	Tween® 80
UV	Ultraviolet
V	voltage

CHAPTER 1.

INTRODUCTION -

BACKGROUND, OBJECTIVES, AND STRUCTURE OF THE THESIS

1. Background

Following World War II, the industrial revolution drastically altered lifestyles. Modern lifestyles rely heavily on the benefits provided by numerous industries, with chemical industries and their derivatives contributing significantly to various life branches. However, the disproportional increase is observed when evaluating the relationship between modern lifestyle "civilization" and the environment, followed by significant tension on many environmental components (Schwab, 2017). Anthropogenic contamination from human operations in global businesses, together with other natural contaminants, feeds this tension and poses a severe environmental concern (Naidu et al., 2021).

All terrestrial ecosystems depend on soil, which is the most fundamental of all natural resources because it supports all life on Earth. But soil resources are scarce, mostly non-renewable, and dispersed unevenly among ecological regions. Agricultural use, poor management, contamination, the discharge of urban industrial waste, and other forms of pollution can all cause soil quality to decline or degrade, despite the soil's inherent resilience (Sayara et al., 2015; Lal, 2015). Contaminated sites are a global concern and can be considered a substantial barrier to long-term development because soil pollution is a frequent side effect of industrial activities. Despite limited efforts to address the problem, soil pollution is growing increasingly widespread (Mohammadi et al., 2022).

The contamination rate is increasing, even though modest attempts are still being made to address the issue. It is estimated that the number of potentially contaminated sites in Europe is estimated between 2.8 and 3.5 million sites. 899,000 (40%) are assessed to require remediation; among them, 650,000 have been identified, only 235,000 have been remediated, and 14,000 are under remediation (Pérez and Eugenio, 2018). Moreover, the report shows that Each European Union member state currently has contaminated or potentially polluting industries (E.U.). It's also worth mentioning that the maximum size of potentially polluted sites is usually one order of magnitude more than the area of contaminated sites found. The most industrialized (for example, Germany) and urbanized (for example, the Netherlands and Denmark) countries are most afflicted by soil pollution (Pérez and Eugenio, 2018). As shown in figure 1.1, oil and gas industries, mining waste disposal, chemical and military activities, gas and coking plants, and other industrial activities are implicated in the expanding number of polluted sites. Aromatic solvents such as hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), chlorinated and inorganic (heavy metals), dioxins, and polychlorobiphenyls are

listed as toxic contaminants. They have diverse toxic, carcinogenic, mutagenic, or genotoxic effects on the ecology of the environment. The most common pollutants in Europe are heavy metals and mineral oil, accounting for 60% of contaminated sites (Pérez and Eugenio, 2018).

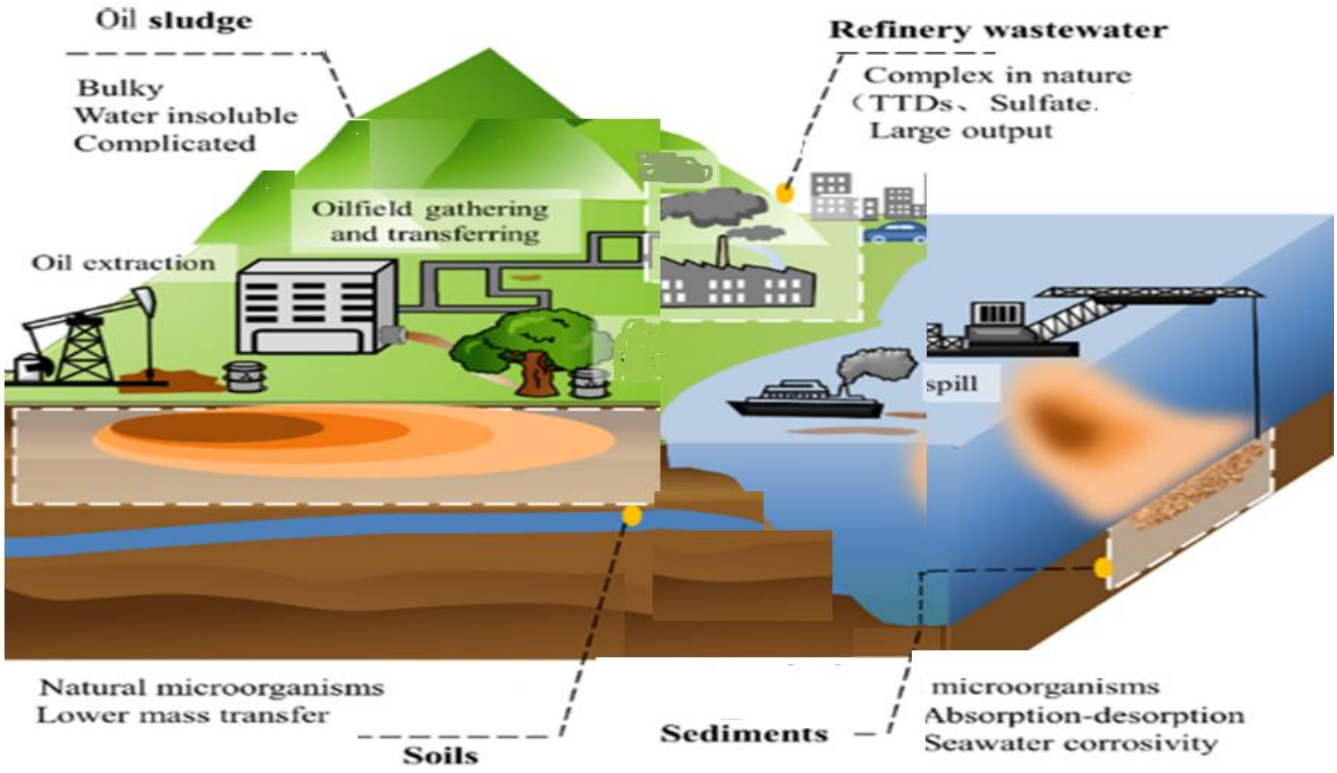

Figure 1-1. Sources and characteristics of petroleum hydrocarbon pollutants in the environment

The number of potentially contaminated sites in Italy is estimated at 22100 (only 6754 inventoried), posing significant health risks. The most common contaminants in Italy are chlorinated hydrocarbon (11%), mineral oil (46%), hydrocarbon (11%), and heavy metals (16%) (Pérez and Eugenio, 2018). Furthermore, pesticide contamination of soils due to agricultural activities is a significant concern. Furthermore, polluted soils are an important long-term source of groundwater contamination.

These figures demonstrate the scope of the problem and the necessity for appropriate remediation techniques. Since many of these compounds are both carcinogenic and toxic to the ecosystem and people, the problem has gotten worse as public awareness and concerns about the compounds in the environment have grown. Among the significant challenges to soil function in Europe, according to the European Commission's Communication "Towards a Thematic Strategy for Soil Protection" is soil contamination (Sayara et al., 2015; Pérez and Eugenio, 2018).

2. Why do we need to focus on polluted soil with Petroleum hydrocarbon?

Naturally, petroleum hydrocarbons are deposited underground under the earth's surface in sedimentary rocks. The primary petroleum hydrocarbon source is commercially exploited fossil fuels (Adelaja et al., 2015). However, it is also generated during petrochemical production, oil and gas leakages from tankers, and accidental oil spills. Environmental Protection Agency (2005) reported that it could pollute the environment during petrochemical loading, transport, storage, and distribution. Furthermore, discharge of Petro-industrial effluent, leakage from aged underground pipelines burst, fugitive emissions, sabotage, and massive natural disasters create huge petroleum hydrocarbon pollution problems. Annually, 1.7–8.8 million metric tonnes (MMT) of petroleum hydrocarbon are discharged into the environment, with almost 90 % related to human activities (Dadrasnia et al., 2013, Wang and Stout, 2010). In addition, they can negatively impact aquatic and terrestrial life (Ambaye et al., 2022). All fossil-based petroleum hydrocarbons are classified into three main classes: heterocyclic, aromatic, and aliphatic hydrocarbon (Speight, 2014).

2.1 Aliphatic hydrocarbons

Aliphatic hydrocarbon compounds subdivide into alkanes, cycloalkanes, and alkenes, in which carbon and hydrogen atoms are linked by a single or double bond. Furthermore, they are less reactive and can be quickly oxidized at the terminal carbon position by microorganisms (Varjani, 2017). Another type of aliphatic hydrocarbon is cycloalkane, which has higher densities, melting points, and boiling points than alkanes (Mulligan, 2002). Alkenes contain a long chain of carbon atoms, in which one carbon atom is a double bond. Besides, they are also encompassed in three configurations: cyclic, branched, and straight.

2.2 Heterocyclic hydrocarbons

This class of cyclic organic compounds contains two different elements, such as oxygen, sulphur, nitrogen, and other metals, in their ring. Moreover, based on the number of heteroatoms present, the compounds' type and ring structure differ from 3 to 8-membered ring compounds. Some examples included in the class of heterocyclic hydrocarbons are furfural, pyridine, dioxane, pyrrole, etc.; furthermore, depending upon the type and nature of heterocyclic, it can also classify into unsaturated and saturated. Thus, they are highly reactive and can easily be attacked by biological and chemical methods (Johnson et al., 2003).

2.3 Aromatic hydrocarbons

Aromatic hydrocarbon compounds contain only C and H atoms, and the ring in their structure

leads to different physical and chemical properties from aliphatic hydrocarbon. In addition, these organic compounds have two or more double bonds in their structure. For this reason, they are unsaturated hydrocarbons. However, its toxic effect on the environment attracts scientists to study its degradation mechanism (Abbasian et al., 2015).

2.4 Monoaromatics – BTEX

These monoaromatics aromatic hydrocarbons include benzene, toluene, ethylbenzene, and xylene. They have one parent benzene ring in their structure, are primarily used in various raw materials across multiple petrochemical products and are a source of fuels in the aviation industry. However, neurotoxic and carcinogenic toward human beings cause acute deterioration to the water environment because they are partially soluble in water and make it more difficult to remediate the BTEX compounds from the contaminated site (Foght, 2008).

2.5 Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are a class of aromatic chemical compounds found in fossil fuels, including gasoline, coal, and garbage-containing plastics. They are also naturally present in many aromatic plants (Biache et al., 2015). PAHs have low solubility due to their lipophilic properties (Biache et al., 2015). PAHs are highly resistant to different transformations and degradation and have high intrinsic chemical stability; they are also considered pollutants (Ambaye et al., 2022). Hence, it needs more research and investigation about its fate and transport in the environment to reduce its negative impacts.

2.6 Transport of petroleum hydrocarbons in the environment and their effects

Petroleum hydrocarbons are generally transported to the environment, often starting from production, transportation, storage operations, refining, and consumption, then moving to the air, water, soil, and entire terrestrial and aquatic ecosystems. Its environmental concentration increased with various types of petrochemicals and industrial effluents. As a result, they may affect various environmental and biological processes and create health issues for humans and other living organisms. They can also enter the environment through biological transformation (Abdel-Shafy and Mansour, 2016). However, the effect of petroleum hydrocarbon on the environment and soil depends on the chemical and physical characteristics and the various hydrocarbon components, primarily affecting the soil properties through adsorption, biodegradation, and leaching (Logeshwaran et al., 2018).

Using a physical abiotic weathering process, petroleum hydrocarbons with lower molecular weight can evaporate and pollute the soil environment. The other component of petroleum

hydrocarbons can be degraded through biodegradation, Tarbell formation, resurfacing, photo-oxidation, foamy mousse formation, emulsification, sinking, dissolution, evaporation, dispersion, and spreading process to pollute the soil. However, due to their hydrophobicity property, most petroleum hydrocarbons pollute the soil through adsorption. As a result, they may enter the food chain and create toxic effects on humans (Clay, 2014). Hence it is essential to investigate the consequences of petroleum hydrocarbon exposure on human health. There is also a need to select and develop a sustainable remediation approach to reclaim polluted soil from hydrocarbon petroleum.

3. Why are Soil bioremediation processes a way to enhance the processes?

Although public knowledge of preventative and sustainable development techniques is increasing, developing country industrialization and present solid waste disposal practices ensure that polluted sites will continue to be an issue. As a result, contaminated land is a global issue that might significantly impede long-term development. As a result, developing and implementing efficient remediation solutions to lessen the hazards posed by such contaminants is vital and urgent (Ambaye et al., 2022). For this reason, different treatment ex-situ or in situ methods include separation and destruction through ultrasonic, acoustic, electromagnetic and electric, heat and thermal, physico-chemical, chemical, and biological treatment methods are involved as strategies to restore soil polluted through petroleum hydrocarbon and use in agriculture purpose. However, most of the treatment methods are expensive, environmentally unfriendly, and ineffective. Still, there is a high health risk to society due to the pollution of petroleum hydrocarbon. This leads scientists and researchers to develop and select new strategists to remediate the contaminated environment through risk-based analysis (Ambaye et al., 2022). In detail, the drawback of most widespread soil treatment processes is explained in chapter two.

This thesis focuses on bioslurry reactors and bioelectrochemical systems (BESs) to enhance the degradation process.

A bioslurry reactor is one of the best ex-situ bioremediation technologies used to remediate soil contaminated with high oils, and compared to other ex-situ technologies, it increases the mass transfer rate induced by soil particles in water, thereby increasing the pollutant's bioavailability, which leads to a high biodegradation rate with a shorter bioremediation period (Tomei and Daugulis, 2013; Ambaye et al., 2022). However, the availability of hydrocarbons

for microorganisms to consume as a carbon and energy source is one of the most significant factors influencing their biodegradation because petroleum hydrocarbons are more stable in the matrix of soil as well as their bioavailability is hindered by physical contact (Ambaye et al., 2022, Ambaye et al., 2021). Adding nutrients N, P, and organic carbon sources as biostimulation, inocula as bioaugmentation, increasing pollutant availability through the use of surfactants or solvents, or inducing biosurfactant production inside the biolsurry reactor may appear to be a more appropriate treatment strategy to improve pollutant removal rates (Ambaye et al., 2021).

The integration of electro-fenton and biological processes has been used to break down various dangerous compounds in wastewater (Moussavi et al., 2012). However, the combined method's efficacy in wastewater remediation is expected to differ from that in contaminated soil remediation. Furthermore, soil characteristics vary during the electro-fenton process, which may reduce pollutant breakdown efficiency during the biological approach (Xu et al., 2015). As a result, electro-fenton and biological processes must be combined in this thesis to ensure that the amount of pollutants broken down during the electro-fenton process is adequate and that the soil properties are suitable for biological processes.

Another technology in this thesis is a bioelectrochemical system, a process that converts organic compounds present in wastewater and soil into biogenic electricity for soil and wastewater treatment. One of the most interesting things about this technology is that the microorganisms involved can transfer electrons to solid anode electrode materials outside of their cells. They produce less sludge than microbial metabolism using intracellular mechanisms and low-cost conventional technologies. They can also create various high-value fuels and chemicals such as polyhydroxyalkanoates, methane, and ethanol. In addition, compared with anaerobic digestion technology, it can operate in oligotrophic and psychrophilic conditions (Bajracharya et al., 2016, Kumar et al., 2017, Wagner et al., 2010).

For this reason, in the past ten years, a bioelectrochemical system such as microbial fuel cells can be used for the treatment of inorganic and organic pollutants wastewater discharge from a variety of sources of industrial and municipal such as the chocolate industry, sewage, brewery, and swine with having high removal of nitrate and sulfates as well as a high reduction of chemical oxygen demand to about 95% (Mohyudin et al., 2022; Prathiba et al., 2022). This opens a new chapter to using this system for the remediation of soil polluted with petroleum hydrocarbons by using the electrochemically active microorganism that can be put on the anode of the microbial fuel cells that can catalyze organic electron donor oxidation,

such as hydrocarbons, and transfer electrons to the anode extracellularly. Electrons are moved to a cathode via an external circuit, where O_2 is usually reduced to H_2O. In this process, the microbial fuel cell's anode can act as the non-exhaustible electron acceptor to transfer electrons from the polluted site to the source having high terminal electron acceptors (Ambaye et al., 2022).

Furthermore, the bioelectrochemical system's electrode can enhance the degradation of petroleum hydrocarbons without any energy input or chemical addition, so the operating cost may be significantly lower than other methods of remediation, as it is used to generate an electrical current, which can not only act as a bio-remediation indicator in real-time but also drive wireless sensors for remote online tracking (Gambino et al., 2021; Li et al., 2021; Wang et al., 2021). Nevertheless, as a promising and emerging bioremediation technology, the bioelectrochemical system still faces challenges. One of the challenges is substrate non-bioavailability, bottlenecks in scale-up operations, ohmic losses, and a lack of robust understanding of the microbiology of microbes regarding electron transfer. However, biosurfactants added to the anodic MFC compartment may increase biofilm formation by making the electrode surface more hydrophilic, facilitating bacterial cell attachment to the electrode. This, in turn, led to an increase in BES performance (Gomaa et al., 2021). From this standpoint, adding biosurfactants and biochar to anodic bacteria can cause permeabilization, leading to mediated electron transfer, or attachment as biofilm that would lead to direct electron transfer. Another mechanism to increase the performance of BESs can be enhanced by establishing conductive networks of exoelectrogenic bacteria and conductive materials. For example, Li et al. (2016) reported that conductive carbon fiber could significantly enhance the bio-power generation and remediation efficiency of petroleum hydrocarbon-contaminated BES by improving soil conductivity. In general, biocathodes, biochar, biosurfactants, electrochemically active microorganisms, and redox mediators can solve the problems encountered by the bioelectrical system to remediate soil polluted with petroleum hydrocarbons. However, using materials integrated with the bioelectrochemical system is still an initial step. Therefore, it needs more research and investigation in the future to be applied on a large scale.

Finally, in this thesis, an environmental life cycle assessment for petroleum hydrocarbon-

polluted soil remediation technologies was performed to compare the effectiveness and sustainability of the tested strategies with literature studies on conventional methods and to assess the technique's full-scale applicability. A new proposed strategy for sustainable remediation and management of soil contaminated with petroleum hydrocarbons is also developed using Simapro 9.1.

4. Objective of the thesis

The main objective of this study was to investigate the bioremediation of soil that had been contaminated with PHCs through bioslurry reactors and bioelectrochemical systems. Due to this, the research content can be separated into four key categories. The first is about the state-of-the-art and current understanding of scientific challenges for identifying the most suitable approaches for the remediation of PHC-contaminated soils. The second one was about bioslurry reactor treatment carried out by adding various mixed stimulants as an enhancement and integrating the bioslurry reactor with an advanced oxidation process to deal with the pollutants. The third section was the bioelectrochemical treatment of the same contaminated soil by adding stimulants such as biosurfactants and biochar to enhance the process. Finally, the fourth section evaluates the environmental impact under various conditions involved in the bioremediation of soil polluted with petroleum hydrocarbons using primary data as input in the using SimaPro 9.1 and its feasibility to apply on a large scale.

To clarify the impact of various parameters regulating the bioremediation process, the overall objective was carried out through a number of subsequent studies in this context. In light of this, the specific objectives of the bioremediation procedure through bioslurry reactors and bioelectrochemical systems can be summed up as follows:

- To review state-of-the-art and understand the scientific challenges of the most suitable approaches for remediation of PHC-contaminated soils and treatment.
- To develop a laboratory-based bioelectrochemical system that assists with biosurfactant and biochar for the removal of petroleum hydrocarbon with the generation of electricity simultaneous that is efficient and effective for various treatment conditions, as well as dynamic microbial community and phytotoxicity and applicable to any environmental condition
- To develop a laboratory slurry bioreactor assisted with various externals stimulant as well as integrated with an advanced oxidation process for the bioremediation of the oil-

contaminated soil that can be applied in any environmental condition

- To compare the effectiveness and sustainability of the chosen technologies using primary data for feasibility and full-scale applicability on a large scale.

5. Organization of the Thesis

There are eight chapters in this thesis, as described in Figure 1.2, with chapters 3–7 presenting the findings and discussion (which supports the particular objectives) and concluding remarks about significant findings. At the end of each chapter, a list of references is given.

Here is a short description of the 8 chapters following this general introduction:

- Chapter 1 describes the thesis's background, objectives, and structure.
- Chapter 2 is a state-of-the-art and current challenge for understanding scientific challenges for selecting the most suitable approaches for the remediation of PHC-contaminated soils.
- Chapter 3 is dedicated to comparing several external supplements (bioaugmentation, biostimulation, surfactants, and biosurfactant) for the degradation of petroleum hydrocarbon-contaminated soil in the bio-slurry reactor investigated and discussed. A new mesocosm treatment strategy is proposed and tested for the degradation of petroleum hydrocarbon.
- Chapter 4 investigates the use of electro-fenton and biosurfactant-assisted biolsurry processes in combination. Electro-fenton was initially used as a pre-treatment in conjunction with the bioslurry process. The optimum conditions for the combination process are determined by changes in intermediate petroleum hydrocarbons, pH, hydroxide, and soil organic matter (SOM) composition that occur in the individual degradation efficiency processes of the electro-fenton-and-biological process.
- Chapter 5 investigates the results and discussion on the biodegradation of petroleum hydrocarbon and generation of electricity in dual-chambered microbial electrochemical treatment (MET) using various doses of biosurfactants fed with petroleum hydrocarbon and the performance of MET.
- Chapter 6 reports the results and discussion of the biodegradation of petroleum

hydrocarbon and generation of electricity in dual-chambered microbial fuel cells using rice-based biochar fed with petroleum hydrocarbon, as well as its effect on the performance of MET.

- Chapter 7 is a study focused on the life cycle assessment to remediation technologies for soil polluted with petroleum hydrocarbon. A new proposed strategy for sustainable remediation and management of soil contaminated with petroleum hydrocarbon is developed.
- Chapter 8 is a general conclusion on the main results obtained during this thesis. Recommendations are given for treating petroleum hydrocarbon-contaminated soil by bioslurry and bioelectrochemical systems. The scientific challenges and perspectives that still must be faced for the scale-up of petroleum hydrocarbon-contaminated soil by bioslurry and the bioelectrochemical system are also discussed.

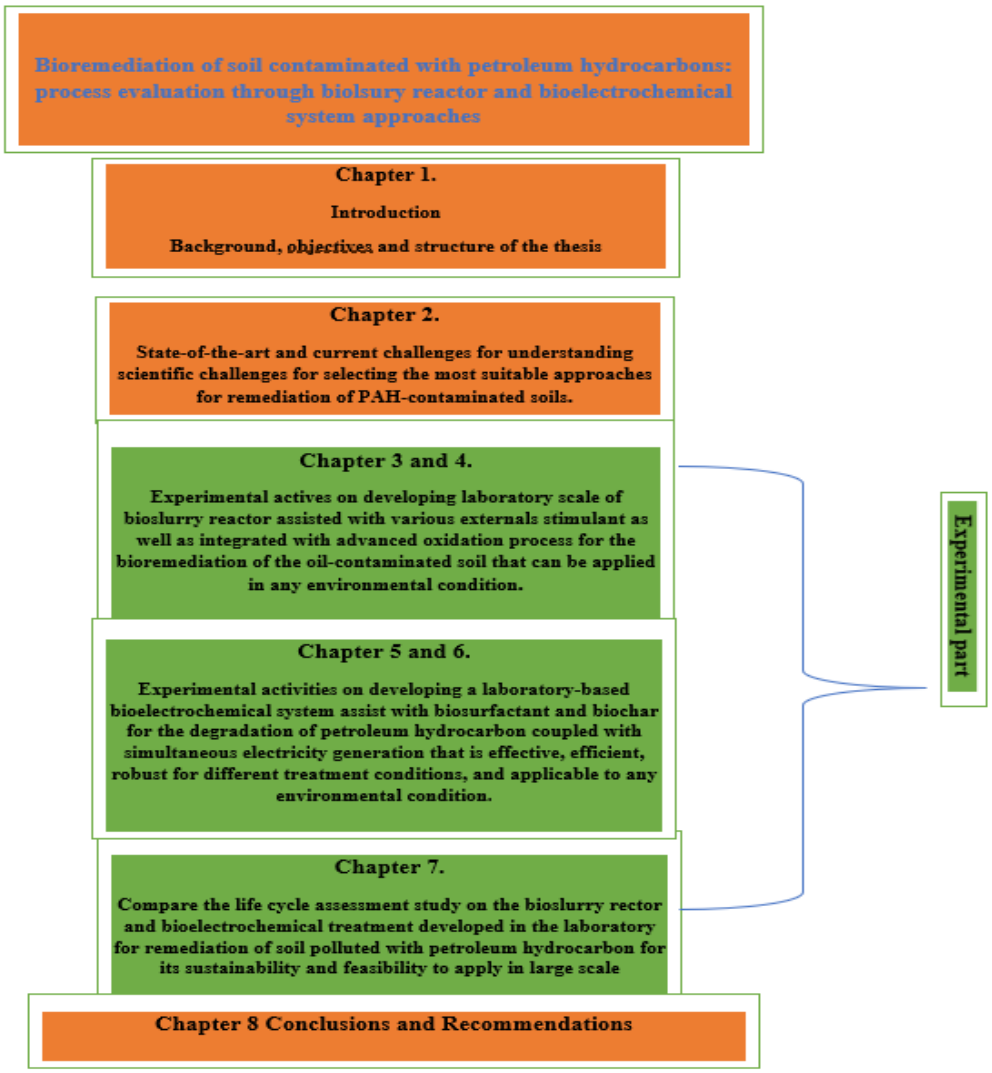

Figure 1-2. Structure of the thesis.

6. Novelty of the thesis

For environmental engineering firms, contaminated soils containing petroleum hydrocarbons and their treatment are challenging problems. This thesis research endeavor focused on a comprehensive examination of these concerns, ranging from the characterization of contaminated soils to selecting the most appropriate treatment options and the removal of target pollutants from the contaminated soil. These factors must be examined simultaneously to make relevant and meaningful changes.

The following chapters describe and discuss the new findings and scientific problems that this thesis has encountered. In Appendix 1, you will find a record of all the conferences I have

attended, and papers written (published, accepted, or submitted) about this thesis.

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CHAPTER 2.

LITERATURE REVIEW -

REMEDIATION OF SOIL POLLUTED WITH PETROLEUM HYDROCARBONS, AND THEIR REUSE FOR AGRICULTURE: RECENT PROGRESS, CHALLENGES, AND PERSPECTIVES

This chapter has been published as a review article:

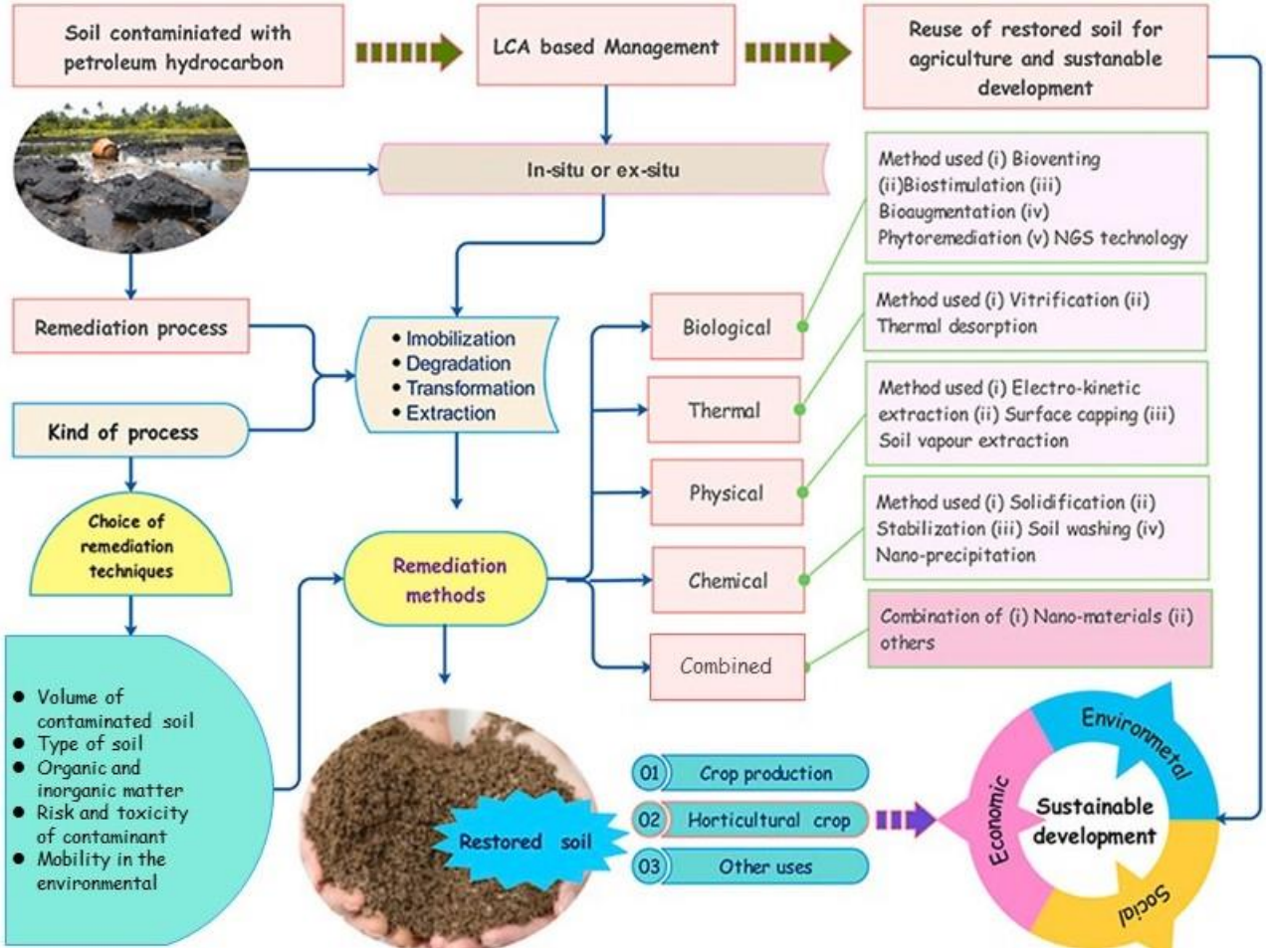
Ambaye, T.G., Chebbi, A., Formicola, F., Prasad, S., Gomez, F.H., Franzetti, A. and Vaccari, M., 2022. Remediation of soil polluted with petroleum hydrocarbons, and their reuse for agriculture: Recent progress, challenges, and perspectives. Chemosphere, p.133572. doi: 10.1016/j.chemosphere.2022.133572.

Abstract

Petroleum hydrocarbons (PHCs) are used as raw materials in many industries and as primary energy sources. However, excessive PHs act as soil pollutants, posing serious threats to living organisms. Various *ex-situ* or *in-situ* chemical and biological methods are applied to restore polluted soil. However, most of the chemical treatment methods are expensive, environmentally unfriendly, and sometimes inefficient. That attracts scientists and researchers to develop and select new strategies to remediate polluted soil through risk-based analysis and eco-friendly methods. This review discusses the sources of PHCs, their properties, distribution, transport, and fate in the environment, internal and external factors affecting the soil remediation and restoration process, and its effective re-utilization for agriculture. Bioremediation is an eco-friendly method for degrading PHCs, specifically by using microorganisms. Next-generation sequencing (NGS) technologies are being used to monitor contaminated sites. Currently, these new technologies have caused a paradigm shift by giving new insights into the microbially mediated biodegradation processes by targeting rRNA, which are also discussed in detail. The recent development of risk-based management for soil contamination and its challenges and future perspectives are also discussed. Furthermore, nanotechnology seems very promising for effective soil remediation, but its success depends on its cost-effectiveness. This review paper suggests using bioslurry and bio-electrochemical systems that utilize electro-chemically active microorganisms to remediate and restore polluted soil with PHCs that would be eco-friendlier and help tailor-made effective and sustainable remediation technologies.

Keywords: Polluted soil, Biodegradation, Petroleum hydrocarbons, Agriculture utilization NGS technologies.

Graphical abstract



1. Introduction

Many industries use PHCs as raw materials for manufacturing various goods and consumer products. PHCs consumption is forecasted to increase from 85 million barrels in 2016 to 106.6 million barrels at the end of 2030 (Igunnu and Chen, 2005). However, its use varies from 32% for Europe and Asia and 53% for the Middle East. Petroleum hydrocarbons include alkanes, cycloalkanes, polycyclic aromatic hydrocarbons, and many other organic pollutants. They are classified as major environmental pollutants due to their stability, and durability in the environment (Song et al., 2021; Uddin et al., 2021). For instance, Dadrasnia and Agamuthu. (2013) reported that globally, about 8.8 million metric tonnes of oil are annually discharged into the aquatic environment. About 90 % is directly linked to human activities due to the oil spill in the terrestrial and marine environment.

Excessive use of petroleum hydrocarbons leads to high pollution in the water and soil environment (Song et al., 2021; Uddin et al., 2021). Spillage of crude oil contamination is now common during offshore oil drilling, transport, and transfer to onshore in many parts of the world. The inherent problem is its complex composition with alkanes, aromatic hydrocarbons, resins, and asphaltenes. They are very toxic to organisms due to their environmental transport, chemical properties, and pollutant nature (Mirjani et al., 2021). Their fate in the environment is not fully understood and is still very challenging. Consequently, the characterization of petroleum hydrocarbon is also a crucial factor for evaluating and estimating the pollutant's behavior and chemical properties with the long- and short-term impact on the contaminated environment to decrease the animal's exposure humans to the contaminant (Keramea et al., 2021).

Petroleum hydrocarbon and oil spill dissolution, dispersion, photo-oxidation, and biodegradation naturally occur very slowly, and these toxic pollutants keep going with adverse outcomes in contaminated exposed areas. Hence, they cause high potential risks and hazardous impacts on humans and other living organisms surrounding the polluted aquatic and terrestrial ecosystems (Haider et al., 2021). In addition, soil contamination with petroleum hydrocarbon has been reported to alter top-soil and sub-soil Physico-chemical properties and phytotoxic impact on seed germination, crop growth, and yields (Grifoni et al., 2020; Haider et al., 2021). Therefore, to lower the danger of exposure, restore soil function, provide ecosystem services, and remediation contamination, it is required to implement risk-based remediation strategies for the restoration and reclamation of affected environments for various uses, including

agriculture (Zhang et al., 2021). For this reason, different treatment ex-situ or in situ methods include separation and destruction through ultrasonic, acoustic, electromagnetic and electric, heat and thermal, physio-chemical, chemical, and biological treatment methods are involved as strategies to restore soil polluted through petroleum hydrocarbon and use in agriculture purpose. However, most of the treatment methods are expensive, environmentally unfriendly, and ineffective. Still, there is a high health risk to society due to the pollution of petroleum hydrocarbon. This leads scientists and researchers to develop and select new strategists to remediate the contaminated environment through risk-based analysis (Dubey et al., 2021; Koshlaf, 2020; Nwankwegu et al., 2022; Popoola et al., 20200; Ruley et al., 2022).

This review focused on emerging novel technologies for the remediation of petroleum-contaminated soils and their effective utilization for growing crops and other plants. The internal and external factors that affect the restoring process, treatment methods, source, properties, distribution, and transport in the environment are discussed precisely. Furthermore, the mechanism of various petroleum hydrocarbon degradation, plants, microorganisms, their relationships, and interactions, including the methods for monitoring the remediation process such as chemical, biological, and NGS methods, are also presented. Finally, challenges and future perspectives are discussed in the recent development in petroleum hydrocarbons' eco-friendly management and sustainable remediation of soil contaminants.

2. Sources of PHCs, their fate in the environment

Naturally, PHCs are the most commercially exploited fossil fuels (Adelaja, 2015). However, according to Environmental Protection Agency (2005), accidental leakage from oil and gas tankers, leakage during loading, transport, storage, and distribution, or in the form of oil spills can pollute the environment. Annually, 1.7–8.8 million metric tons (MMT) of PHCs are discharged into the environment, almost 90 % related to human activities (Shahzadi, 2021). In addition, they can negatively impact aquatic and terrestrial life s (Adetunji et al., 2021; Chokor, 2021; Dadrasnia et al., 2013; Gao et al., 2022; Haider et al., 2021; Konur, 2021; Marvin et al., 2021; Rahimi et al., 2021; Raja et al., 2022; Sayed et al., 2022). Generally, fossil-based PHCs are classified into aliphatic hydrocarbon heterocyclic and aromatic (Balint, 2021; Bojan et al., 2021; Speight, 2014). These PHCs are usually transported to the environment, often starting from production, transportation, storage operations, refining, and consumption, then moving to the air, water, soil, and entire terrestrial and aquatic ecosystems. The foremost route by which PHCs permeate and contaminate soil is shown in Fig. 2.1.

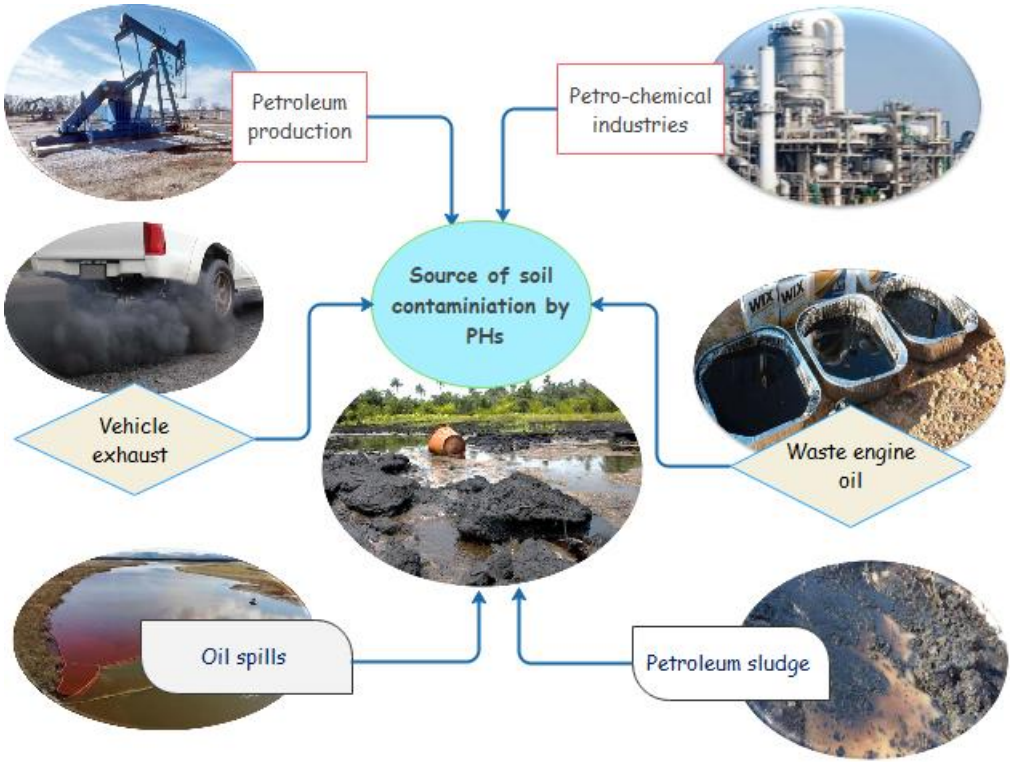


Figure 2-1. Source of soil contamination by PHCs

In addition, the hydrophobicity property of various PHCs enhances their affinity towards water, air, and soil, as shown in Fig. 2.2.

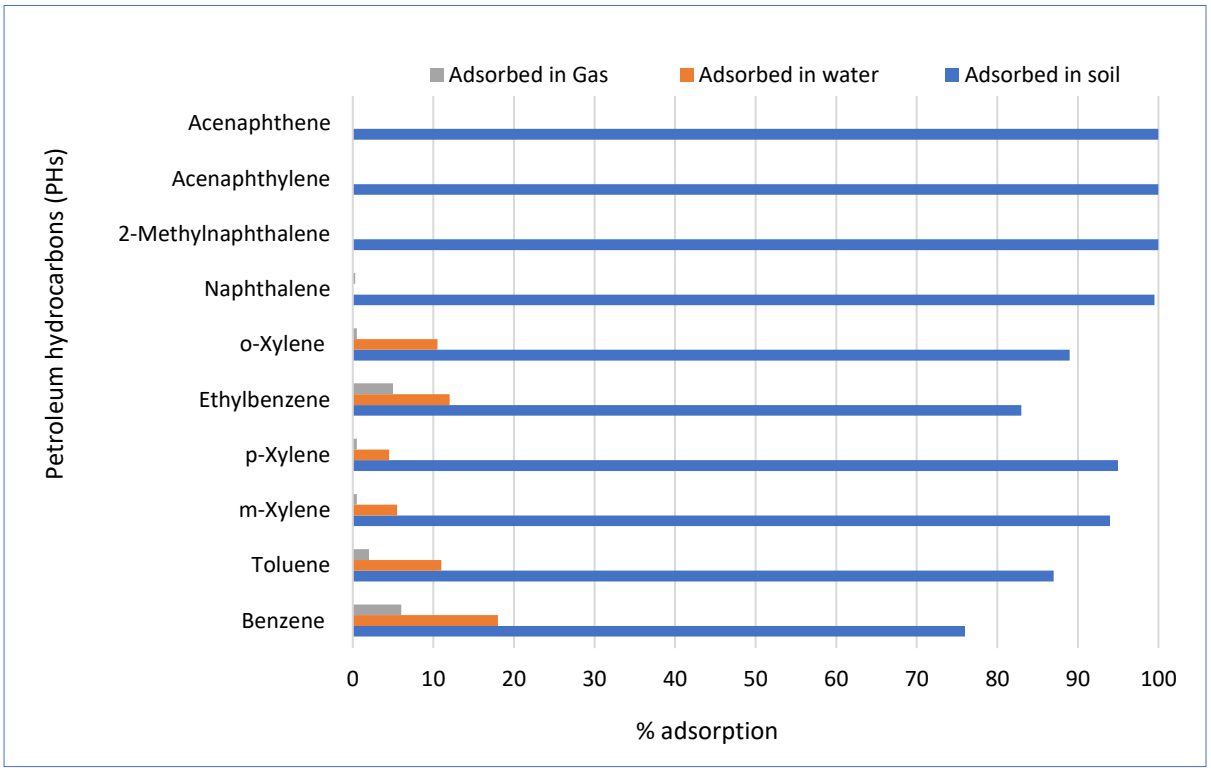


Figure 2-2. Distribution of petroleum hydrocarbons on water, air, and soil. Source: Adapted from Chartered Institute of Environmental Health (https://slidetodoc.com/contaminated-land-dealing-with-hydrocarbon-contamination-remediation-options/)

3. Effect of petroleum hydrocarbon (PHCs) on the environment and human health

PHCs concentration in the environment has increased with various types of petrochemicals used and related industrial waste products and effluents. They are reported to pollute soil and emerging threat to the environment, neurotoxic and carcinogenic to human beings (Abbasian et al., 2015; Acharya et al., 2022; Asejeje et al., 2021). Most PHCs enter the environment through biological transformation (Abdel-Shafy and Mansourt, 2021; Hassanshahian and Cappello, 2021). Moreover, they may also affect various environmental and many living organisms' biological processes. Hence, it needs more research and investigation into the environment's fate and transport to reduce its negative impacts (Abdel-Shafy and Mansourt, 2021; Biache et al., 2014; Wartell et al., 2021; Yadav et al., 2021).

3.1. Effect of petroleum hydrocarbon (PHCs) on agriculture soil

The effect of petroleum hydrocarbon on the environment and soil depends on the chemical and physical characteristics and the various hydrocarbon components, primarily affecting the soil properties through adsorption, biodegradation, and leaching (Clay, 2014; Logeshwaran et al., 2018). They may enter the food chain and create toxic effects on humans. Agriculture soil plays a vital role in providing food for all and maintains balance in the ecosystem environment; however, contamination of soil adversely affects soil fertility and physical and chemical properties (Steliga and Kluk, 2020). The release of petroleum hydrocarbon into topsoil and subsoil has been reported to destroy the soil texture, and structure and decreased pore spaces, and saturated hydraulic conductivity **(**Hou et al., 2021; Steliga and Kluk, 2020). It can also affect the soil's biological properties**,** especially soil microbial population, and enzymatic activities, indirectly affecting plants' nutrient availability**.** Al-Joumaa (2009) conducted a study to evaluate the impact of petroleum hydrocarbons on soil's physical and chemical properties. Results indicated that the polluted soil had a relatively higher bulk density and absolute density than unpolluted soils. Because diesel-contaminated clay soil has more open porosity and thus looser aggregates than an uncontaminated clay sample, the percent clay fraction and calcium carbonate content were found to be low in the contaminated soil. EC values were higher in polluted soil than in unpolluted soil. In unpolluted soil, soluble Ca and SO4-ions predominated.

3.2. Effect of petroleum hydrocarbon (PHCs) on plants/vegetation

A higher petroleum hydrocarbon concentration has been reported as toxic to crop plants and much other vegetation. Soil aeration and water infiltration clogged by oil may severely affect plant growth (Hou et al., 2021; Steliga and Kluk, 2020), primarily reducing **the** root length and

leaf area of sunflower crops (Hou et al., 2021; Steliga and Kluk, 2020). Al-Joumaa (2009) also reported no barley seed emergence after 14 days of exposure to the polluted soil, even after leaching and alleviating salts from the contaminated soil. Petroleum pollution causes deformations of leaf pigments, H_2O_2 accumulation, oxidative stress, and even plant cell death (Al-Joumaa, 2009). Maize root-shoot growth, leaf lengths, and cell expansion have all been reported to be inhibited (Al-Joumaa, 2009; Hou et al., 2021; Steliga and Kluk, 2020). However, the phytotoxicity of petroleum hydrocarbons differs among various plant species. Some crop species, such as wheat, rye, and oat, can tolerate and accumulate these pollutants and are used for phytoremediation, as discussed separately elsewhere (Al-Joumaa, 2009).

3.3. Consequences of PHCs on human health and other organisms

PHCs are characterized by having high boiling points with a wide range of high relative molecular weights, which causes a different degree of toxicological effect on the environment and human and animal health. Depending on their susceptibility and exposure time, petroleum hydrocarbon molecules have diverse toxicity levels to the environment, people, and wildlife. They can damage various human body systems, i.e., nervous, circulatory, immune, and endocrine systems, and cause many metabolic, and hormonal disorders and diseases. However, its lethal bioavailability to a substrate and toxicological effect depends upon the physical state, chemical nature, exposure concentration, exposure time, exposure mode, chemical properties, and compositional fractions of petroleum hydrocarbon (Priyadarshanee et al., 2021).

Consequently, it causes a different range of toxicological effects on human and animal health, such as mutagenicity, hemotoxic, genotoxicity, carcinogenicity, ocular toxicity, cardiotoxicity, hepatotoxicity, nephrotoxicity, immunotoxicity, neurotoxicity, cytotoxicity, and teratogenicity, as shown in Fig. 2.3 (Azeez et al., 2015; Ossai et al., 2020; Premnath et al., 2021; Zheng et al., 2014). Besides, it also affects plants' growth due to inhibiting the uptake of mineral salt and water, which halts plant metabolic processes, further enhances the shortage of nutrients and chlorophyll, and leads to decreased resistance to disease pests. As a result, the plant's growth becomes stunted. In addition, the root becomes deformed, and the leaves and flowers develop necroses and chlorosis (Rusin et al., 2015; Saeed et al., 2022; Shan et al., 2014).

Petroleum hydrocarbons contain a diverse range of chemical constituents. However, the proportion, features, method of degradation and toxicological effect of small fraction chemical compounds on human and animal health have not been well understood and investigated. Hence, needs more research to study the toxicological effects, mechanisms, and degradation of

all the compounds present in petroleum hydrocarbon to develop sustainable remediation technology. Li et al. (2020) studied the ecotoxicological effects of petroleum-polluted soil on the earthworm. They reported that high petroleum concentrations in contaminated soil might significantly damage the cocoon and negatively impact DNA in the earthworm's seminal vesicles and reduce their body weight. The biological and some of the enzymatic activities like peroxidase, superoxide dismutase (SOD), and catalase activities were inhibited that indicating petroleum pollution could induce oxidative stress.

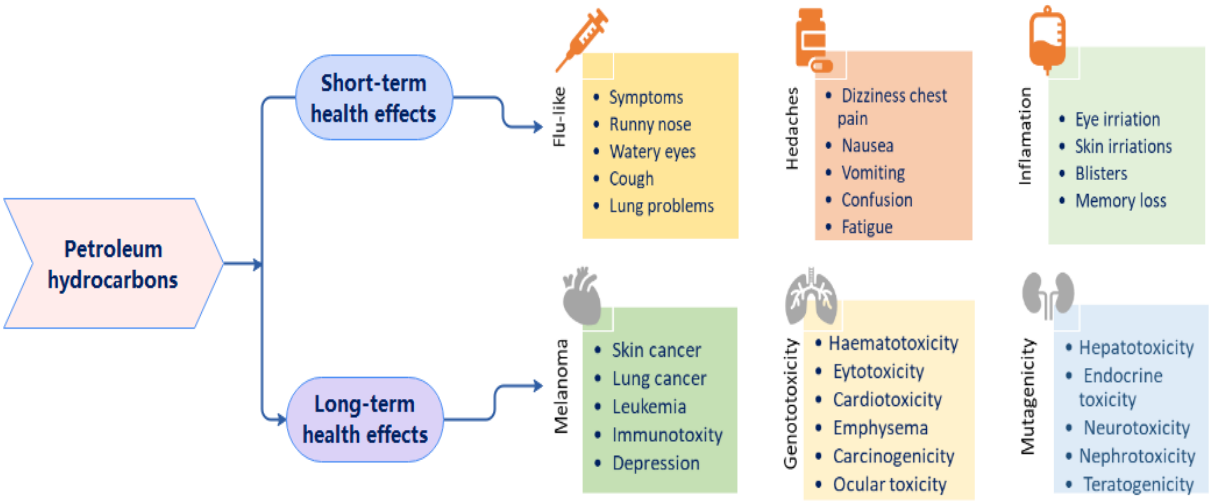


Figure 2-3. Consequences of petroleum hydrocarbon exposure on human health adapted from (Ossai et al., 2020)

4. Remediation technologies for soil polluted with petroleum hydrocarbon

As discuses above, petroleum hydrocarbons have a massive impact on the environment and human health. It is necessary to clean and remove the contaminated environment's pollutants through suitable and sustainable remediation approaches. Hence the best remediation approach that can remove petroleum hydrocarbons from the polluted environment must be selected. Variables such as contaminate composition and type, polluted environment, biological and chemical conditions, and microbial species required for augmentation, as well as time, cost, regulatory requirements, and procedures, must all be considered. Moreover, particular attention must be given to adapting novel sustainable approaches towards reducing major risks of soils contaminated with toxic petroleum hydrocarbons. In this context, various physical, chemical, biological, and other equivalent technologies are also discussed below.

4.1. Physical methods for petroleum hydrocarbon removal from soil

Worldwide several physical treatment methods to degrade and remove petroleum hydrocarbon from polluted soil have been reported. These methods include methods of separation and recycling **i.e.,** soil flushing, soil washing, solidification, vitrification, stabilization, thermal desorption, incineration, and vapor extraction **(**Dadrasnia and Agamuthu, 2013; Raja et al., 2022). Petroleum-hydrocarbon polluted soil can be generally reclaimed and decontaminated by widely used (i) *in-situ* and (ii) *ex-situ* methods. Here, in-situ means in the original place, while ex-situ means outside the original place. Generally, the in-situ method is practiced on-site such as unmodified in their natural setting. In contrast, the ex-situ method is practiced off-site, such as using biodiversity outside their natural habitats in a laboratory or aquarium. To remove lighter molecules, especially BTEX, polluted soil air extraction, air sparging, **i**gnition, or combinations of these methods are used. Ex-situ soil cleaning treatment methods are carried out off-site by excavation, detoxification**,** and destruction technology and can be applied to remove petroleum hydrocarbons from soil and groundwater (Abdelhafeez et al., 2022).

4.1.1. Excavation of contaminated soil for petroleum hydrocarbon removal

Excavation is a conventional and mechanical **removal** of petroleum hydrocarbon from polluted soil subsurface. It consists of (i) excavation and (ii) removal of the contaminants from polluted soil, primarily from industrial and military bases. After excavation, polluted soil is transported to treat or destroyed at remediation sites. This method has been reported to create problems during the excavation due to the polluted soil's risks through handling and transportation. However, it can be avoided by adequately delineation the site before excavation. Sometimes, finding new landfill sites for the material's final disposal is more expensive and challenging (Ferronato and Torretta, 2019). Therefore, this method is considered an interim solution to decontaminate polluted areas because it constantly monitors potential risks, costs, and other related liabilities (Sohoo et al., 2022).

4.1.2. Surface capping of the contaminated soil

Surface capping is used to conserve the contaminated soil with petroleum hydrocarbon by the physical barrier between the polluted media and the surface. It helps to isolate petroleum hydrocarbon-polluted soil from potential receptors. It also helps to prevent water pollution from rainfall water infiltration due to scientific surface soil capping. Contaminated media is covered with sufficiently thick and impenetrable barriers that minimize the transport of toxic petroleum

hydrocarbons. Thus, surface soil and water pollution can be controlled, and direct or indirect contact between humans and animals with the contaminated media also be minimized to ensure their health safety (Liu et al., 2022).

4.1.3. Prevention of soil contamination by physical barriers

Physical barriers are applied to minimize and control the transport of PHCs in the soil profile. They also limit pollutants' flow away from the contaminated site and restrict their flow to groundwater and other surface water bodies (Wagner and Yarmak, 2017). Physical barriers are installed in front (down-gradient) of the polluted region. In a novel strategy, vertical barriers are used. It includes layering slurry, making walls and curtains, plastic cut-off walls, steel shoring, cement, and bentonite on top of background soil to create a barrier. In the case of softer soil, primarily slurry walls are applied. This system mainly consists of a trench having a down-gradient around a polluted zone. They are usually filled with bentonite, cement, or hard clay soil. Sheet-piling cut-off walls are water-retaining structures installed into the impacted polluted soil to a depth of around 12 to 30 meters. However, physical barriers do not eliminate pollutants but restrict the leakage and lateral movement of petroleum hydrocarbons (Wagner and Yarmak, 2017).

4.1.4. Contaminated soil-washing

It is a crucial ex-situ method applied based on the physicochemical separation and leaching processes. In this method, the dispersion of toxic PHCs occurs between soil clay particles and the washing solution (Tran et al., 2022). Some chemical additives like surfactants are also applied (Fabbricino et al., 2018; Kang, 2014). A study conducted by Murena and Gioia (2009) showed 97% petroleum hydrocarbon removal. To remediate contaminated soil, extraction solvents such as chlorinated organic compounds, hexane, and acetone (ethyl acetate-acetone) are used as washing solutions.

4.1.5. Thermal/heat desorption treatment methods

Under ex-situ methods, low-temperature thermal desorption (LTTD) is crucial to remediate toxic petroleum hydrocarbons. In this method, heat is used to separate and disperse pollutants from excavated contaminated soils physically. This method has a specific design for heating excavated polluted soils to volatilize organic constituents, entirely or partially, at 1200 °F. The vaporized hydrocarbons are treated in a catalytic oxidation chamber before discharge into the air. Then, soils are kept to cool and stabilized, then prepared for disposal or reuse in other possible work. However, soil types, such as coarse to fine soils, need proper, proven treatment and are accepted universally. LTTD is a practical remedial alternative to volatilize, stripping

pollutants. However, its efficiency is reported as site-specific and depends on various characteristics of petroleum pollutants, locations, contaminated soil volume, and layout. Its success is determined by the regulatory framework, logistical infrastructure, and economic considerations (Kuppusamy et al., 2017; Riser-Roberts, 2020).

4.1.6. Petroleum hydrocarbon removal by microwave heating

Microwave heating is reported as a cost-effective and time-saving method. It involves homogeneous heating of PHCs polluted soil by electro-magnetic irradiation, especially infrared and radio frequencies, ranging from 300 MHz to 3000 GHz. Its mechanisms solely depend on the interaction of dielectric materials and electromagnetic irradiations (Aguilar-Reynosa et al., 2017). Heat proportion during polluted soil heating is produced by bipolar rotation and ionic conduction. However, its efficiency depends on soil permeability and particle size, which play a significant role in the vaporization of hydrocarbons. In the ionic conduction process, free ions are the key factor in the remedial process, which is placed by ionic motion produced via electric fields that lead to fast heating (Falciglia et al., 2018). Most of the chemical methods, including microwave heating, are energy-consuming and technologically complex and lack public acceptance.

4.2. Chemical methods for petroleum hydrocarbon removal from soil

Chemical methods used for the removal of petroleum hydrocarbon from the soil are described as follows.

4.2.1. Mechanisms of PHCs degradation through a chemical process

Mechanisms for PHC degradation from contaminated soils through the chemical process take place through various abiotic and biotic transformations (Aeppli et al., 2014). In this process, these molecules are broken down into smaller parts via abiotic chemical changes without microorganisms' help through oxidation-reduction and hydrolysis (Almutairi, 2022; Cabral et al., 2022; Kim et al., 2022). Whereas the biotic chemical transformations include both biotransformation and biodegradation processes. They might occur under aerobic conditions in which the degradation is activated by the oxygenase enzymes and oxidized into water and carbon dioxide. It can also take place in anaerobic conditions (Hsia et al., 2021).

4.2.2. Chemical oxidation-reduction

This is an advantageous technique based on in-situ chemical oxidation reactions. Various chemical oxidants like MnO_4 -, •OH-, •SO_4, O_3, Fe_2^+, $S_2O_8^{2-}$, H_2O_2, and O_2 are used in this

method. They react on contaminated soil subsurface after application, where PHCs are converted into non-hazardous, less toxic, less mobile, and inert compounds (Besha et al., 2018). However, oxidants are used up by dissolved heavy metals and soil organic substances instead of PHCs during chemical oxidation reactions, resulting in ineffective treatment. Chen et al, (2016) conducted a chemical oxidation study to remediate diesel from contaminated soil and reported 48 to 93% diesel removal efficiency using MnO_4^-, $K_2S_2O_8$ H_2O_2, under 1, 3, 5, and 10% concentrations. The removal efficiency for the PHCs was in the order of < MnO_4^-, < $K_2S_2O_8$, < H_2O_2.

4.2.3. Ultraviolet and photocatalytic oxidation of petroleum hydrocarbon

UV and photocatalytic oxidation methods involve UV irradiation and chemical oxidants like O_3 and H_2O_2 to degrade and mineralize petroleum hydrocarbon compounds (Pisharody et al., 2022). High-intensity UV irradiation is used by employing Hydrogen peroxide (H_2O_2) to oxidize contaminants into water, CO_2, and salts. In UV-photocatalytic oxidation methods, UV irradiation reacts with H_2O_2 by activating atomic bonds. It makes the molecules very oxidizable to produce reactive OH•, which is highly reactive to the petroleum hydrocarbon for subsequent destruction. Hu et al, (2008) conducted photo-catalytic remediation of methyl tert butyl ether (MTBE). UV- irradiation on titanium dioxide (TiO_2) was found to catalyze and generate reactive oxidizing OH• in aerobic conditions and achieved 80% MTBE removal efficiency from contaminated media within 48 hours. Gholami et al. (2020a) studied the effect of biochar on the photocatalytic performance of Zn-Co-LDH nanostructures. They found that 92.7% of the antibiotic gemifloxacin was degraded. A novel biochar-incorporated Fe-Cu-LDH nanocomposite was used, best sonocatalytic efficiency of 97.6% cefazolin sodium degradation was achieved (Gholami et al., 2020b). A newly synthesized 3-dimensional photocatalyst, WO_3 nanorods, and N-Doped Magnetic WO3- x@Mesoporous Carbon (NM-WO_3–x@MC) nanocomposite degraded 90% of antibiotics and 76% of total organic carbon, respectively (Gholami et al., 2021). In another study, Moradi et al, (2021) used photocatalytic $FeTiO_3$/GO nanocomposite and showed significant phenol degradation under visible light irradiation.

4.2.4. Activated carbon adsorption of petroleum hydrocarbon

Activated carbon adsorption was also found helpful in the decontamination of petroleum hydrocarbon compounds from surface soil. Many researchers have reported its efficiency in the removal of organic pollutants and reducing their bioavailability. It was reported as the most versatile adsorbent due to its micro-porous structure, large internal surface area, and hydrophobicity, making it a very strong, highly effective, and suitable *adsorbent* for petroleum

hydrocarbon removal (Wang et al., 2022). The adsorption process is controlled by the van-Der-Waal force, and pollutants desorption occurs at an equilibrium state. For instance, Kalmykova et al, (2014) conducted a study on PAHs sorption using activated carbon. They showed around 50-63% sorption and removal of PAHs from leachate-collected landfill sites.

4.2.5. Electrochemical systems for petroleum hydrocarbon removal

Electrochemical systems have been reported to remediate cyclic and aromatic *hydrocarbon from polluted media, including soil.* In this system**,** electric current serves either as an electron donor or acceptor for oil spill remediation. Electro-osmosis, electromigration, and electrophoresis are the primary mechanism of transport and mobilization of pollutants. Electrical potential or electromagnetic waves are green technologies for effective remediation and low molecular weight hydrocarbon volatilization. Wang et al, (2022) conducted an experiment using single-chamber air-cathode microbial fuel cells for the degradation of pyrene and found it very effective. Also, the addition of pyrene influenced the electrical properties of micro fuel cells and this leads to an increase in the density of the anodic biofilm.

4.3. Biological methods for petroleum hydrocarbon removal

Biological methods for petroleum hydrocarbon removal are grouped into (i) Bioremediation. Microorganisms, especially bacteria and fungi, are applied to remove petroleum hydrocarbons, and (ii) Phytoremediation, where plants and symbionts species are used to remove PHCs from the contaminated environment. It also facilitates their removal via amendments of soil and agronomic practices (Ruley et al., 2022). A principal mechanism involved in phytoremediation i.e., phytovolatilization, phytodegradation, phytoextraction, phytostabilization, Phyto stimulation, and rhizofiltration, are shown in Fig. 2.4.

Due to the complex nature of petroleum hydrocarbons, the degradation process depends on the concentration, composition, and nature of the petroleum hydrocarbon present in the contaminated environment. Therefore, its degradation process results from biodegradation and chemical transformations (Ruley et al., 2022). As shown in Fig. 2.5, various physical and biological factors affect the biodegradation rate of petroleum hydrocarbons. Therefore, the physicochemical qualities and nature of the pollutant are some of the essential variables to consider when developing appropriate remediation methods for PHC-polluted soil. For example, aliphatic hydrocarbons have a higher degradation rate than aromatic hydrocarbons such as PAH and BTEX compounds due to having less structural complexity and low

molecular weight because the molecular weight and structure of the petroleum hydrocarbon increase leads to less biodegradable compounds. However, the addition of nutrients or co-substrates such as biosurfactants to complex hydrocarbons can enhance their degradation rates and other environmental factors, e.g., temperature and pH, salinity, and pressure (Ambaye et al., 2021a; Cui et al., 2022).

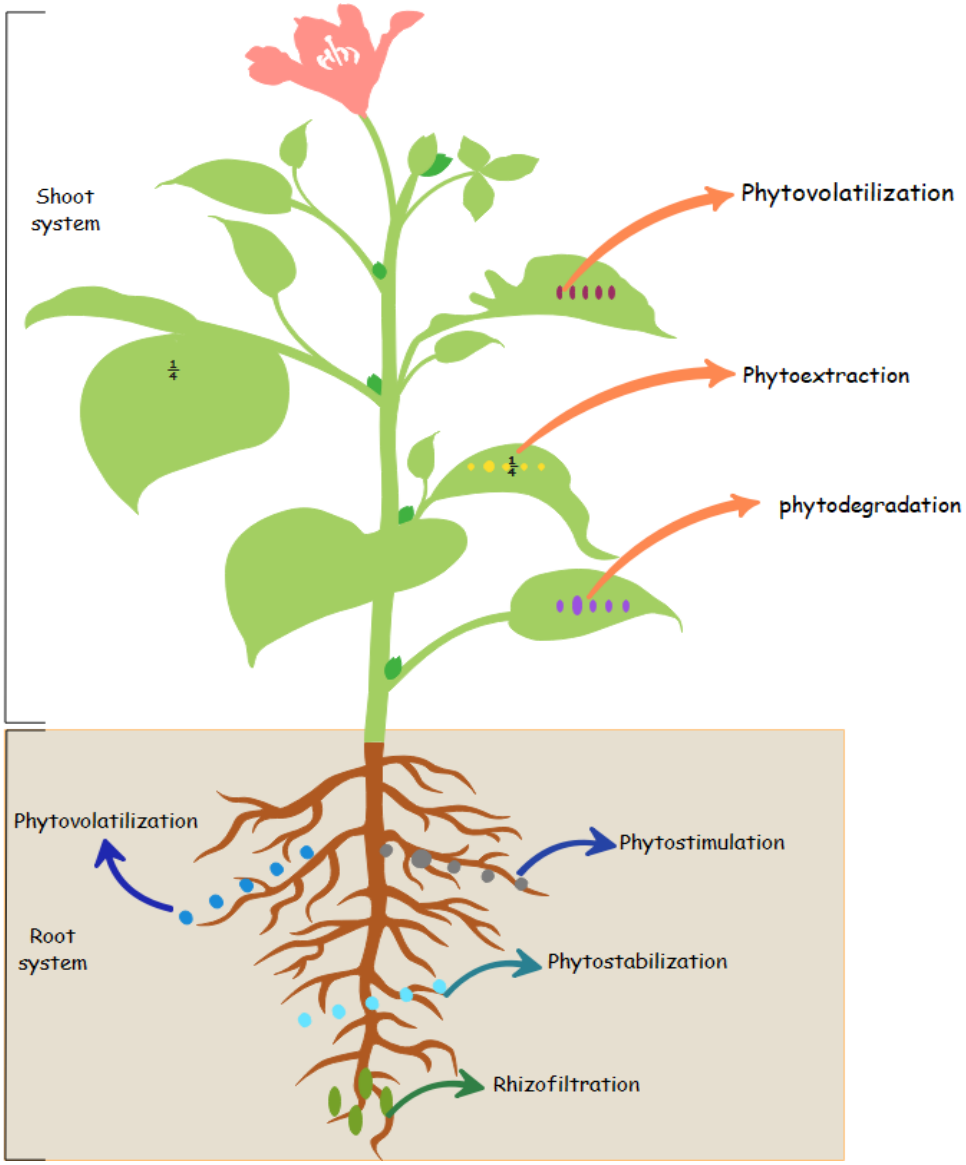


Figure 2-4. Mechanisms involved in phytoremediation of petroleum hydrocarbon

In addition, PAH degradation rates at anaerobic conditions are affected by the availability of terminal electron acceptors like SO_4^{2-}, NO_3^-, and O_2 (Sun et al., 2022). Especially in the area where the availability of oxygen is very low leads to a decrease in a continuous supply of nutrients like SO_4^{2-}, NO_3 as well as decreases the availability of terminal electron acceptors,

which can be used for microbial metabolism during the biodegradation process (Sun et al., 2022). Due to this, several efforts have been made to supply alternative terminal electron acceptors to the soil, showing increased mineralization of petroleum hydrocarbons.

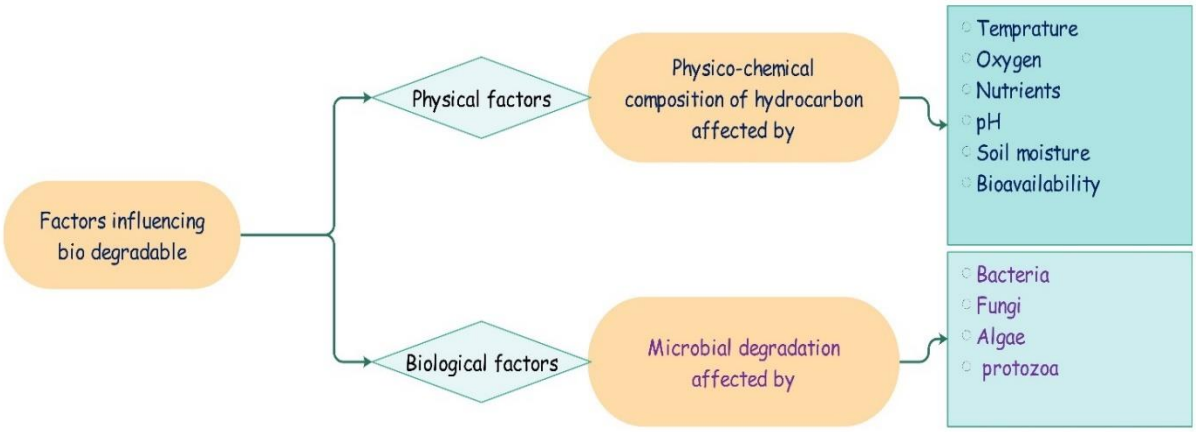


Figure 2-5. Physical and biological factors affecting the biodegradation rate of petroleum hydrocarbon from the contaminated environment

Temperature is another environmental component that could affect the PHC-biodegradation rates of petroleum hydrocarbons. It ranges from (-10 to 55 °C), affecting the diversity of microbial communities, physiology, and chemistry of the pollutant, and the pace of contaminating biodegradation (Li et al., 2022; Okonkwo et al., 2021). Moreover, the degradation rate and solubility of polycyclic aromatic hydrocarbons also decrease with decreasing in temperature.

4.3.1. Phyto-degradation of PHCs

Recent studies on phytoremediation of soil contaminated with PHCs are reported in Table 1. Phytoremediation conventional studies have been associated with biostimulation via the addition of fertilizers (Tang, 2019; Tang et al., 2019) but lately linked to bioaugmentation with bacteria or fungi (Asemoloye et al., 2017; Hou et al., 2015). There is also interest in examining the effect of Se in increasing the phytoremediation plant's efficiency (Huang et al., 2019). *Sorghum bicolor, Zea mays, Helianthus Annuus, and Lolium multiflorum* are typical species utilized in phytoremediation investigations. New phytoremediation-capable plants are being added to the list as a result of screening investigations. *Epipremnum aureum, Azolla filicuilodes, Mucuna bracteata, and Epipremnum aureum* are the most recent additions.

Table 2.1 shows some potential methods and plants that have been used, and the duration of phytoremediation for hydrocarbon removal, ranging from 20 d to 150 d. They have a more significant influence on the reduction of hydrocarbon. For instance, Baoune et al (2019) reported 70% of crude PHCs and 61% of phenanthrene removal from the soil in 14 d. Kösesakal et al, (2016) reported 94% of total aliphatic hydrocarbons removal from liquid medium contaminated.

In general, "phytoremediation" is a term that refers to a group of technologies that use plants' natural capacity to clean up soil that has been contaminated by petroleum hydrocarbons. Phytoremediation is the most successful remedial strategy for reducing persistent organic compounds, and it has recently attracted a lot of interest due to its ability to degrade contaminants across a large area and its broad applicability, eco-friendliness, and cost-effectiveness. However, only a few plants can absorb these organic chemicals from the soil, and plants cannot break down or detoxify persistent organic compounds due to a lack of fixed enzymes. Only a few plant species, such as Cucurbita species, have demonstrated a unique ability to absorb a significant amount of persistent organic chemicals from the soil and are considered persistent organic compound hyperaccumulators (Malik et al., 2022). Furthermore, genes encoding and expressing persistent organic compound-degrading enzymes have been discovered and extracted from persistent organic compound-degrading microorganisms. Transgenic hyperaccumulator plants encoding persistent organic compound-degrading enzymes could help with the phytoremediation of persistent organic pollutants (Malik et al., 2022).

Table 2-1. Phytoremediation (using plant species) of soil contaminated with petroleum hydrocarbons

Soil contaminant	Plant species	Methods	Removal efficiency	References
Crude petroleum oil	*Phragmites australis and Juncus maritimus*	Attenuation, bio stimulation in marshy sediment	PHCs removal efficiency achieved best with P. australis (16%) by bio stimulation within 150 d	Ribeiro et al. (2014)
Petroleum hydrocarbons in crude oil contaminated soil	wheat *(Triticum aestivum),* maize *(Zea mays),* white clover *(Trifolium repens),* alfalfa *(Medicago sativa), and* ryegrass *(Lolium multiflorum)*	soil amendments (biochar and compost) on plants belonging to Poaceae and Fabaceae families.	The highest TPH removal (68.5 %) by ryegrass with compost, and white clover with biochar (68 %). Without any soil amendment, ryegrass and alfalfa showed 59.55 and 35.21 % degradation of TPHs, respectively. Biochar and compost alone removed 27.24 % and 6.01 % TPHs, respectively, within 62 d	Yousaf et al. (2022)
Crude oil	*Imperata cylindrica; Mucuna bracteata; Pteris vittata; and Epipremnum aureum;*	Screening of plants in Malaysia at 5% crude oil contamination by weight	*Mucuna bracteata* (31 %), *Pteris vittata* (36%); *Imperata cylindrica* (40 %); *Epipremnumaureum* (50 %); to remove total PHCs within 42 d	Tang and Angela. (2019)
Petroleum hydrocarbons	*Medicago sativa L*	*microcosm experiments involving Medicago sativa L. and isolated bacterial consortium Acidocella aminolytica* and *Acidobacterium capsulatum*	*M. sativa* + Consortium resulted in 91% removal of diesel hydrocarbons in just 60 d	Michael et al. (2022)

4.3.2. Microbial degradation of petroleum hydrocarbons

Various researchers have widely reported microbial contribution in degrading hydrocarbons from soil and water in the last few decades (Cabral et al., 2022; Mohapatra et al., 2022; Rafeeq et al., 2022). Hydrocarbon-degrading microorganisms play an essential role in effective bioremediation methods, cleaning polluted soil, water, sediments, and other contaminated media (Mohapatra et al., 2022). Oleophilic microorganisms for degrading and cleaning petroleum pollutants from terrestrial and aquatic systems are considered eco-friendly and economical. Therefore, microbial degradation is the ultimate natural mechanism for maintaining ecosystem and environmental sustainability (Cabral et al., 2022). One of the widest mechanisms that can be used for the degradation of petroleum hydrocarbon is biodegradation. In this process, the compounds are degraded via specific anaerobic and aerobic enzymes and use emulsifiers and biosurfactants, at a certain condition, to attach microbial cells to the substrates. The general process for the aerobic degradation of petroleum hydrocarbon using microorganisms is shown in Fig. 2.6 (Koshlaf and Ball, 2017).

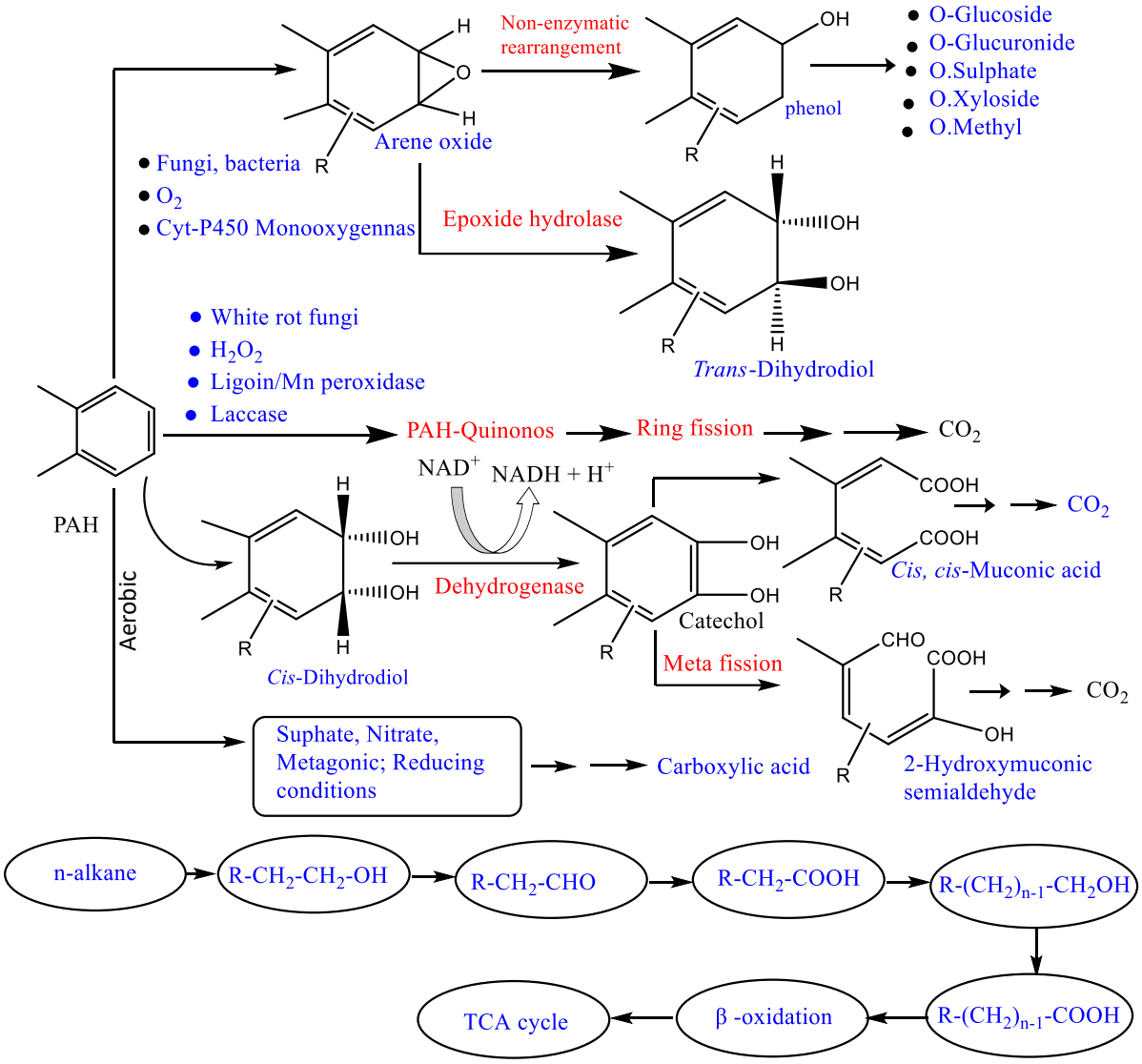


Figure 2-6. Graphical flowchart for petroleum hydrocarbon degradation using Microbial degradation adapted from (Koshlaf and Ball, 2017).

In aerobic transformation, first, the petroleum hydrocarbon is attacked by using an enzyme that mediated the oxidative process (oxygenase), then it will be followed by the cleavage of the ring. Polycyclic aromatic hydrocarbons can also be oxidized into phenolic and quinone derivatives using fungi by the cation of peroxides (Koshlaf and Ball, 2017). The enzymes use the molecular oxygen produced from the aerobic transformation process to oxidize the substrate of polycyclic

aromatic hydrocarbons to hydroxylation rings and other forms of intermediate oxidized products (Imam et al., 2022). The list of some potential microorganisms which can degrade petroleum hydrocarbon is shown in Table 2.2.

Table 2-2. Summary of some potential petroleum hydrocarbon-degrading bacteria and fungi

Microorganisms	**Compound**	**Removal rate**	**References**
Aspergillus spp	Crude oil	59–87.7% of crude oil recovery	Zhang et al. (2016)
Aspergillus ochraceus CBMAI 849	Benzo[a]pyrene	76.6 to 99.7% bioremediation from saline environments	Passarini et al. (2011)
Penicillium sp. RMA1 and RMA2	Crude oil	Degradation achieved by RMA1-57% and RMA2 55%	Al-Hawash et al. (2018)
Aspergillus sp. RFC-1	Different PHCs	60.3- 97.4% removal of PHCs from aqueous environments	Al-Hawash et al. (2019)
Ochrobactrum sp.	crude oil	83.49% degradation of crude oil equivalent to 3% v/v	Varjani et al. (2015)
Candida tropicalis	Diesel	Degradation of 83% diesel	Adams et al. (2015)
Pseudomonas sp.	Petrol, diesel, and waste engine oil	Degradation achieved petrol-76%, diesel-83%, and waste engine oil -69%	Ruley et al. (2020)
Bacillus soreness D11, *Pseudomonas stutzeri* D13, and *Bacillus cereus* D12,	Petroleum- diesel TPH-DRO.	Strains consortium removed up to 80% petroleum- diesel, extract at 31.1 mg g^{-1} TPH-DRO.	Alsayegh et al. (2021)

Degradation of PHCs is reported faster by bacteria. However, fungi take a long time to acclimate to the environment and have low, competitive capabilities. Thus, degradation by fungi depends on the species' capacities and is mostly limited to the soil environment, which is oxic (Magan et al., 2022). The other mechanism used for the degradation of BTEX and polycyclic aromatic hydrocarbons in anaerobic degradation. There is carboxylation of the substrate in this process, followed by the cleavage aromatic ring of the phenanthrene aromatic compound. This pathway shows how the degradation route of PHCs using an anaerobic process differs from the aerobic process route (Mohapatra et al., 2022).

Tsai et al, (2009) conducted research to degrade phenanthrene and fluorene through an anaerobic bio-transformation route coupled with sulfate-reducing bacteria. Results showed the formation of molybdate, which indicates that phenanthrene and fluorene are bio-transformed via hydration and hydrolysis processes followed by decarboxylation with p-cresol and phenol formation. Hence, these metabolites' formation can be considered a new biotransformation route of phenanthrene and fluorene. However, most petroleum hydrocarbons' degradation mechanism areas are not fully understood; more research and investigation are needed in the future, especially by considering the complexity of the field scale, as shown in Fig. 2.7.

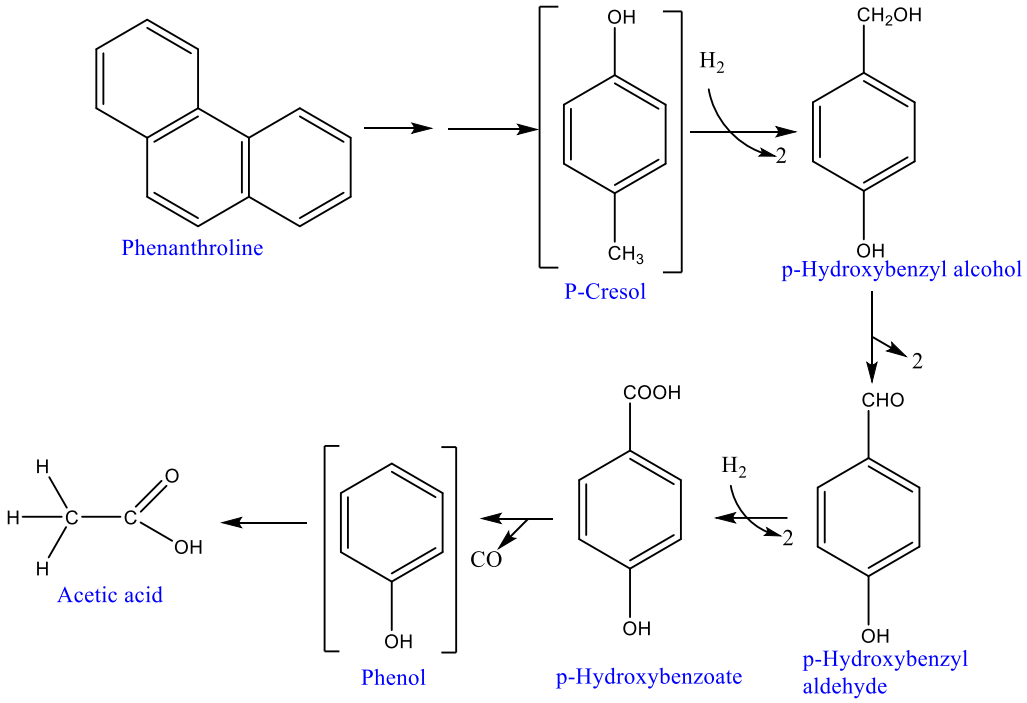


Figure 2-7. Biotransformation route of phenanthrene using anaerobic and sulfate-reducing bacteria adapted from (Tsai et al., 2009).

4.4. Other Bioremediation technologies

4.4.1. Land farming

This is a process in which the soil polluted with petroleum hydrocarbon is aerated and regularly spread out over thin layers, facilitating the degradation of the contaminant species, and adding nutrients. In addition to this, this treatment is a solid-phase approach in which the contaminated

soil is immobilized and biodegraded using microbiological processes and oxidation, which can be done either ex-situ or in situ (Brown et al., 2017; Guarino et al., 2017).

4.4.2. Composting

Compositing can mainly be used to degrade targeted soil pollutants by combing the cured organic material (mature) with contaminated soil. This technology uses composted organic matter such as fishbone compost, spent mushroom, and bark compost supplemented with urea. Hence, adding urea-enriched composted organic matter to the contaminated soil gives a source of carbon supplements of nutrients to the microorganisms, increasing the metabolic capability and, therefore, the biodegradation of the organic compounds found in the soil matrix (Chen et al., 2015; Leech et al., 2020).

4.4.3. Bio slurry system

It is one of the best ex-situ bioremediation technologies used to remediate soil contaminated with PHCs under controlled conditions. To aid in pollutant biodegradation, the soil is mixed with water to make a slurry, which is typically 10 to 60% by weight by volume. Indeed, increasing the mass transfer rates and the contact between contaminants, nutrients, and microorganisms present in a slurry phase is achievable. Biolsurry reactors are typically run-in batch mode to make slurries easier to handle and increase process yields. (Avona et al., 2022; Geng et al., 2022; Kuppusamy et al., 2016b; Thenmozhi et al., 2022). However, the hydrocarbon availability for microorganisms to consume as a carbon and energy source is one of the most significant factors influencing their biodegradation rates because PHCs are more stable in the soil matrix, and their bioavailability is hindered by physical contact. Moreover, due to the partial oxidation of PHCs, they form more toxic intermediates, which leads to poor performance during the treatment processes by microorganisms (Abena et al., 2019). To increase the bioavailability and solubility of hydrocarbons, it is necessary to have a liquid phase in the slurry bioreactors and add additives, e.g., biosurfactants, that can improve the bioremediation of soil polluted with PHC. The optimizations of the operational parameters deserve in-depth investigation in the future, including pH, oxygen, nutrients, the type of additive used, and autochthonous bacterial diversity towards commercial scales and appropriate management of its effluents (Wu et al., 2017). Some recent studies on the remediation of PHCs-contaminated soils using bio-slurry are summarized in Table 2.3.

Table 2-3. Studies on remediation of PHC-contaminated soils using bio-slurry

Type of Pollutant	Working conditions and type of bacteria	Removal efficiency	Reference
PAHs	Soil contaminated with PAH: 10973 mg kg^{-1}. with soil RO water ratio of 30%; microbial consortium	93.4% removal of PAH in 12 d	Lewis. (1993)
PAHs	Microbial consturim, PAHs: 4494 mg kg^{-1}. Soil/water: 20%, Temperature: 21 ± 2 °C; pH: 6.5-8	90% removal of PAH in 35 d	Lee et al. (2001)
PAHs	Indigenous soil bacteria, PAH: 13 g kg^{-1} Soil/water: 25%, Temperature: 20 ± 2 °C; pH: 7.5	58.2.% removal of PAH in 150 d	Fava et al. (2004)
PAHs	Natural soil bacteria, HCs:500 µg g^{-1} PAHs: 6.1 µg/g, Soil/water: 20%; Temperature: 30 °C	>70% removal of HCs and 40% for PAHs removal in 35 d	Beolchini et al. (2010)
Phenanthrene	Microbial consortium, Phenanthrene: 75 mg kg^{-1}. Soil/water: 18%, Temperature: 25 °C	100% removal of phenanthrene in 6 d	Woo et al. (2004)
PAHs	Microbial consortium, Pyrene: 100 mg L^{-1} Soil/water: 50%, Temperature: 25 °C	77% removal of PAHS in 60 d	Thavamani et al. (2012)
PAHs	Microbial consortium, PAH: 3967 mg L^{-1} Soil/water: 50%, Temperature: 25 °C	90% removal of PAHs in 60 d	Kuppusamy et al. (2016c)
Total petroleum hydrocarbons (TPHs)	Microbial consortium, Soil 1: 9.45 g kg^{-1} Temperature: 27 °C	72.7 % removal of TPHs in 54 d	Bento et al. (2005)
TPHs	Microbial consortium, TPH: 10 g kg^{-1}	79% removal of TPHs in 70 d	Suja et al. (2014)
TPHs	Microbial consortium, TPH: 38.08 g kg^{-1} Temperature: 25 °C	79.8% removal TPHs in 84 d	Hesnawi et al. (2013)
TPHs	Microbial consortium, TPH: 10 g kg^{-1} Temperature: 30 °C	61.9% removal TPHs in 60 d	Zhao et al. (2013)
TPHs	Microbial consortium, TPH: 42 g kg^{-1} Temperature: 25-30 °C, pH: neutral	76% removal of TPHs in 120 d	Farahat, and El-Gendy. (2007)
TPHs	Microbial consortium, TPH: 39-41 g $kg^{-1,}$ Temperature: 25 ± 2 °C	74% removal of TPHs in 210 d	Ramadass et al. (2018)

4.4.4. Bioventing and Biosparging

This is a method of degrading polluted soil by supplying air or oxygen to existing soil microorganisms and removing pollutants in-situ. This promotes microbial activity and leads to the removal of organic pollutants from soil or wastewater (Hussain et al., 2022). In addition to this, nutrients can also be applied when required to promote the indigenous microbial community's growth. Constructed bioventing wells may be mounted horizontally or vertically, depending on the location's depth and geology (Hussain et al., 2022). While biosparging has a similar working principle and design to bioventing, they differ in the active region. That is, biosparging is mainly used below the groundwater level in the saturated zone, and compared to the bioventing systems, it has a high air injection flow rate. Moreover, in actual use, biosparging and bioventing systems are combined with various treatment approaches such as permeable reactive barrier technologies and soil vapor extraction (Chatterjee et al., 2022).

4.4.5. Permeable Reactive Barriers

It's a subterranean wall designed to clean up polluted groundwater. The wall is "permeable," meaning it allows groundwater to move through it. To be treated, water must flow through the permeable reactive barriers. The wall's "reactive" materials either trap or decrease the toxicity of harmful pollutants. The groundwater is treated and then discharged on the other side of the wall (Obiri-Nyarko et al., 2014). Compared to the other traditional groundwater remediation technologies, permeable reactive barriers have the following benefits: having low maintenance cost and treating the pollutants in-situ approach and rejecting groundwater, and absence of above-ground infrastructure. On the other hand, permeable reactive barriers are the only suitable approach for shallow aquifers and need more capital-intensive costs (Andrade and dos Santos, 2020).

4.4.6. Natural attenuation

In natural attenuation, indigenous microbes degrade PHCs as they live solely on carbon (C), readily available even in polluted media. When a site is contaminated, indigenous PHC-degrading microbes multiply rapidly and adapt to the newly introduced petroleum hydrocarbons, resulting in the entire degradation of PHCs (Zhang et al., 2022). This remediation process arises naturally in polluted sites, particularly those with lower light PHC, usually used in zones where other restoration mechanisms are unsuccessful (Okparanma et al., 2017). Natural attenuation is suitable for treating petroleum hydrocarbon-contaminated places because it acts without human

intervention and requires limited monitoring. Natural attenuation is effective in managing petroleum hydrocarbons-polluted sites in a biodegradation study. For instance, Lv et al. (2018) stated an about 60% decrease in PHCs pollution plumes due to the natural attenuation of polluted soil. Natural attenuation was used to treat 25% of PHC-polluted soils worldwide (Marić et al., 2018). Natural attenuation is more effective than other bioremediation strategies because it reduces the bulk, toxicity, mobility, volume, and concentration of pollutants in soil without requiring human involvement (Šrédlová and Cajthaml, 2022).

4.4.7. Reductive dechlorination and dehalogenation

In this process, PHCs are removed by the reductive chemical processes. The dechlorination and dehalogenation process is used to lower toxic compounds' levels through progressive chlorine replacement, in which halogen atoms are replaced by hydrogen atoms either by decomposition or partial volatilization (Zhu et al., 2022). Various studies have reported that bacteria and a few mesophilic and thermophilic methanogens can act during reductive dehalogenation at anaerobic conditions. For example, according to Zanaroli et al. (2015), their microbial dehalogenation of organohalides showed that 98% of debromination was effective in the degradation of the marine in mangrove sediments after 90 days of incubation. In general, halogen compounds can exacerbate the harmful biological effects of living compounds by increasing their toxicity, mutagenicity, and other harmful forces, as well as affecting their hidden as bioremediation lacks certain halo-organic compounds that are generally biodegradable, moderately, as a completely broken apart body. Despite facing metabolic hurdles posed by halo-organic substances, bacteria have developed a strong desire to biodegrade them.

5. Emerging remediation technologies for soil polluted with petroleum hydrocarbon

5.1. Nanotechnology applications for petroleum hydrocarbon removal

Nanomaterials have unique properties such as high anion and cation adsorption capacity, high porosity, and specific surface area, as well as high thermal and chemical stability. These properties make nano-materials good candidates for environmental remediation technologies (Ambaye et al., 2021b; Ambaye et al., 2018). These exceptional properties can also solve drawbacks of corresponding bulk material of surface/interface, quantum size, and high surface reactivity. This unique property makes them best suited for in situ remediation applications (Rani et al., 2017; Rani

et al., 2021). Datsyuk et al. (2008) claimed acidic pre-treatment could increase hydrophilic functional groups, leading to nano-material dispersion and immobilization, which enhanced the performance efficiency of carbon nanotubes as adsorbent material. Oxidation and adsorption are the two main processes in which nanoparticles remove PHCs from the contaminated soil matrix (Usman et al., 2022).

In general, since their reaction is much more efficient than that of other materials, nanosized particles provide a solution for quicker and more cost-effective site remediation. For instance, Jiemvarangkul et al. (2011) reported that about 88 % of PHCs are removed from contaminated water using nanosized oxide material prepared from iron and extract of *vaccinium floribundum*. However, 32 hours of treatment with the same nanoparticles can remove about 82% of petroleum hydrocarbon from the soil (Murgueitio et al., 2018). Pavía-Sanders et al. (2013) used hybrid nanoparticles to remove polycyclic hydrocarbon from contaminated soil made from combining hydrophobic polyacrylic acid block copolymers with oleic acid-stabilized Fe_2O_3 nanoparticles with tetrahydrofuran as PAH sequestering agents from crude oil and hydrophilic polystyrene material, showing that the hybrid material can remove the pollutants more than ten times compared its weight. Hu et al. (2014) showed that compressible carbon nanotube-graphene hybrid aerogels could be used for oil sorption and removal from polluted media.

Cobalt and manganese nanoparticles facilitated the degradation of PHCs in soils within 3 hours (Nador et al., 2010; Zhou et al., 2019). In UV radiation, Saien and Shahrezaei. (2012) used TiO_2 metal nano-oxide to remediate pollutants from petroleum refinery wastewater. In UV, 100 mg/L catalysts degraded 78 % PHCs within 60–90 minutes. Alizadeh et al. (2013) successfully degraded PHCs using TiO_2 nanopowder film, while Ziolli and Jardim. (2002) used colloidal TiO_2 metal nano-oxide and successfully photo-catalyze and degraded crude oil from sea seawater. In general, nanotechnology gives more benefits in the bioremediation of soil contaminated with petroleum hydrocarbons.

The use of nanotechnology in bioremediation is playing a vital role (Table 2.4). However, it also has some concerns regarding living biota, including human health. it is essential to evaluate their ecological risk and consequences. Currently, many studies emphasize the need to understand the toxicity, mobility bioavailability, and potential risks of nanoparticles. The applicability of nanoparticles to large-scale remediation of PHCs from polluted soil is another issue due to the recalcitrance nature and stability of manufactured nanoparticles (Vázquez-Núñez et al., 2020).

Before these nanoparticles are used on a broad scale, it is important to understand their environmental fate better (Cecchin et al., 2017). Furthermore, nanotechnology's successful and long-term implementation would be dependent upon the use of low-cost, environmentally friendly nanotechnology in fields as diverse as biomedicine, agriculture, and bioremediation.

Table 2-4. Application of Nanomaterials for removal of petroleum hydrocarbons from soil

Type of PHs	Nanomaterials	Conditions	Remediation Efficiency (%)	References
Total PHs	Biogenic iron oxide	Total PHs are degraded in 14 h and 5 d at pH 6.0 and 0.74 M oxidant.	95	Bolade et al. (2021)
Total PHs	Biogenic iron oxide	Total PHs are degraded in 14 h and 5 d at pH 6.0 and 0.74 M oxidant.	95	Bolade et al. (2021)
Total PHs and PAHs n-alkanes	Ferrous sulfate enriched with biochar and activated persulfate	It removes the difference at pH 7 with a shaking incubator at 25 °C in 7 d	62 78 92	Xia et al. (2021)
Total PHs	Iron nanoparticles coated with mortiño berry (Vaccinium floribundum)	It removes the total PHs in 32 h at pH between 9 and 109 with initial concertation of 94.20 mg L^{-1}	88	Murgueitio et al. (2018)
Total PHs	Green mango peel-nanozero valent iron	Total PHs removal over one-week treatment at the temperature of 20 °C on a rotating shaker (200 rpm)	90	Desalegn et al. (2018)
Diesel-oil	nanoscale zero-valent iron activated with peroxymonosulfate	It removes the aged petroleum hydrocarbon in 2 h	61	Bajagain et al. (2018)
Petroleum Hydrocarbons	Goethite-chitosan Nanocomposite	The nanocomposite increases the biodegradation process in 3 d of incubation	93	El-Sheshtawy et al. (2021)

5.2. Electro-bioremediation (EB)

EB is a technology where electromigration or electroosmosis is used. It improves in situ pollutants biodegradation using a low voltage applied by electrodes positioned inside polluted soil. This technology is incredibly successful in polluted soil with a high surface area and low hydraulic conductivity (Camacho et al., 2021). For this reason, various biological growth parameters such as microbial population, nutrients, and surfactants can all be moved in various orientations within the groundwater matrices and soil (Li et al., 2020). Despite its effectiveness, this technology has

many disadvantages, including energy consumption, only applicable to specific contaminated soils, low hydraulic conductivity, contamination availability, and soil matrix heterogeneity (Li et al., 2020). However, some studies are currently boosting these techniques by using additives, e.g., biosurfactants, to increase the availability of contaminants during the treatment processes. Even though other biological methods have some advantages, they also have disadvantages like lack of terminal electron acceptors, lower biokinetics, pollutants toxicity and partial degradation of materials, and high demands of capital and maintenance costs. Hence, they required more effective and long-term approaches to removing hydrocarbon pollutants from the environment (Baniasadi and Mousavi, 2018).

5.3. Bio-electrochemical systems (BESs) applications

BES is evolutionary new bioengineering technology that converts organic compounds present in wastewater into biogenic electricity for wastewater bioremediation. One of the exceptional properties of this technology is the microorganism that participated have the ability transferring of electrons extracellularly to solid anode electrode materials, compared to microbial metabolism using intracellular mechanisms as well as compared to low-cost conventional wastewater technologies, they produce less sludge, and it can also produce various high-value fuels and chemicals such as polyhydroxyalkanoates, methane, and ethanol (Baniasadi and Mousavi, 2018; Espinoza-Tofalos et al., 2020; Daghio and Franzetti, 2018).

As a result, in the last ten years, bioelectrochemical systems such as microbial fuel cells have been employed to treat wastewater discharge from several industrial and municipal sources, including the chocolate industry, sewage, brewery, and swine. They have a high elimination of nitrate and sulfates and a 95 percent reduction in chemical oxygen requirement (Mohyudin et al., 2022; Prathiba et al., 2022). This has opened a new chapter to use these systems to remediate soil polluted with PHs by the electro-chemically active microorganism: the anode of the microbial fuel cells that can catalyze organic electron donor oxidation, such as hydrocarbons, and transfer electrons to the anode extracellularly. Electrons are moved to a cathode via an external circuit, where O_2 is usually reduced to H_2O. The microbial fuel cell's anode can act as the non-exhaustible electron acceptor to transfer electrons from the polluted site to the source having high terminal electron acceptors.

Furthermore, the BESs electrodes could enhance petroleum hydrocarbon degradation without any energy input or chemical addition. That could reduce the operating costs than other methods of

remediation as well as it is used as generates an electrical current, which can not only act as a bio-remediation indicator in real-time but also drive wireless sensors for remote online tracking (Gambino et al., 2021; Li et al., 2021; Wang et al., 2021). Several research studies have been carried out on bioelectrochemical systems to remove petroleum hydrocarbon from contaminated soil in the past ten years, which are summarized in Table 2.6. Accordingly, much attention should be dedicated in the future to setting those technologies at larger and green scales with more standardized engineering protocols.

Besides the above studies on PHs remediation from contaminated soil, for instance, Yu et al. (2021) have investigated the degradation of petroleum hydrocarbon polluted soils as substrates using double microbial fuel cells and showed that the pollutant degradation has increased by about 59% compared to the open circuit control within 115 d. In another study, Morris and Jin. (2012) reported that using a modified single microbial fuel cell can increase by about 12 % the removal rate of total petroleum hydrocarbon from contaminated soil compared to the initial biodegradation rate. Finally, some reports showed that the use of microbial fuel cells and BESs could improve the degradation of petroleum hydrocarbons, particularly asphaltene fractions, and aliphatic and aromatic compounds (Jin and Fallgren, 2022; Wang et al., 2022).

In both ex-situ and situ methods, microbial fuel cells and BESs can be used to remediate soil polluted with PHs. These technologies are reported biocompatible, robust, versatile, and eco-friendly to bioremediate various pollutants. However, due to diversion and competition among sulfates and nitrates electron acceptors, low microbial growth rate, low bioavailability of the substrate, high cost of the catalyst material, and overpotential losses at electrodes are considered the main obstacle that restricts its efficiency for bioremediation processes at higher scales (Singh and Kumar, 2022). For example, Zeppilli et al. (2021) reported that phosphate buffer in the anode could maintain the neutrality of the solution's pH for short periods. However, this develops overpotential at the anode, resulting in acidification, and leading to microbial activity inhibition. The other problems in which BESs hindered application on a large scale are the ohmic losses, low substrate bioavailability, and a clear understanding of the microorganism with the transfer of electrons (Gupta et al., 2022). The use of bio-cathodes, biosurfactants, electro-chemically active microbes, and redox mediators can solve the electrical problems encountered to remediate soil polluted with PHs. However, the application of using materials integrated is still in the initial stage and needs more research and investigation.

Table 2-5. Application of BESs for removal of petroleum hydrocarbons from soil

Type of pollutant	Configuration of the BESs and operating condition of the reactor	Microorganisms	Remediation efficiency rate (%)	Reference
Petroleum hydrocarbons	Petroleum hydrocarbons act as electron donors and glucose, with Tween 80 in single-chambered microbial fuel cells	Activated sludge	45	Zhao et al. (2019)
Nitrobenzene $C_6H_5NO_2$	Acetate acts as an electron donor and nitrobenzene as an accepter in continuous dual-chambered microbial fuel cells	Mixed cultures	1.3	Mu et al. (2009)
Petroleum hydrocarbons	Petroleum hydrocarbons act as electron donors and oxic water as an electron acceptor in Bottle-type dual-chamber reactors microbial fuel cells		29	Wang et al. (2020)
Furfural $C_5H_4O_2$	Furfural acts as an electron donor and oxygen as an electron acceptor in batch Single-chambered microbial fuel cells	Mixed cultures	95	Luo et al. (2010)
Diesel oil	Diesel act as an electron donor and oxygen as an acceptor in Tubular microbial fuel cells with air-cathode	Microbial anaerobic consortium	59	Wang et al. (2019)
1,2-Dichloroethane $C_2H_4Cl_2$	Dichloroethane act as an electron donor and oxygen as an acceptor in continuous dual-chambered microbial fuel cells	Microbial anaerobic consortium	95	Pham et al. (2009)
Diesel oil	Diesel act as an electron donor and oxygen as an electron acceptor in batch dual-chambered microbial fuel cells	Mixed cultures	83	Cheng et al. (2017)
4-nitrophenol	Glucose acts as an electron donor and 4- nitrophenol with H_2O_2 in batch dual-chambered microbial fuel cells	Sludge from anaerobic	100	Zhu and Ni. (2009)
Petroleum hydrocarbon	Petroleum hydrocarbon act as an electron donor, and oxygen act as an acceptor in continuous dual-chambered microbial fuel cells	-	17	Li et al (2019)
Petroleum hydrocarbons	PHs act as electron donors and oxygen as an electron acceptor in batch U-tube Single-chambered microbial fuel cells.	Microbial flora is mixed and extracted from the soil contaminated with saline and petroleum	15.2	Wang et al. (2012)
Phenanthrene	Phenanthrene acts as an electron donor, and oxic water acts as an electron acceptor in batch sediment microbial fuel cells	Mixed microbial extracted from the polluted site	99	Yan et al. (2012)

6. Characterization of remediation processes

The effectiveness, predictability, and reliability of remediation technology are key factors in its performance. These criteria must be monitored regularly to be adopted on a broader scale. In addition, Treatment-specific monitoring protocols that are statistically sound are needed. Also, replication, proper sampling, and data processing are critical components of monitoring. There is a range of main biological and analytical approaches available today to track soil remediation processes (Kour et al., 2021).

6.1. Biological methods

Environmental remediation technologies have grown in prominence as a long-term strategy for restoring contaminated areas. To enable the proper implementation of such protocols, efficient and accurate techniques for monitoring environmental clean-up must be created. Several analytical approaches have been established over time to monitor the environmental clean-up process. These approaches, however, have several flaws, including poor efficacy and the inability to discern between biological and non-biological deterioration. As a result, biological or molecular methods are considered excellent tools for microbial identification. It also provides information on the concentration of substrates and their fate. They are primarily dependent on the metabolic and abundance activities of microorganisms involved in bioremediation. Hence, there are two types of methods that can be used for this: culture-based and culture-independent (Kour et al., 2021). Culture-dependent methods like plate count, carbon source utilization tests, most probable number, transcriptomics, and proteomics are also considered methods for microbial identification and bioremediation (Daverey et al., 2021). The enzyme-related immunosorbent assay is the most widely used immunochemical tool for identifying and monitoring bioremediation techniques. For instance, according to Ko et al. (2019), their research to analyze and identify the microcystin formed by the cyanobacteria during the bioremediation process of eutrophic water using immunochemical tools shows high efficiency for controlling the bioremediation process. Culture-dependent methods cannot analyze the entire population at contaminated sites because only about 1% of all microorganisms are culturable. As a result, a modern technique known as the culture-independent approach replaces conventional recognition methods. These fingerprinting approaches do not need to cultivate and include a complete profile of microbial communities and have a high throughput, effective and repeatable such as metagenomic approaches. In addition to this, polymorphic DNA random amplification, restriction fragment length polymorphism,

sequence typing, single-strand conformation polymorphism, fluorescence in situ hybridization, repetitive sequence-based PCR, reverse sample genome probing, PCR, and phospholipid fatty acid analysis can also use for monitoring and characterization of bioremediation process that includes microorganisms (Srivastava et al., 2021). However, a lack of standardization and reliability is also noted among molecular techniques when applied to complex and long-term contaminated soils and wastewater. Another effective alternative widely used to characterize the bioremediation process is the enrichment method. The basic idea behind bromodeoxyuridine methods enrichment is that metabolically active organisms can integrate a branded nucleotide bromodeoxyuridine method into their DNA, which can then be extracted based on the inserted mark and mainly used to enrich microbes that have been grown on xenobiotic compounds (Bhadury and Ghosh, 2022). Besides, another enrichment process is stable-isotope probing, which includes soil bacterial species providing a 13C-labeled substrate. This proves that the microorganism using substrate integrates 13C in their DNA, making it denser than standard DNA containing 12C, allowing the metabolically active cells to be identified. The technique of stable isotope probing is also applied to monitor biological effects (Wang et al., 2022). For instance, Kasanke et al. (2019) used this technique to identify and characterize *Rhodoferax sp*, degrading sulfolane successfully. Recently, microbial biosensors and microbial fuel cells are also used to assess bioremediation processes (Jadhav et al., 2021). Tang et al. (2008) developed a laccase enzyme-based biosensor to monitor catechol in bioremediation. Hence more research and investigation need in in synthetic biology and genetic engineering to facilitate the design and development of microbial biosensors for efficient and accurate monitoring and assessment of bioremediation as well as to employ a sustainable tool for the rehabilitation of environmental remediation technologies.

6.2. NGS technology

Next generation sequencing technology (NGS) provides unique opportunities in PHs bioremediation and biodegradation research (Mangse et al., 2020). It offers new insights into the ecology of microbially mediated processes that influence the degradation of pollutants in the soil. NGS technology enables parallel analysis of DNA sequence information about nucleic acids, used for environmental quality assessment. It is applied to identify novel genes, pathways, and even microbial shifts involved in the degradation of hydrocarbons. In addition, NGS technology provides reliable information about critical enzymes involved in various detoxifying types of

pollutants. Thus, they became a valuable tool for quality monitoring and data management based on contaminated soil sites (Chandran et al., 2020). NGS technology consists of library preparation, sequencing, base calling, established genome alignment, and assorted annotation. Library preparation starts with multiple DNA fragments, and enzyme digestion, followed by sequences (López, 2020). However, they are typically more costly and need more complex devices and reagents than other advanced statical and bioinformatics tools.

6.3. Analytical methods

Analytical methods are at the core of remediation science, and it is difficult to assess without them. In situ monitoring of bioremediation with electrochemical electrodes is the preferred process, particularly in anoxic environments (Kour et al., 2021). They may detect several cations and as well anions (Yan and Reible, 2015). However, this technique was less successful in the case of hydrophobic organic compounds, such as PAHs (Jasmine and Mukherji, 2019). Pollutants and various intermediates contaminants can be quantified using spectrometry spectroscopic, and chromatographic techniques. This is another way to track the bioremediation method. These entail thorough chemical and physical characterizations of the target molecules and their degraded intermediates (or final products), with high specificity, sensitivity, and reproducibility (Kour et al., 2021).

Gas chromatography (GC), and high-performance liquid chromatography (HPLC) is among the chromatographic methods that are widely used to track the remediation of pesticides, petroleum products, oily sludge, hydrocarbons, and heavy metals (Ambaye et al., 2021a). The combination of GC and mass spectrometry is an excellent way to identify pollutants and their intermediates with both standardized and appropriate methods of purification of soil samples (Ambaye et al., 2021a; Patil et al., 2022). In addition to this, various spectroscopy techniques like nuclear magnetic resonance, atomic absorption Fourier transform infrared, 3D-fluorescence spectroscopy, X-ray diffraction, spectroscopy, and Raman spectroscopy are used to monitor and characterize various pollutants, including PAHs, PHs, and petroleum sludge (Ambaye et al., 2021; Patil et al., 2022; Shrivastava et al., 2019). From the above spectroscopy techniques, atomic absorption spectroscopy and Fourier transform infrared spectroscopy are two of the most cost-effective, responsive, and reliable but less accurate methods available. Another way to classify the pollutants is by using the pollutants' physical and chemical properties due to thermal treatment (Ziglio et al., 2019). In

general, petroleum pollution has a significant negative impact on humans and the environment. Researchers from all over the world are looking into bioremediation to minimize this type of pollution safely. Analytical scientists can provide a variety of answers in this area with the use of modern analytical techniques. Various analytical techniques have evolved because of the extensive research conducted in this area, and it has become clear that proper sample preparation is critical in determining the validity of quantitative hydrocarbon assay results. The use of GC-based analysis for monitoring analytes and their metabolites is obvious. Based on metabolomic profiling through Gas chromatography-mass spectrometry and Liquid chromatography-mass spectrometry, degradation pathways are determined. Other analytical techniques such as Fourier-transform infrared spectroscopy, Nuclear magnetic resonance, Liquid chromatography-mass spectrometry, and high-performance liquid chromatography are being used more frequently in bioremediation research to determine petroleum pollutants.

7. The challenges in adopting sustainable remediation technology

Selecting and adopting an appropriate sustainable remediation technology is dependent upon various factors that can affect the remediation process, efficiency, and suitability, including abiotic and biotic, scientific, and non-scientific factors, as well as a shortage of clear scientific evidence regarding these factors and how they can affect the efficiency and remediation process during real-world application (Ossai et al., 2020). As a result, physicochemical properties, contaminate and environmental properties, biological properties, source, composition, age, type, heterogeneity, concentration, site conditions, process, geohydrologic location, monitoring difficulties, space requirement, extended treatment time, required level of clean-up standard, remedial approach and strategy, risk management strategy, life-cycle analysis, cost-benefit analysis, and cost-benefit analysis are all required (Kuppusamy et al., 2016a; Ossai et al., 2020). In addition to the above factors, some non-technical approaches to selecting sustainable remediation technology are human resource management, liability, and techno-economic regulation to meet the required clean-up standard (Ossai et al., 2020). Proposed strategies for sustainable remediation and management of soil contaminated with petroleum hydrocarbons are shown in Fig. 2.8 (Kuppusamy et al., 2020; Thomé et al., 2020).

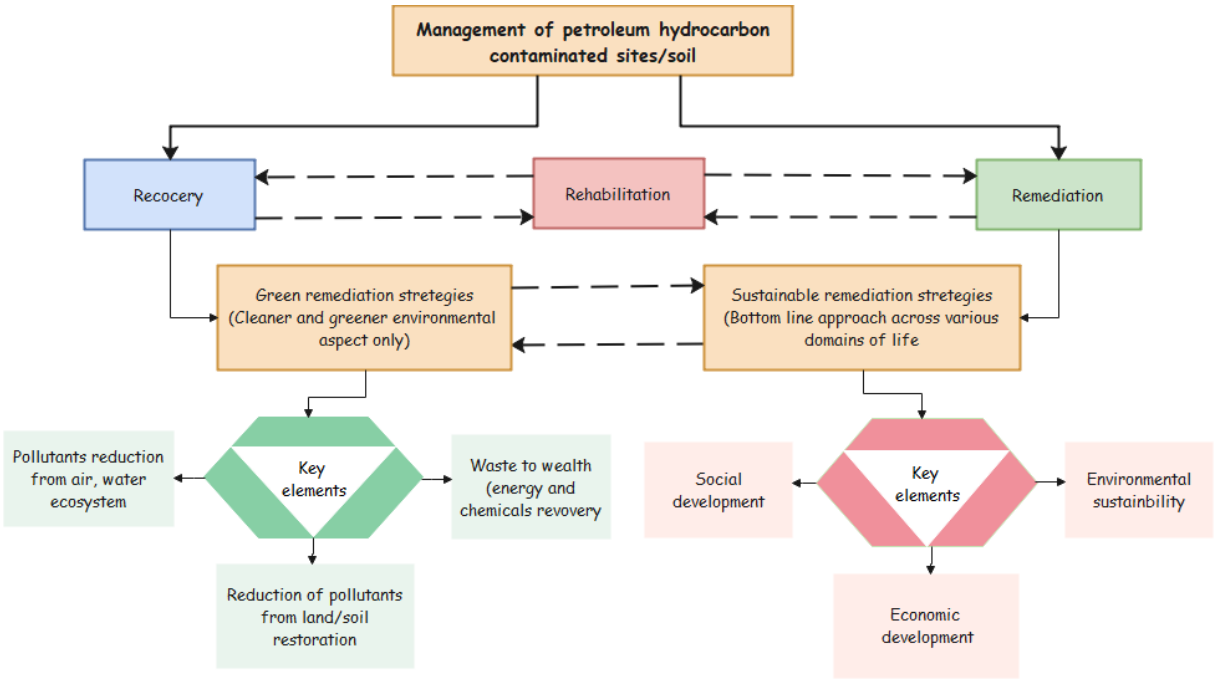


Figure 2-8. Proposed approaches for sustainable remediation and management of petroleum hydrocarbon contaminated soil (Kuppusamy et al., 2020; Thomé et al., 2020).

8. Conclusion and future perspective

This review paper addresses the leading available soil-remediation technologies to restore soil used at both lab and field scales. Various remediation technologies are being practiced, but no single approach can be applied to all environmental conditions to reduce PHs from polluted soil and other contaminated sites. Hence, this review paper provides, first, a better understanding of PHs compositions, properties, main transportation routes, and their fate in soil and environment. It is essential to mention that all remediation process parameters considerably impact the decision-making process and enhance remediation effectiveness. Therefore, nowadays, eco-friendly approaches and inventing novel PHs-degrading mechanisms for restoring contaminated soils with cost-effectiveness are highly requested. Essential strategies for managing PHs and the design-making process are also needed for removing pollutants from the contaminated sites to achieve long-term sustainability goals. Parallelly, the development of risk-based management tools based on databases, machine learning platforms, LCA and LCCA studies, the use of next-generation sequencing (NGS), the cost-effectiveness of nanotechnology and electrochemically active microorganisms-based, bio-electrochemical systems must be given more emphasis to design the

appropriate solutions for soil contamination and with better future perspectives. More focus should be given to assessment approaches to determine the efficacy of remediation in the development of new *in-situ* remediation technologies and as well as in the development of new open-source software (for low-income countries). That can be applied to reduce PHs from polluted soil in all environmental conditions as sustainable and eco-friendly soil remediation technologies that can be used at several sites in the world with different contamination levels.

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CHAPTER 3.

EX-SITU BIOREMEDIATION OF PETROLEUM HYDROCARBON CONTAMINATED SOIL USING MIXED STIMULANTS: RESPONSE AND DYNAMICS OF BACTERIAL COMMUNITY AND PHYTOTOXICITY

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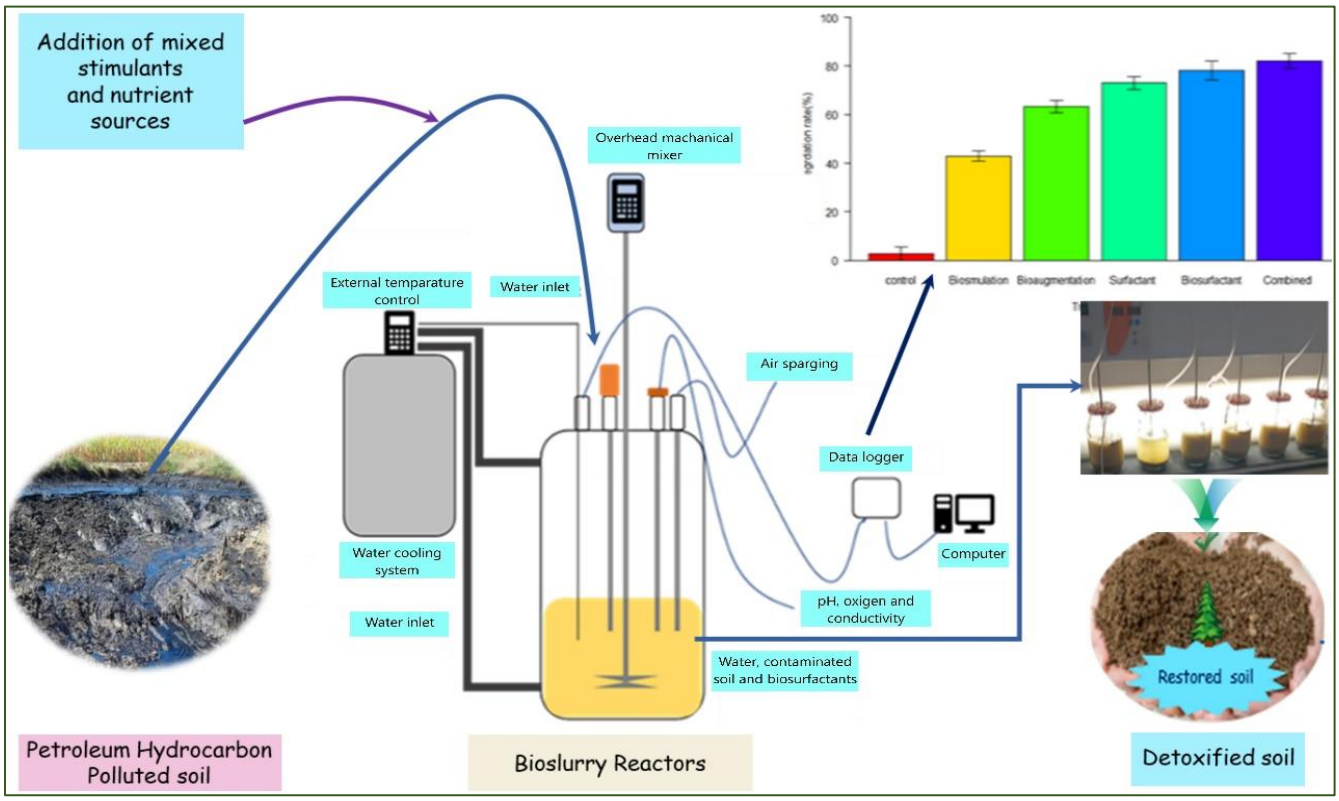
Abstract

Pollution of agricultural soils by excessive petroleum hydrocarbons (PHCs) has recently become a severe worldwide environmental issue. Several approaches have recently been used to biodegrade PHs and detoxify soils. In the present study, an experimental mesocosm was developed in a bioslurry reactor. Six treatments were applied to degrade PHs and detoxify contaminated soil by adding external stimulants such as nutrients, activated sludge, synthetic surfactants, and long-chain rhamnolipids. The comparative analysis revealed that the combination of activated sludge, nutrients, and biosurfactant showed a 79.4% reduction, while the lowest reduction in total PHs (48.5%) was attained in the nutrient-supplied mesocosms in total petroleum hydrocarbons (TPHs) compared to the mesocosm experiment performed without the addition of stimulant within 10 days. The consortium's kinetic analyses of PHs revealed the lowest half-life time ($t1/2 = 4.1$ days), the highest biodegradation rate constants ($k = 0.17$ day^{1}) and the reaction rate data from each bioremediation method was fitted with a first-order reaction rate model. The 16S rRNA gene amplicon sequencing revealed that *Streptomyces, Nocardioides, Arthrobacter,* and *Pseudomonas* are the main petroleum hydrocarbon degrading bacteria during crude oil biodegradation, and the top highest relative abundances of the genera are *Arthrobacter* and *Pseudomonas, which* presented a positive correlation to the biodegradation of petroleum hydrocarbons. The phytotoxicity studies proved that there was a significant decrease in the toxicity of the soil after the biotreatment. It can be deduced from the findings of this study that the combination of activated sludge, nutrients, and biosurfactants in bioslurry promotes the degradation of PHs in the soil and is a promising technology for large-scale application.

Keywords: Petroleum; Soil; Pollution; Stimulants; Bioremediation; Phytotoxicity

Graphical abstract



1. Introduction

Petroleum is an inseparable component of socioeconomic and industrial development (Wu et al., 2022). Various petroleum hydrocarbons (PHs) are released into the environment during the extraction, storage, transportation, refining, and consumption of PHs products, causing water and soil contamination (Ambaye et al., 2022; Vaccari et al., 2020). PHs as pollutants are a very toxic mixture of paraffinic, aliphatic, and aromatic components and have become a severe environmental concern (Zhang et al., 2020) due to their capability to pollute soil and water and have become an important research topic (Ambaye et al., 2022).

Traditionally, excavation and landfill disposal have been used for PH contamination of soils. However, these strategies have become less practicable as landfills have become scarcer and more expensive (Flood, 2010). Solvent extraction, incineration, chemical oxidation, thermal desorption, immobilization, nanostructured materials, and electrochemical sludge are used to treat and clean up contaminated soil (Ghanbari et al., 2020; Daniel et al., 2022; Karim et al., 2022). Moreover, these treatment methods dilute PHs or shift them from one environmental place to another and are typically costly, energy-consuming, and unsustainable in terms of environmental implications, as they produce secondary pollutants and do not solve the problem (Ambaye et al., 2022). These constraints need a more cost-effective and ecologically friendly approach to contaminated soil remediation.

Microbes like heterotrophic bacteria and fungi can metabolize and transform complex PHs pollutants into simple, less toxic forms (Srivastava and Kumar, 2022) and are widely used as bioremediation agents to remove PHs from the environment (Waychal et al., 2022; Irfan et al., 2002). However, bacterial bioremediation efficiency depends on the availability of nutrients, organic carbon, soil pH, temperature, texture, and PHs bioavailability, the abundance of indigenous and exogenous microbial communities, and the selection of the desired bioremediation processes (Gielnik et al., 2020; Lumia et al., 2020; Wang et al., 2021; Pradhan et al., 2021).

Bioslurry reactors are among the most efficient ex-situ bioremediation systems for restoring PHs contaminated soils, particularly under controlled conditions. Lumia et al., 2020 conducted a study where polluted soil was mixed with water to make a slurry; and showed that 10 to 60% (w/v) is sufficient to maintain homogeneous conditions. Fed-batch bioslurry reactors have high bioremediation efficiency (Mohan et al., 2008) due to mixing devices that maintain the suspension

constant throughout the period, while an aeration pump creates a suitable condition for anaerobic metabolism (Robles González et al., 2008). However, partial oxidation of PHs produces more intermediate compounds, lead poor soil bioremediation performance. Moreover, PHs bioavailability to microbes enhances PH biodegradation (Ambaye et al., 2022; Collivignarelli et al., 2018).

Currently, many reports have shown that the addition of nutrients, activated sludge, surfactant, and biosurfactant enhances the biodegradation of PHs in oil-polluted soil (Yaman, 2020; Kuppusamy et al., 2020; Ossai et al., 2020; Agamuthu et al., 2013; Daniel et al., 2022; Chebbi et al., 2021). However, the effects of these external stimulants are case-specific, and results are inconsistent and vary with activated sludge, nutrients, surfactants, and biosurfactants (Rathankumar et al., 2022; Karthick et al., 2019, Chebbi et al.,2022, Nwankwegu et al., 2022; Obayori et al., 2009). Understanding how nutrients, activated sludge, surfactants, and biosurfactants influence the populations of hydrocarbon degraders, and the diversity and activity of the microbial community to adapt to new environmental conditions is critical in ensuring effective bioremediation of petroleum-contaminated soil (Mohan et al., 2008; Rahbarisisakht et al., 2017; Syafruddi et al., 2017).

A recent study also demonstrated the impact of long chains of rhamnolipids on the remediation of contaminated soil individually in bioelectrochemical systems (Ambaye et al., 2022a). However, no literature has reported the effect of long chains of rhamnolipids combined with nutrients and sludges on the bioremediation effect of TPH-polluted soil as well as its relationship between petroleum hydrocarbon degradation efficiency and microbial community dynamics and microbial activity. Hence, combining the amendment of long-chain rhamnolipids with nutrients and sludge may be an excellent approach to remove TPHs and improve the polluted soil condition. Therefore, the degradation of PHs contaminated soil using added external stimulants such as nutrients, activated sludge, surfactants, and long-chain rhamnolipids were studied individually and in combinations in mesocosm experiments with bioslurry reactors to investigate the relationship between hydrocarbon degradation efficiency and microbial community dynamics in petroleum-polluted soil as well as its phytotoxicity assays were also done using *Lepidium sativum* seeds.

2. Materials and methods

2.1 Materials and chemicals

The soil was collected from a remediation site located in northern Italy. For an increased content of total petroleum hydrocarbons (TPH), the soil was spiked with a mixture of hexane and diesel (v/v = 1:1) in plastic vessels to prepare the soil substrate (Silva-Castro et al., 2013), incubated under a chemical hood for solvent evaporation, and incubated in the dark at room temperature (22.5 °C) for 24 h. Physicochemical characterizations of soil samples were analyzed using the standard ALPHA 5310 (Napoli et al., 2017). The feasibility of biostimulation was tested using analytical grade nutrients such as phosphorus and nitrogen (1.5g NH_4Cl and 0.1g KH_2PO_4) were added at the beginning of the study to achieve a carbon: nitrogen: phosphorus (C: N:P) molar ratio of 100:10:1 to ensure that there is no nutrient limitation during the assay (Maddela et al., 2016). The bioaugmentation was tested using activated sludge (5g/L) obtained from a municipal wastewater treatment plant in Brescia (Italy), and the added concertation was selected based on prior studies (Juteau et al.,2003). In addition, the effect of a non-ionic synthetic surfactant was tested with Tween 80 (polyoxyethylene (20) sorbitan monooleate) purchased from Sigma Aldrich (Cecotti et al., 2018). The broths containing long chain rhamnolipids used in the bioslurry reactor were previously produced by a safe bacterium E264 at a large-scale fermenter (10 L) using winey residues, a sole carbon, and energy source, as described by Chebbi et al., 2021.

2.2 Mesocosm tests

The experimental setup included a variety of mesocosm conditions to determine the best one for use in a bioslurry reactor. The preliminary microcosm investigation was conducted using collected from a remediation site located in northern Italy with initial concentration (3500 mg/kg_{dw}). The physicochemical analysis of the contaminated soil reveals a high total carbonate content (148 g/kg), organic carbon (26.44 g/kg), moisture content (8.14%), total N (2.02 g/kg), total P (27 mg/kg), C/N (13.1 g/kg), pH (7.6), and conductivity (113.2µS/Cm). The soil particle size investigation showed the presence of the sand fractions (469 g/kg), followed by silt (401 g/kg) and clay (130 g/kg) fractions in petroleum hydrocarbons (PHs), as shown in Table 3.1.

Table 3-1. Main Physico-chemical parameters of PHs contaminated soil

Parameters	**Value**
pH	7.6 ± 0.3
Total carbonates (g/kg)	148± 2.1
Organic C (g/kg)	26.4± 1.2
Total N (g/kg)	2.02± 0.1
Total P(mg/kg)	27± 1.1
C/N (g/kg)	13.1± 0.4
Sand (g/kg)	469± 3.1
Silt (g/kg)	401± 4.2
Clay (g/kg)	130± 3.1
Conductivity (µS/cm)	113.2± 1.2
Moisture (%)	8.14± 0.5
Total petroleum hydrocarbon (TPH) (mg/kgdw).	3500

Note: Mean values with a standard deviation

Compared to the abundant carbonaceous content, the soil was described as a sandy loam with a neutral pH and a phosphorus and nitrogen deficiency. Given these circumstances, biostimulation with nutrients may be beneficial to the soil. Carbon, nitrogen, pH, and moisture conditions within each soil did not impede biodegradation because they were all within acceptable levels. Degradation studies were conducted in microcosms consisting of 6 bioslurry reactors using 100 g of soil polluted with diesel oil suspended in 500 mL of distilled water with a 1:5 soil-to-water ratio. 1L Erlenmeyer /conical flasks were used as bioreactors with constant mixing by mechanical mixers at 150 rpm to ensure substrate availability and oxygen transfer for about 10 days. Treatment details for mesocosm experiments for bioslurry reactors with various amendments are provided in Table 3.2. A schematic diagram of the bioslurry reactor design is presented in Fig. 3.1.

Table 3-2. Treatment (T) details for mesocosms experiment with various amendments

Treatments	**Details**
T1	soil + water
T2	soil + water + bio stimulated with 1.5 g NH_4Cl and 0.1 g KH_2PO_4
T3	soil + water + supplemented with 0.0014g/L Tween 80 (polyoxyethylene (20) sorbitan monooleate)
T4	soil + water +supplemented with 5 g/ L of activated sludge
T5	soil + water + supplemented with crude rhamnolipids biosurfactant (500ml).
T6	soil + water + bio stimulated with 1.5 g NH_4Cl and 0.1 g KH_2PO_4+ 5 g /L of activated sludge + supplemented with crude novel rhamnolipids (275 mL)

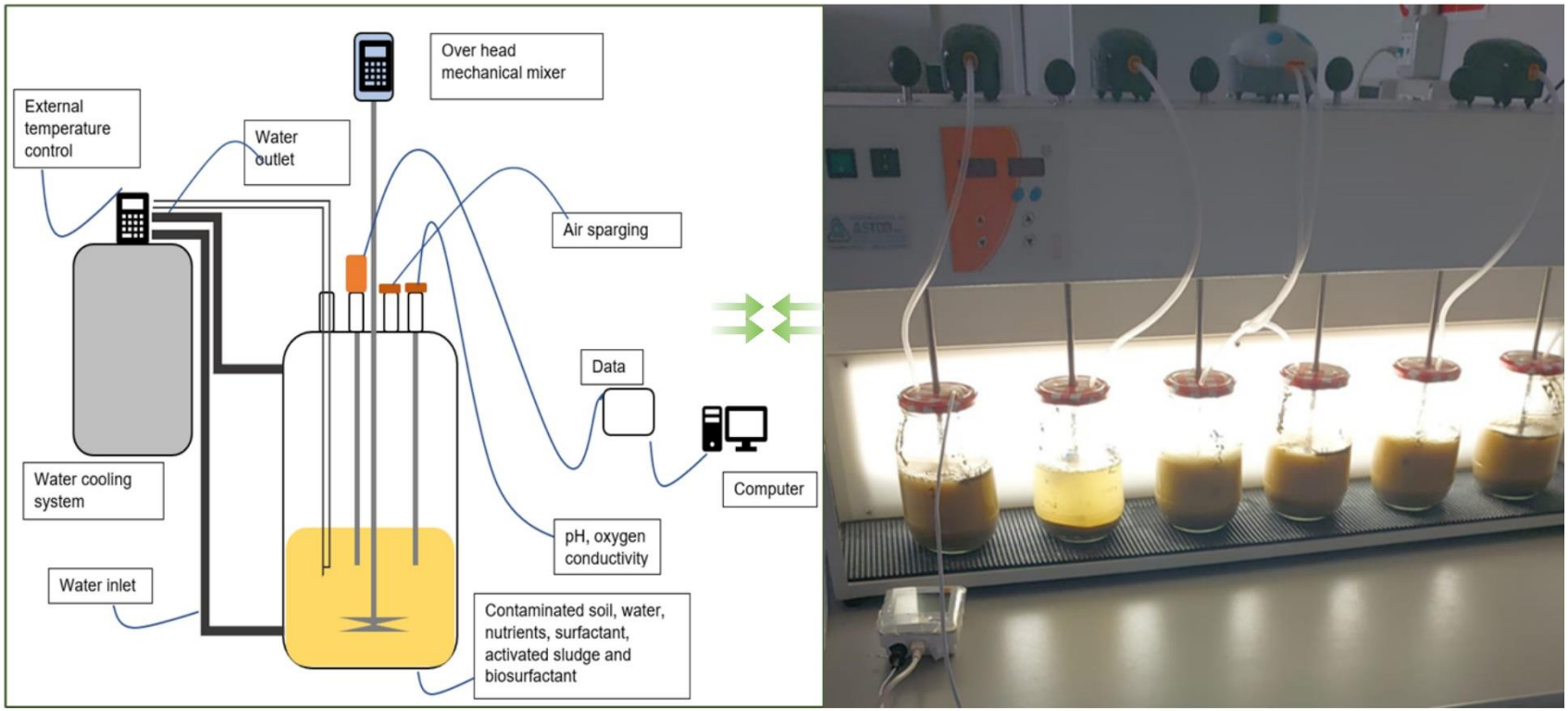


Figure 3-1. Schematic diagram of bioslurry reactor design and photographs of real lab reactor.

2.3 Analytical methods

2.3.1. Extraction of petroleum hydrocarbons (PHs) from soil and its analysis

All collected soil samples were freeze-dried at 20 °C for 24 h under vacuum conditions. Before petroleum hydrocarbon (PHs) extraction, samples were crushed to 2 mm. The following processes were used to extract PHs from soil samples:1.5 g of soil samples, 1 mL ***o***-Terphenyl, and 14 mL n-hexane: acetone (70:30, v/v) were placed in a PTFE vessel. To extract of PHs, the vessel was sealed carefully and placed in a 1000 W microwave oven at 110 °C for 20 minutes, after shaking it for a homogeneous combination. The extracts were filtered using a 0.1 mm cellulose fiber filter. The filtrate was collected and dried with a vacuum hood evaporator before being dissolved in 2.0 mL of n-hexane for purification.

2.3.2. Petroleum hydrocarbon (PHs) purification

The concentrated PHs extract was purified using the solid-phase extraction technique. The conditioned Florisil column was loaded under vacuum with 10 mL of concentrated extract in n-hexane. The PHs were then eluted using a 2 mL n-hexane solution. Following elution, all effluents were collected before chromatographic analyses (Mogashane et al., 2022).

2.3.3. Petroleum hydrocarbons (PHs) analysis by GC

The petroleum hydrocarbon (PHs) concentrations were measured using a GC-FID. The DB-5 MASS capillary column (with a film thickness of 30 m, 0.32 mm ID, 0.25 µm df) was used at a flow rate of 1 mL/min, and N_2 gas (99.99% purity) was used as a carrier gas. The injector temperature was first adjusted to 50 °C for 2 min, then gradually increased to 200 °C at 10 °C/minute. It was then elevated linearly to 250 °C at a rate of 5 °C/min for 5 minutes after being kept at 200 °C for 4 min. There was no split in the sample. PHs were identified using retention time. Their amounts were quantified by using the peak area approach using an external reference method (Mogashane et al., 2022).

At various periods (from zero to 10 days), residual TPH was collected from different experiments: The reduction rate of TPH was removed from the soil calculated using the following equation: Eq. (1)

$$Degradation\ of\ TPH\ (\%) = (Initial\ TPH - Final\ TPH) / Initial\ TPH \times 100 \quad \text{Eq. (1)}$$

2.4 Kinetics of biodegradation and Estimation of biodegradation half-life time

The kinetic study of polluted soil bioremediation was performed as described by Matthies et al., 2008. PHs biodegradability is typically explained by first-order kinetics, as shown in Eq. (2):

$C=C_0e^{-kt}$ Eq. 2

where C is the residual petroleum hydrocarbons (PHs) content in the soil at time t (mg kg^{-1}); C_0 is the initial petroleum hydrocarbons (PHs) content in the soil (mg kg^{-1}); *k* is the constant rate biodegradation (day^{-1}); *t* is time (day). From the plot slope In C vs. *t*, the biodegradation rate constant (*k)* can be derived.

The biological (biodegradation) half-life periods (*t*1/2) of petroleum hydrocarbons (PHs) were computed by Eq. (3) as described by Matthies et al., 2008 as follows:

$t_{1/2}=\ln 2/k$ Eq. 3

where ln2 is 0.693; *k* is the constant biodegradation rate (day^{-1})

The half-life model assumes that the rate of degradation of petroleum hydrocarbons (PHs) is proportional to the size of the PHs pool in the soil (Oleszczuk and Baran, 2003).

2.5 DNA extraction and analysis of Microbial community

The standard protocol was followed to extract DNA from bioslurry-treated soil samples. The procedure was performed using the model FastDNA™ SPIN Kit for Soil (MP Biomedicals, Solon, OH) (Matturro et al., 2017). The samples proceeded with amplicon sequencing of the 16S rRNA gene. All these samples were finally proceeded using the Illumina-MiSeq® platform for sequencing using the 783F 1027R primer to amplify the V5-V6 hypervariable region. The output of Illumina sequencing is a FASTQ file. First, quality-filtered reads are assembled into Operation Taxonomic Units (OTU) using a 97% similarity cut-off. Then, representative sequences of the assembled OTUs are classified (phylum to genus) using the Ribosomal Database Project database. (RDP; http://rdp.cme.msu.edu/).

2.6 Germination index (GI) measurement

To determine the germination index, *Lepidium sativum* L. seeds were grown on the raw and treated soil samples. The experiment was performed by taking 5 g of dry soil in Petri dishes, and the appropriate amount of deionized water was added to keep it moisturized. After about 24 hours, these Petri dishes were covered with Whatman filter paper # 1. In each Petri dish, 10 undamaged and healthy *Lepidium sativum* seeds were placed. After that, all seeded Petri dishes were incubated at 27 °C for 72 hours in the darkroom. After that, germinated seeds (G) were counted. All treatments were measured, including control relative root length (L). All the seed germination tests were carried out in triplicate. The germination index GI (%) was calculated as follows: (G*L)/(Gc*Lc) x100 = GI (%), as described by Avona et al., 2022.

2.7 Statistical analysis

All experiments are performed in triplicate, and results, with the exception of the diversity indices, are reported as the mean ± one standard deviation (SD) using R-package software.

3. Results and discussion

3.1 Reduction of petroleum hydrocarbon

This study evaluates the effectiveness of several bioremediation techniques in improving the biodegradation of petroleum hydrocarbons (PHs) in soil. Compared to the unamended controls, Biostimulation, bioaugmentation, surfactant, and long-chain rhamnolipids-modified treatments reduced total petroleum hydrocarbons (TPH) and shaped the soil bacterial population. Fig. 3.2 depicts the effect of treatments on soil TPH decrease. After 10 days of bioremediation, most treated bioslurry experiments showed a significant drop in TPH levels as compared to untreated controls (79 %TPH reduction). This indicates that increased biodegradation is responsible for decreasing TPH concentration.

3.2 Effect of Biostimulation on the degradation of petroleum hydrocarbons (PHs)

Nutrient limitation in the soil is one of the most severe issues in petroleum hydrocarbon (PHs) bioremediation. As a result, inorganic nutrient addition is required to accelerate the rate of PHs-biodegradation. The addition of nitrogen and phosphorus fertilizers alone effectively stimulated the indigenous microbial population and significantly improved ($p < 0.05$), with a 45% reduction in TPHs compared to the unamended controls, as shown in Fig. 3.2. This suggests that supplying nutrients stimulates PHs degradation in the soil and the native microorganism's growth, supporting the natural selection of diverse PHs-degrading populations. The results are in line with those presented by Kauppi et al., 2011; Sayara et al., 2011; and Abed et al., 2014, which are more efficient for bioremediation of oil-contaminated soil. Combining biostimulation with additional treatments such as bioaugmentation, surfactant, and biosurfactant is an effective treatment strategy for PHs breakdown in oil-contaminated environments.

3.3 Effect of bioaugmentation on the degradation of PHs

Bioaugmentation is used to eliminate hazardous compounds effectively by applying bacteria to contaminated soil under microcosm experiments (Abena et al., 2019; Behera et al., 2022; Nwankwegu et al., 2022; Polyak et al., 2022). For biostimulation, some researchers used activated

sludge as a source of nutrients (primarily nitrogen) (Juteau et al., 2003; Muter et al., 2017). Compared to inorganic fertilizer, this approach represents a cost-saving and smart way to valorize waste often disposed of by landfilling or incineration.

The result showed that amending soil with 5 g /L of activated sludge significantly enhanced ($p < 0.05$) bioremediation efficiency and TPH removal by up to 65% compared to control, as shown in Fig. 3.2. This indicates that activated sludges have a double role as a source of nutrients (biostimulation) and also as bioaugmentation agents by increasing microbial diversity. Juteau et al. (2003) also reported a similar finding by adding activated sludge to about 80 % of TPH removal from the soil compared to the control. Despite the environmental pollution of activated sludge, activated sludge can help bioremediation by reducing toxicity and improving the physicochemical qualities of soil. Furthermore, diverting organic waste to bioremediation programs reduces landfill demand and greenhouse gas emissions.

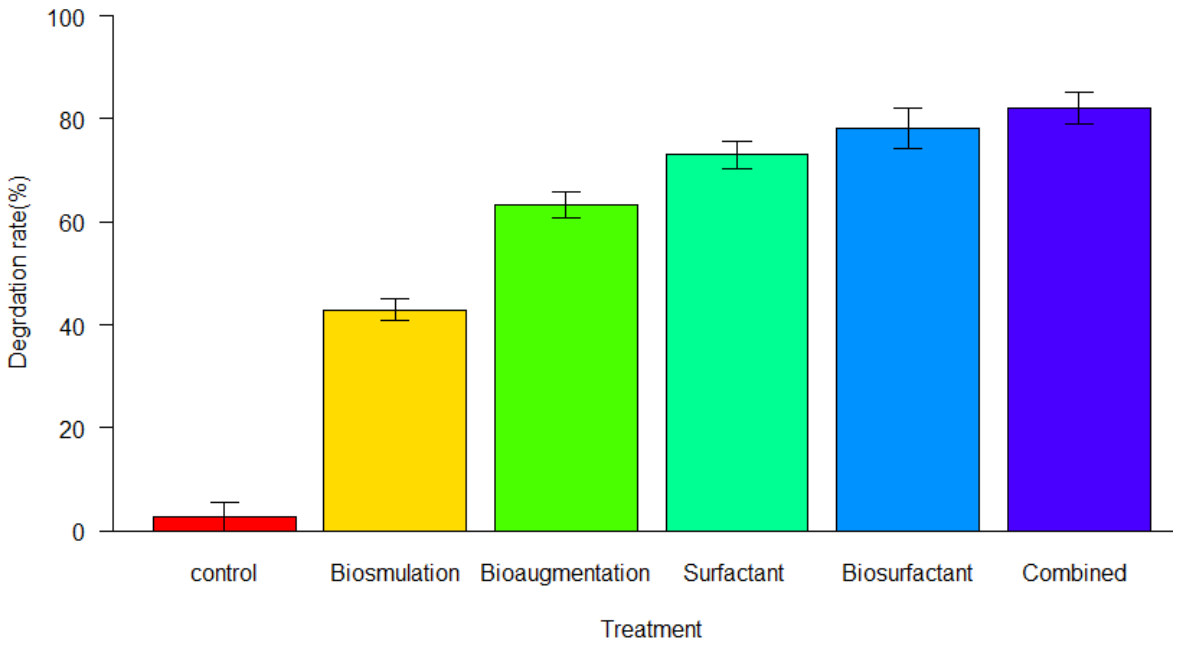


Figure 3-2. TPH degradation by, biostimulation, bioaugmentation, surfactant, and biosurfactant in bioslurry reactor Treatments results were analyzed by employing single-factor ANOVA and are mean of three replicates with standard deviation (mean ± SD, n =2). (**Control** = 100 g soil + 500 mL distilled H_2O; **Biostimulation** = 100 g soil + 500 mL distilled H_2O + 1.5 g NH_4Cl+ 0.1 g KH_2PO_4; **surfactant** = 100 g soil + 500 mL distilled H_2O + 0.0014g/L Tween 80 (polyoxyethylene (20) sorbitan monooleate); **Activated sludge** = 100 g soil + 500 mL distilled

H_2O + 5 g/ L of activated sludge; **Biosurfactant** = 100 g soil + 500 mL distilled H_2O + 500 mL crude Biosurfactant; **combined** = 100 g soil + 500 ml distilled H_2O + 1.5 g NH_4Cl+ 0.1 g KH_2PO_4 + 5 g/ L of activated sludge + 275 mL crude biosurfactant)..

3.4 Effect of surfactant addition on the degradation of petroleum hydrocarbons (PHs)

Surfactant addition may positively impact the reduction in surface tension and increase the solubility of PHs contaminants through micellar solubilization, leading to an increase in the bioavailability of pollutants (Rathankumar et al., 2022). In this study, adding 0.0014g/L of surfactant-amended soil showed a 71% TPH reduction, as shown in Fig. 3.2. Adding the surfactant (Tween 80) enhanced the bioavailability of PHs. The same result also reported by Ortega-Calvo et al. (2013) discovered that adding a surfactant to soil caused bioaccessibility of the contaminants to microbes and enhanced the degradation of polycyclic aromatic hydrocarbons (PAHs) compared to the untreated soil, and this shows that surfactant being utilized as a favored substrate due to its ease of mineralization. However, some studies showed that surfactants could be adsorbed to soil matrices, making them ineffective for the mass transfer of hydrophobic pollutants. Therefore, the utilization of surfactants needs more research in bioremediation (Yan-Zheng et al., 2007).

3.5 Effect of biosurfactant addition on the degradation of PHs

Biosurfactants emulsify the compounds, increase their water solubility, and make the compounds more accessible for the microorganisms, and the addition of biosurfactants plays a role in the bioremediation of soil polluted with petroleum hydrocarbons by increasing the surface area of substrates (Kaczorek et al., 2018; Ambaye et al., 2021).The long-chain rhamnolipids produced on winery residues are investigated for biodegradation assays; the supplementation of rhamnolipids enhanced the rate of bioremediation by up to 75% in terms of TPH removal as compared to the other ex-situ treatments. This research aligns with Bezza and Chirwa (2016), who reported 86.5% PHs removal by adding biosurfactant and nutrients after incubation for 45 days. Because of the hydrophilic-lipophilic balance and the amphiphilic structure of the biosurfactant, Pandey et al. (2022) reported that crude biosurfactant enhanced 77.3% degradation of diesel and 55.9% degradation of n-hexadecane within seven days. The property of the cell surface of the microorganisms leads to high degradation of the PHs and facilitates the uptake of PHs (Chebbi et al., 2021; Saravanan et al., 2020).

3.6 Combined impact of nutrients, activated sludge, and biosurfactant on TPH reduction

The combined impact of nutrients-activated sludge and biosurfactant on the degradation of soil polluted with petroleum hydrocarbons (PHs) was evaluated (Fig. 3.2). The findings indicate that the addition of nutrients, activated sludge, and biosurfactant in a bioslurry reactor improved the degradation of TPH to about 79 % compared to control. These results agreed with previous findings by Jasmine and Mukherji (2014). When a non-ionic surfactant was combined with bioaugmentation and biostimulation, the crude PHs breakdown was increased by 50% compared to natural attenuation (35%). The synergistic effect of these combinations improved the degradation of PHs-contaminated soil. This might be because the biosurfactant boosts PHs-bioavailability, while nutrients and activated sludge help to boost microbial growth and their efficiency in degrading PHs. The minimum reduction (44.9%) was achieved in the reactor having only nutrients. The inclusive performance for TPHs reduction of various treatments was in the order of (combined effect) > (biosurfactant) > (surfactant) > (activated sludge) > (nutrient) > (control).

4. Biodegradation kinetics of petroleum hydrocarbon

The biodegradation rate of the experimental setup includes a variety of microcosm layouts to select the optimal one for use in a bioslurry reactor using a first-order kinetics model equation (Eq. (1) fitted to PHs-biodegradation data. Then, Eq. 1 was applied to compute the half-life durations of the developed microcosm biodegradation (2). Fig. 4 and Table 3.3 show the biodegradation rate constant (k) and half-life durations ($t1/2$) for the various treatment combinations. As shown in fig. 3.3 and Table 3, the combined treatment condition has a higher k (0.1707 days) and lower $t1/2$ (4.1 days) than the control condition with no amendment (k = 0.0115 day1 and $t1/2$ = 60.2 days), despite the inhibitions caused by the combined impact of co-pollutants and nutrient limitations.

Table 3-3. Biodegradation rate constant (k) and half-life durations (t1/2) for the various treatment combinations

Treatment	**Biodegradation constant k (d^{-1})**	**Half-life $t_{1/2}$ (d)**	R^2
Control	0.0115± 0.1	60.2± 0.3	0.774± 0.3
Biostimulation	0.0712± 0.3	9.7± 0.1	0.925± 0.1

Bioaugmentation	0.1158± 0.2	6.0± 0.2	0.959± 0.2
Surfactant	0.1602± 0.2	4.3± 0.1	0.970± 0.2
Biosurfactant	0.1658± 0.1	4.2± 0.2	0.9816± 0.2
Combined	0.1707± 0.1	4.1± 0.1	0.9841± 0.1

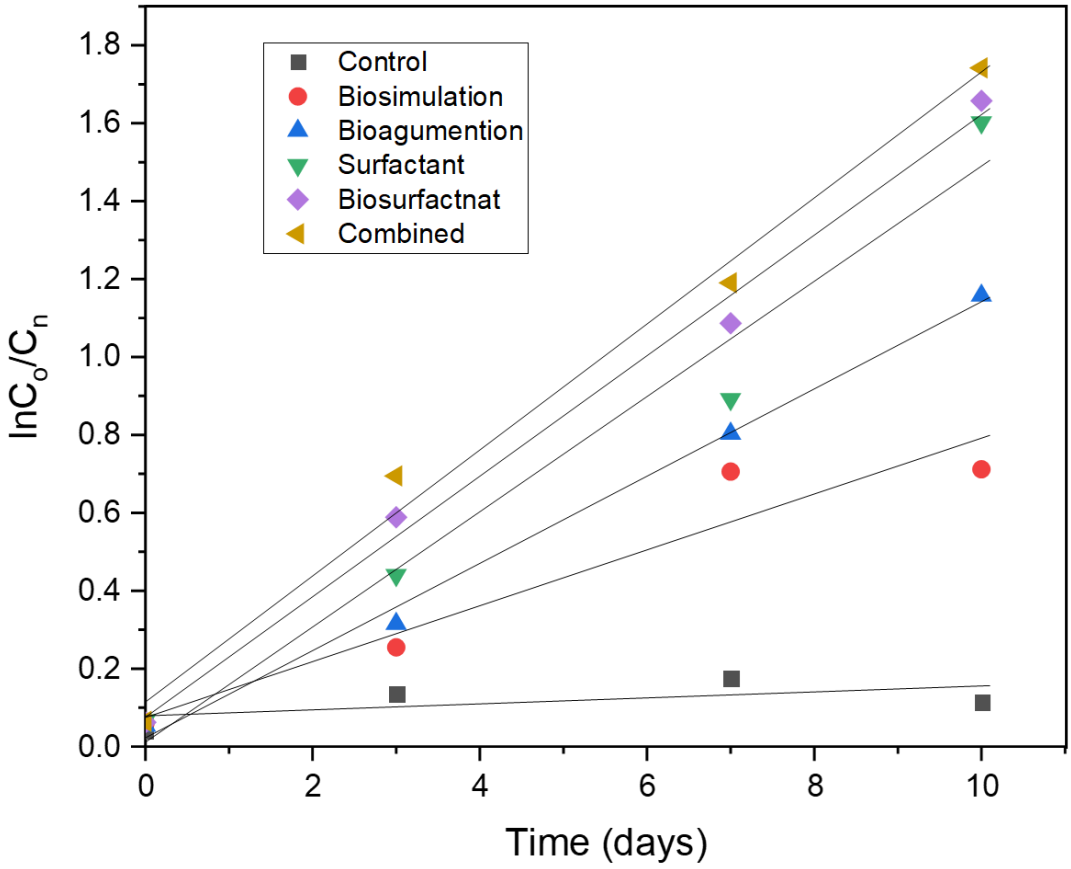


Figure 3-3. Biodegradation kinetics of PHs by biostimulation, bioaugmentation, surfactant, and biosurfactant in bioslurry reactor.

Similarly, Agarry et al. (2013) conducted a kinetic study on the biodegradation of lubricating motor oil in soil by applying natural attenuation, biostimulation, and bioaugmentation. During the process, they reported the values of k of 0.015, 0.033, and 0.030 d^{-1}, respectively. In addition, the values of $t_{1/2}$ were 46.2, 21, and 23 days. As a result, kinetics is critical for determining the equilibrium constant, reaction speed, and half-time of pollutants in PHs in bioremediation studies. Therefore, it is essential to establish an effective cleanup strategy for a PHC-contaminated environment. The kinetics of soil remediation is also essential since it displays the pollutant

concentration at a specific moment and predicts the level that will be present in a future period. (Pi and Bao, 2022).

5. Dynamics of the microbial population during bioremediation

To assess the dynamics of the microbial population during bioremediation, 16S rRNA gene amplicon sequencing was applied. It is imperative to examine the diversity of bacterial community structures by high-throughput sequencing. Fig. 3.4 shows the bacterial community architectures under various genus-level treatments after 10 days of bioslurry treatment. A low abundance of <1% was labeled as others in these sequences. The composition of soil bacterial communities at genus level in treatments and controls were comparable; however, their abundance differed within the treatments. The contaminated soils (control) have a wide range of bacteria at the genus level (Fig. 3.4), including *Streptomyces, Terrabcater, Nocardioides, Pseudomonas, Steroidobacter*, and *Sphingomonas*. The microbial community compositions haven't changed much compared to other treatments, and these bacteria have been found as petroleum hydrocarbons (PHs)-degrading bacteria (Varjani, 2017, Tremblay et al., 2017, Xu et al., 2018).

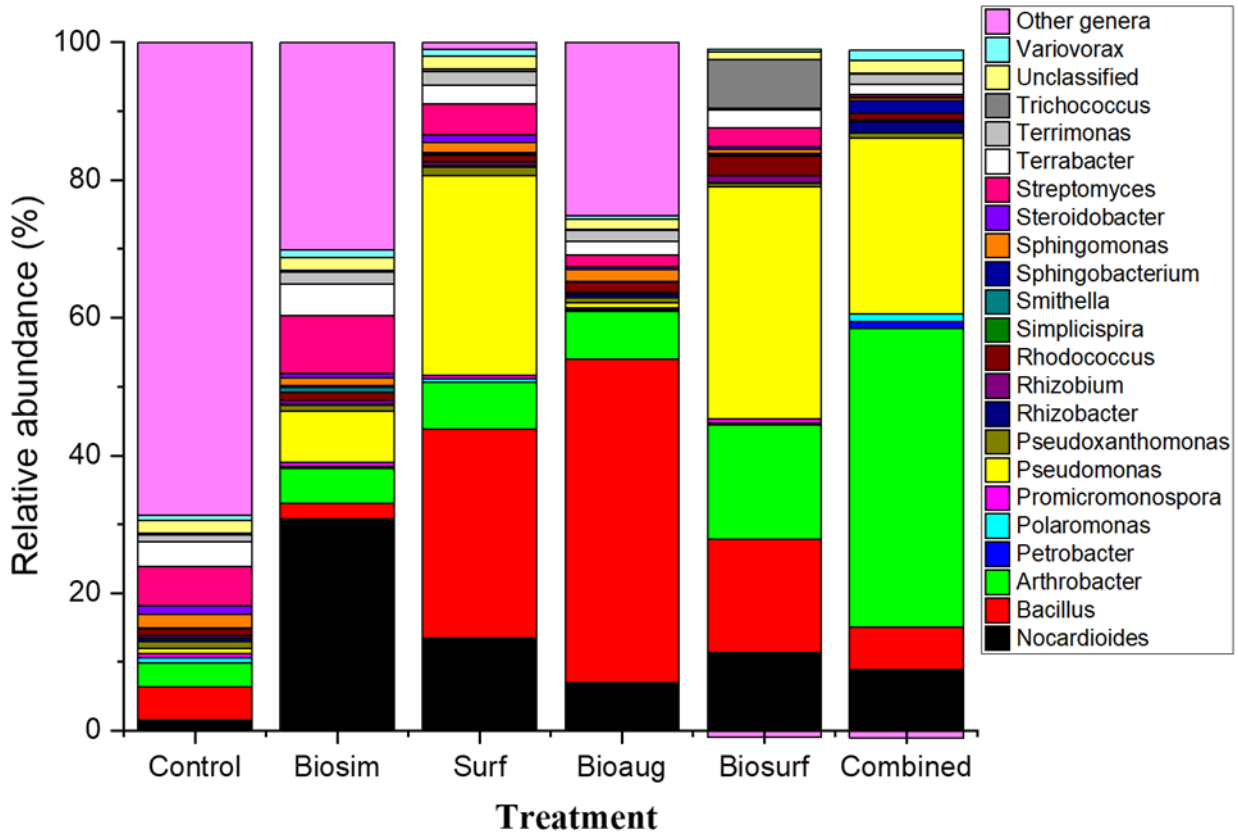


Figure 3-4. Relative abundance and community composition of soil bacteria within treatments

The results shown in Fig. 3.4 show that adding nutrients as biostimulation to PHs-contaminated soil changed the composition of the microbial community significantly compared to the control. However, it also showed that biostimulation resulted in quite different trends for bacterial domination at the genus level, particularly for the genera *Nocardioides, Pseudomonas, Terrabcateria, and Streptomyces*. However, the bacterial community dominance was higher for *Nocardioides* bacteria. At the genus level, the *Nocardioides* genus was predominant and accounted for 35.2% of the biostimulation treatment and 1.4% of the control treatment. A similar report was also reported for *Nocardioides spp.* by Khudur et al. (2019), Lu et al. (2019), and Liu et al. (2020). Furthermore, a decrease in PH levels in biostimulation soils could account for the earlier changes in bacterial diversity. Several studies have found that soils with high PH levels have more varied bacterial populations than soils with lower PH levels (Yang et al., 2022; Devi et al., 2022).

Surfactants induced dramatic shifts in the bacterial taxonomic composition in the bioremediation of soil polluted with petroleum hydrocarbon. It can help microbes be more active if surfactants change their surface properties so that bacteria can contact hydrophobic surfaces (Cheng et al., 2022; Yesankar et al., 2022). In this study, the amended surfactants induced an increase in the PAH-degrading bacterial community of Pseudomonas and *Bacillus* by 35.5% and 40.5%, respectively. Jayashree and Vasudevan (2007) also reported a similar finding that the species tree of the bacterial community revealed five abundant families (*Sphingomonadaceae, Pseudomonadaceae, Bacillaceae, Bacteroidaceae, and Mycobacteriaceae*) in response to the presence of the surfactants. Meng et al., 2019 reported that surfactant Tween-80 treatment combined with *Pseudomonas* was very effective in enhancing benzopyrene solubility and its faster degradation. In addition to this, they found that Tween 80 is biodegradable.

Bacillus, a gram-positive bacterium that dominates the bioslurry, supplemented with activated sludge collected from wastewater treatment plants, has been reported to multiply in soil rapidly in oxygen-rich environments (Tao et al., 2017) and live in anaerobic situations by generating endospores (Seo et al., 2022). Furthermore, they may live for a long period even if nutrients are low or they are exposed to extreme environments like temperature or pH fluctuations (Seo et al., 2022).

Bacillus does not have any genes for oil decomposition (Saeed et al., 2022; Peng et al., 2022). They can, however, produce biosurfactants and boost PHs bioavailability, which could aid in improving degrading performance (Ayed et al., 2015; Almansoory et al., 2017). Cooperation between *Bacillus* strains and other oil-degrading bacteria, for example, can improve degradation efficiency (Villela et al., 2019). Adding biosurfactant as an external stimulant to the bioslurry reactor shows a wide range of bacteria at the genus level, including *Arthrobacter, Pseudomonas*, and *Bacillus*, known as PHs-degrading bacteria. However, the treatment is dominant by *Pseudomonas*, as shown in Fig. 3.4.

Pseudomonades are gram-negative, aerobic bacteria with important medical and industrial applications (Villela et al., 2019). They can produce rhamnolipids which help with PHs-degradation; they also contain catabolic genes for PHs (Villela et al., 2019). Furthermore, they can be found in a variety of settings. They're easy to isolate (Wu et al., 2022), laying the groundwork for bioremediation study and application. As a result, many researchers have turned to using *Pseudomonas spp*. to help with soil PH issues due to its widespread acceptance for oil degradation and because it is a more frequently used bacteria than other bacteria in soil bioremediation (Villela et al., 2019).

In the combined treatment, the relative abundance of the *Arthrobacter* and *Pseudomonas* genera significantly increased, which can be attributed to the introduction of nutrients, activated sludge, and biosurfactants in contaminated soil. *Arthrobacter* belongs to the *Actinobacteria* class, has lived in a wide range of harsh conditions, and has shown a high reduction of TPH in polluted locations (Morozova et al., 2012; Wong et al., 2021; Zamora-Quintero et al., 2022). Their extensive hydrolytic enzyme manufacturing proficiencies enable them to degrade a broad spectrum of complex organic and inorganic substances (Urseler et al., 2022). *Arthrobacter sp*. can use nitroglycerin, benzene derivatives, polycyclic aromatic hydrocarbons, haloalcohols, haloalkanes, N-heterocyclic compounds, insecticides, and herbicides, among other chemical molecules that are identified as environmental contaminants (Teng et al., 2021; Subramaniam et al., 2020; Mahmoud and Bagy, 2018; Romaniuk et al., 2018; Ren et al., 2016). The potential of these microorganisms to make biosurfactants is another appealing feature. This is important for absorbing hydrophobic carbon sources, making them accessible and efficient (Kumari, 2022).

Furthermore, most of these bacteria can form hyphae, reaching the farthest reaches of polluted soil. In addition, they produce spores that are resistant to adverse environmental conditions (Soumeya

et al., 2022). These findings suggest that using bacterial consortia with different catabolic genes to improve biodegradation is a viable and practical way to remove PHs from polluted areas. Bioremediation utilizing microorganisms that degrade PHs is primarily recognized as an environmentally beneficial and effective approach. Many bacterial species potentially degrade PHs have been studied and used in bioremediation. However, more research into their practical uses is required.

6. Soil phytotoxicity assessment

The ecological toxicity of soils can be reflected in the growth status of plants because plants need essential nutrients for germination, growth, and root establishment. In addition, seed development and physiological changes may indicate the presence of hazardous chemicals in soils (Della-Flora et al., 2022). The phytotoxic effect in terms of the germination index (GI) without treatment and polluted soil with treatment operated incubated at 27 °C for 72 h in the dark are shown in Fig. 3.5. GI value >100% indicates a plant–stimulating effect, GI ranging between 50 and 80% means moderate phytotoxicity, and GI > 80% suggests that the material present is not phytotoxic. In comparison, GI < 50% indicates a high phytotoxicity effect (Doni et al., 2018).

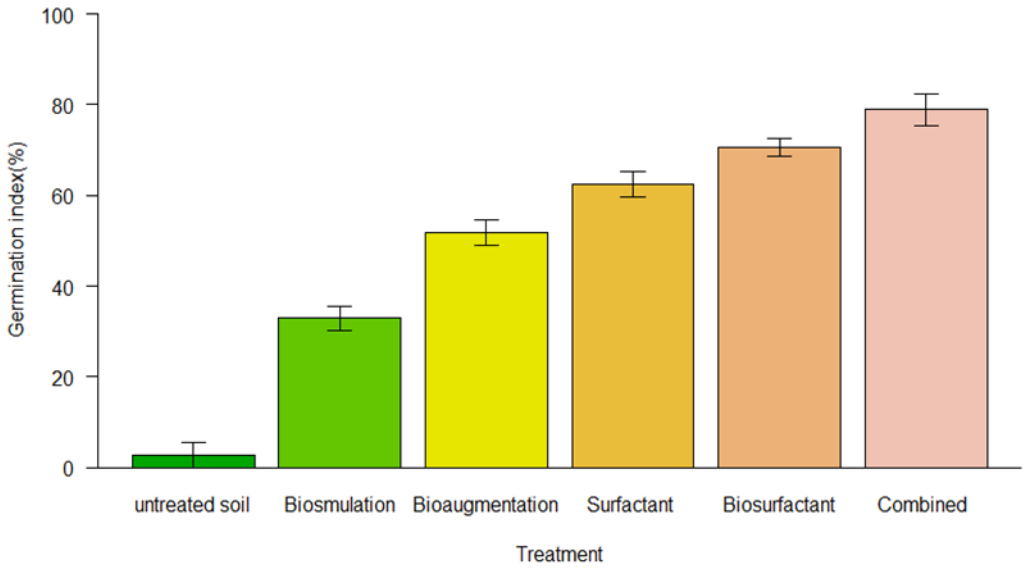


Figure 3-5. Phytotoxicity levels in polluted soil without treatment and polluted soil with treatment operated incubated at 27 °C for 72 h in the dark

The average root length of the *L. sativum* control sample (untreated) was 4.8 mm. For Biostimulation, the average root length was 27.2 mm; for activated sludge, the average root length was 55.2 mm; for surfactant, the average root length was 53.8.2 mm, and the average root length was 61.7 mm, and for a combined treatment it was recorded 59.5 mm. The longest roots characterized *L. sativum* growing in the combined reactor (mean 59.5 mm). The shortest root characterized *L. sativum* growing on soil treated with nutrients only (mean 27.2 mm). In all soil treatments, improvement in quality with a fair germination index of 30.2 % (Biostimulation), 48.9% (activated sludge), 59.7 % (surfactants), 68 % (biosurfactants), and 75.4 % (combined) compared to 5 % (untreated sample) were recorded. Such findings are in line with Avona et al. (2022). They reported that in a bioslurry reactor for bioremediation of crude oil, soil quality was improved within 105 days with an excellent germination index. Research showed that appropriate bioremediation strategies effectively reduce soil toxicity to plants, paving the way for future soil ecological restoration.

7. Conclusions

The use of external stimulants such as activated sludge, nutrients, surfactants, and biosurfactants in bioslurry reactors improved the bacterial community composition and petroleum hydrocarbon (PH) degradation. However, the simultaneous application of activated sludge, nutrients, and biosurfactants resulted in the highest PH degradation, with a half-life time equal to 4.1 d. Furthermore, the results of the 16S rRNA gene amplicon and sequencing revealed that, the combined treatment microbial community composition was more diverse than that in the other treatment. Finally, considering the possibility of repurposing the treated soil, the phytotoxicity assessments revealed that the treatment with the bioslurry reactor produces a matrix with greater quality features than the untreated soil. This phytotoxicity testing could be helpful as an environmental monitoring tool for evaluating the efficacy of soil remediation procedures. However, even though these studies are essential for refining reactor operation and design standardization, they are still in the early stages. As a result, the combination of different bioremediation approaches may lead to an optimized and promising bioremediation method. Based on these studies, this paper suggests more investigation and research is needed in the future on integrating advanced oxidation processes and bioslurry reactors stimulated with novel long-chain molecules.

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CHAPTER 4.

TREATMENT OF PETROLEUM HYDROCARBON CONTAMINATED SOIL BY A COMBINATION OF ELECTRO-FENTON AND BIOSURFACTANT-ASSISTED BIOSLURRY PROCESS

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Abstract

Removing petroleum hydrocarbons (PHCs) from polluted soil is challenging due to their low bioavailability and degradability. In this study, an experiment was carried out to treat soil polluted with petroleum hydrocarbon using a hybrid electro-Fenton (with BDD anode electrode) and biological processes stimulated with long-chain rhamnolipids (biosurfactants). Electro-Fenton treatment was applied as a pretreatment before the biological process to enhance PHC biodegradability, which would benefit the subsequent biological process. The effects of initial pH, hydroxide concentration, soil organic matter composition, PHCs intermediates during the electro-Fenton process, and total numbers of bacteria in the biological process were analyzed to determine the optimum conditions. The results showed that the optimized electrolysis time for the electro-Fenton was 12 hours. The change induced during pretreatment at a specified time was found suitable for the biological process stage and led to 93.6% PHC degradation in combination with the electro-Fenton-and-biological process after 72 hours. The combined system's performance was almost 40% higher than individual electro-Fenton and biological treatments. GC-MS analysis confirms the formation of 9-octadecen-1-ol (Z), 2-heptadecene, 1-nonadecene, 1-heneicosene, and pentacosane as fragmentation during the PHCs degradation process. Thus, the electro-Fenton process as pretreatment combined with a biological process stimulated with rhamnolipids (biosurfactants) could be effectively applied to remediate soil polluted with PHCs. However, the system needs further research and investigation to optimize electrolysis time and biosurfactant dose to advance this approach in the soil remediation process.

Keywords: Petroleum hydrocarbons, Electro-Fenton process, Boron-doped diamond, Rhamnolipids, Bioslurry process, Soil remediation

Graphical abstract

1. Introduction

Petroleum hydrocarbons (PHCs) are known organic pollutants; particularly petrogenic products, waste from the coking industry, and other anthropogenic activities are primary sources of PHCs, which pollute soil adversely (Ambaye et al., 2022; Ambaye et al., 2022a). Many studies have shown that it has a high octanol-water partition coefficient and hydrophobicity (Saber et al., 2021), and as the benzene number of rings in PHCs increases, they become more difficult to degrade (Vaccari et al., 2020). Therefore, soil contamination by PHC is a major international issue due to its low degradability, high carcinogenicity, teratogenicity, and ecotoxicity. Moreover, PHC rich in toxic and carcinogenic compounds not only pose a threat to intertidal environments and aquatic organisms but also to human health owing to their accumulation in foods (Ambaye et al., 2023). Therefore, the removal of PHC pollutants from soil has become an important research topic.

Among various remediation strategies, advanced oxidation processes (AOPs) (Hassani et al., 2021) and various electrode materials-based electrochemical advanced oxidation processes (EAOPs) have recently been widely used and reported to be the most efficient approach to restoring deteriorated natural resources, particularly for PHC removal from soil (Huang et al., 2015; Ahmadi et al., 2021; Hassani et al., 2022). H_2O_2/UV, Fe^{2+}/H_2O_2, hetero- and homogeneous Fenton-like processes, ozonation, and photocatalysis are all examples of AOPs (Huang et al., 2017; Hassani et al., 2021; Hassani et al., 2022). By creating hydroxyl radicals ($^{\bullet}OH$), Fenton processes (Fe^{2+}/H_2O_2) can oxidize contaminants (Li et al., 2022). Hydroxyl radical ($^{\bullet}OH$) is the essential reactive intermediate in the oxidation of organics and is recognized as the most important oxidant (Periyasamy et al., 2022).

Electro-Fenton processes have emerged as the most appealing AOPs due to their fast reaction time, high efficiency, and wide range of targeted pollutants degradation (Hassani et al., 2021; Wei et al., 2022; Hassani et al., 2022). However, despite its higher removal efficiency, the Fenton process uses H_2O_2 and Fe^{2+} as catalysts, making it expensive in large-scale applications. Furthermore, Fe^{2+} consumption results in secondary contamination and needs further treatment (Wei et al., 2022). In order to address these flaws, electro-Fenton has been thoroughly investigated with the goal of in situ H_2O_2 creation, cathodic Fe^{2+} regeneration, or a combination of the two. The boron-doped-diamond (BDD) electrode is applied as an anodic material in the Fenton process to avoid secondary pollution. It has drawn attention due to its long-term response stability, higher potential for O_2

evolution reactions, strong corrosion resistance, high chemical inertness, low background currents, large potential window, and chemical stability (Muddemann et al., 2021).

The water stability zone (E 2.3 V relative to the standard H_2 electrode (SHE)) and the potential area of water disintegration (E > 2.3 V relative to the SHE) are both the ranges in which BDD oxidation takes place. As a result, free and slightly adsorbed hydroxyl radicals (BDD (˙OH)) can develop on its surface. These radicals are non-selective in destroying tenacious organic contaminants (Muddemann et al., 2021). However, electro-Fenton PHC reduction using a BDD anode electrode still has some challenges, such as the high cost of electrodes and energy consumption.

On the other hand, the bioslurry process is considered an environmentally friendly method for pollutant treatment, but the reaction conditions must be carefully regulated (Huang et al., 2017; Xiao et al., 2015; Wan et al., 2013).

Moreover, due to the limited resistance of microorganisms to toxicity, bioslurry technologies may not be efficient at high pollution levels (Kundu et al., 2012). Nevertheless, adding a stimulant such as a biosurfactant or integrating bioslurry with other methods is a potential remediation strategy since it increases pollutants' bioavailability to microbes (Ortega-Calvo et al., 2013). Furthermore, considering the disadvantages and advantages of biological treatment and advanced oxidation, combining advanced oxidation and biological treatment is now considered a more feasible and practical option (Aboudalle et al., 2022; Baiju et al., 2018; Xu et al., 2015; Hassani et al., 2021; Hassani et al., 2022). That opens a new horizon for researchers to use the Fenton process in the combination system to improve the biodegradability of PHCs, which is beneficial to bioslurry treatment. while the same bioslurry treatment combination system can stabilize waste and reduce the use of Fenton chemicals (Liu et al., 2020).

In the combined process, chemical oxidation as an initial step would convert the persistent organic pollutants into more biodegradable intermediates, which could be easily removed in a subsequent biological step (Ambaye et al., 2020). For example, Xu et al. (2015) used a directly electro-fenton approach and reported higher pyrene removal in combined treatment (91%) than treatment alone (50%), indicating that using a combination approach to remediate polluted soil is effective. However, its application for soil remediation is at the initial stage and requires further research and investigation to be applied on a large scale. To the best of our knowledge, the use of electro-

Fenton degradation of PHCs using a BDD anode electrode and a biological process stimulated with long-chain rhamnolipids in a hybrid system has not been investigated previously.

The current study aims to investigate the efficient degradation of petroleum hydrocarbons (PHCs) from polluted soil by combining electro-Fenton and biological processes. In addition, the effects of other parameters that affect the optimum conditions for the combination process were determined.

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2. Materials and Methods

2.1. Materials and analytical chemicals

Analytical research grades of potassium dihydrogen phosphate (KH_2PO_4), Potassium nitrate (KNO_3), Salicylic acid($C_7H_6O_3$), Ferric sulafte ($FeSO_4 \cdot 7H_2O$), Sodium sulfate (Na_2SO_4), Sulfuric acid (H_2SO_4), Alkane mix (C6–C35) and hexane (C_6H_{14}) were procured from USA-Sigma-Aldrich, Inc. (St. Louis, Missouri). A titanium net/mesh electrode was provided by Politecnico di Milano (Italy), and a BDD electrode came from De Nora (Milan, Italy). Oxygen (O_2) was provided externally from a compressed air facility. In all experiments, chemical solutions and other reagents were prepared using ultra-millipore pure water with simplicity >18.2 MΩ cm. A real-time power supply system (MX100QP, AIM-TTI instruments) was used to manage current and voltage during entire experiments.

2.2. Soil preparation and physico-chemical analysis

Soil contaminated with crude oil used in the experiment was collected from a polluted site in northern Italy. The soil sample was taken from 5 to 10 cm below ground level, sealed, and transported to the lab. After being partially air-dried, the soil is sieved to a diameter of less than 2 mm. The main properties of initial soil samples were analyzed for various physicochemical parameters using the standard method of ALPHA 5310 (Napoli et al., 2017). The initial total petroleum hydrocarbon hydrocarbons (TPH) were 4500 mg/kg before being added to the reactor. Polluted soil samples were characterized as sandy-loam soil containing 80% sand, 11 % silt, and 9 % clay. Soil physicochemical properties were as follows: pH 7.6; organic matter content, 12 g/kg; carbon to nitrogen ratio (C/N) 48 g/kg; cationic exchange capacity, 113.2 µS/Cm. The broths containing long chain rhamnolipids used in the bioslurry reactor were previously produced by a safe bacterium B. thailandensis E264 at a large-scale fermenter (10 L) using winey residues, a sole carbon, and energy source. It has yielded 1070 mg/L after extraction, reduced the surface tension

of water from 71 mN/m to 34.5 mN/m, and had a CMC value of 500 mg/L and three congeners with the predominance of Rha-Rha-C14-C14 (MS[M − H = 761]) has been described to have two identical genes containing copies of RL synthesis genes (rhlA, rhlB,rhlC) present at different sites in its genome (Chebbi et al., 2021).

2.3. Experimental design

Seven tests were performed in an experimental setup following standard protocol. Details of the experimental design are given in Table 4.1. Bioslurry and electro-Fenton processes were individually evaluated for degrading PHCs in treatments 1 and 2, respectively. Treatment 3 was performed to measure •OH concentrations during the electro-Fenton process. Treatments 4, 5,6, and 7 were initially kept in electro-Fenton reactors and then transferred to bioslurry reactors after different incubation times to investigate the remediation efficiency of combined electro-Fenton and biological processes. The electrolysis time in the electro-Fenton stage in treatments 4, 5,6, and 7 was 12, 24, 36, and 48 hours, respectively. A graphical representation of the designed experiment is shown in Fig. 4.1.

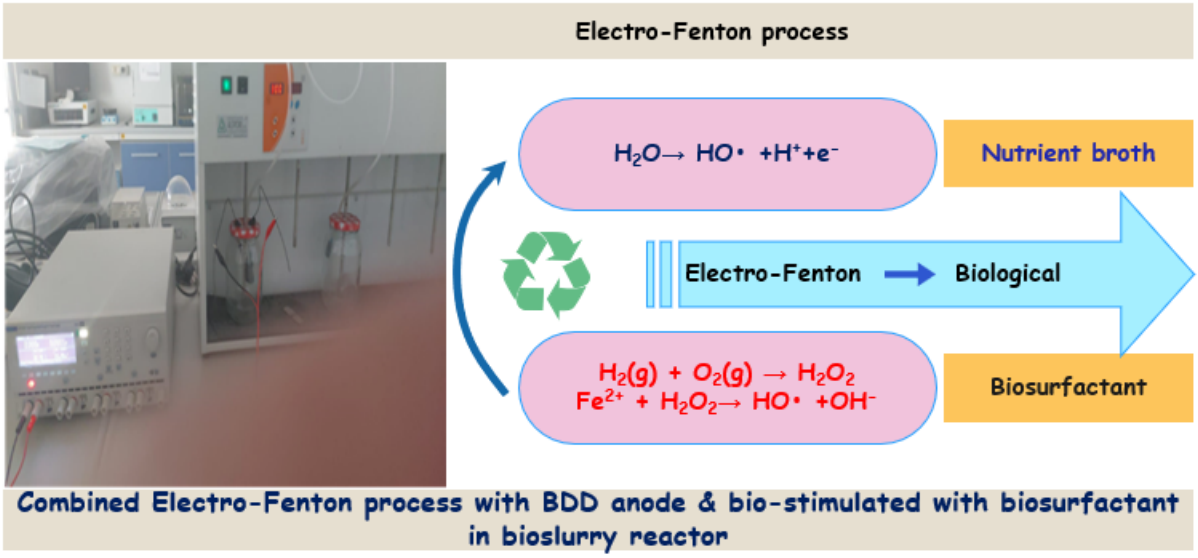


Figure 4-1. Graphical representation of the combined process for degradation of PHs.

Table 4-1. Experimental conditions used for combined EF and bioslurry batch experiments

Treatments (T)	Types of reaction	Working conditions
T1	Biotreatment	275 mLof distilled water and 100 grams of soil (soil-to-water ratio of 1:5) should be mixed and aerated at a rate of 10 mL/minute while stirring at 200 revolutions per minute. Based on PHCs loading as a C measure (Smith et al. 2015), nutrients 1.5 g KNO_3 and 0.1 g NH_42HPO_4 were added at the outset of biotreatment to achieve C: N:P molar ratio-100:10:1; additionally, biosurfactant was added to 275 ml to boost the bioavailability of PHCs (Chebbi et al., 2021).
T2	Electro-Fenton **(**EF)	1-liter capacity glass cylinder with a removable, adjustable lid. A direct current of 2 V is run between a graphite felt cathode (width = 5 cm, length = 6 cm) and a BDD plate anode (length = 6 cm, width = 4 cm). These electrodes have a 2-cm separation (Xu et al. (2015) To Avoid high salinity, 1 g /L of Na_2SO_4 as a supporting electrolyte was added, mixed with 100 g of PHCs -contaminated soil (dry weight) in 500 mL distilled water. Sulfuric acid was added to lower the slurry pH and was adjusted to pH 3. reactors were continuously stirred at 300 revolutions per minute to prevent concentration gradients. Ferric sulfate 0.1 mM used as catalyst (Feng et al., 2014). Aeration of the solution was initiated 5 minutes before electrolysis, and 1L/minute compressed air was bubbled by a solution to saturate it in O_2 while stirring reaction media.
T3	Quantification of hydroxide produced during EF	Salicylic acid with 60 mol/L was used as a trapping agent and added to the reactor before sampling; the soil was sterilized in an autoclave at 120 °C for 60 minutes. Everything else was similar, as shown in Treatment 2.
T4	12 h EF + 60 hours bio-slurry	Treatment using EF & bioslurry was performed in sequence in the same reactor. When the timer went off, the current was turned off, and stirring was continued as bioslurry. Based on PHCs loading as C-measure (Smith et al. 2015), nutrients 1.5 g KNO3 and 1 g NH_42HPO_4 were added to biotreatment to achieve C: N:P molar ratio- 100:10:1; additionally, biosurfactant was added to 275 ml to boost PHCs bioavailability (Chebbi et al., 2021). All other EF and bioslurry operating parameters are the same as in treatments 1 and 2, respectively.
T5	24 h EF + 48 hours bioslurry	
T6	36 h EF + 36 hours bioslurry	
T7	48 h EF + 24 hours bioslurry	

2.4. Analytical methods

Each reactor's slurry was sampled in a 30 mL specialized syringe. Samples were centrifuged at 8000 rpm for 15 minutes, and precipitates and supernatants were extracted using solid and aqueous phases, respectively. To determine PHs degradation rate in a solid phase, the precipitate was air-dried, crushed into a fine powder, and homogenized. 2 g precipitate was added in 20 mL hexane in 100 mL Teflon tubes. After 10 minutes of vigorous agitation, ultrasonic extraction was performed at 35°C on each solid-phase sample for 30 minutes. Next, tubes were centrifuged at 5000 rpm to clean the extract for 10 minutes. Finally, petroleum hydrocarbons in the aqueous phase were analyzed using liquid-liquid extraction with 20 mL of hexane on 1.10 mL supernatant. Triple extractions were performed in both solid and liquid phases. Solid-phase and aqueous-phase extracts were mixed. Samples (20 ml) were evaporated to dryness with a gentle stream of nitrogen, resuspended in 400 µl of n-hexane, and stored at 4°C until analysis.

The extent of petroleum hydrocarbon degradation was determined using a GC with flame-ionization detection (FID). DB-5 MASS capillary column (with a film thickness of 30 m, 0.32 mm ID, 0.25 µm df) was used at a flow rate of 1 mL/min, and N_2 gas (99.99% purity) was used as a carrier gas. The injector temperature was adjusted to 50 °C for 2 minutes, then gradually increased to 200 °C at 10 °C/minute. It was then elevated linearly to 250 °C at a rate of 5 °C/min for 5 minutes after being kept at 200 °C for 4 minutes. Sample injection was operated in spitless mode. PHs were identified using retention time. Their amounts were quantified using an external reference method using the peak area approach.

The quantitative determination of hydroxylated derivatives of SA, 2,3-dihydroxybenzoic acid, and 2,5-dihydroxybenzoic acid was performed by HPLC to determine the concentration of OH. A C18 reversed-phase column (250 mm x 4.6 mm, 5 mm) was used in this case. This method's detection limit was 0.1 mol L^{-1}. Separation was performed with a mobile phase of 30 mmol L^{-1} acetate (pH 4.9), a flow rate of 1 mL/min, and a column temperature of 25°C.

To investigate and verify oxidation and biodegradation by-products qualitatively, the GC-MS, Model 6890 N (Agilent Technologies, CA, USA) equipped with Elite-5MS (5% phenyl methyl polysiloxane) capillary column of (30 m × 0.32 mm i. d.) and film 0.32 µm thick was used. The gas at a flow of 1 ml/min was used as a carrier. From each sample, 1µL aliquots were injected in split mode (30:1). Oven temperature was kept at 60°C for 5 min, then increased to 290°C. The ion

source temperature was kept at 250°C at 5°C/min, and the transfer line was at 300°C. Acquisition range from 40 to 500 amu in electron impact (EI) with an ionization voltage of 70 eV.

2.4.1 Fragmentation identification in GC-MS

For fragmentation identification, GC-MS was used, components retention indices (RI) and mass spectra comparison done with a NIST database library, and literature [Stein, S.E. NIST/EPA/NIH Mass Spectra Database; V2.1; Perkin-Elmer, Hong Kong, China, 2000]. Retention indices were calculated by Elite-5MS capillary columns using an n-alkane series (C6–C35) under the same GC conditions as for the samples. Each component's relative amount obtained from GC/MS was expressed as % peak area relative to the total peak area. The analyses were performed in triplicate.

2.5. Microbial analysis

Real-time polymerase chain reaction (PCR) analysis of the 16S rRNA gene was used to determine bacterial abundance in each soil sample, as guided by Ahn et al. (2009). Furthermore, the total number of bacteria was calculated as the number of copies per milliliter slurry.

2.6. Soil organic matter (SOM) analysis

SOM quality was evaluated using uncontaminated soil. The potassium dichromate-external-heating technique was used to calculate the SOM percentage. Sodium pyrophosphate ($Na_2H_2P_2O_7$) extraction and potassium dichromate ($K_2Cr_2O_7$) outside heating method was used as described by Jarukas et al., 2021, to determine SOM composition (humin, humic and fulvic acid).

2.7. Statistical Analysis

Origin Lab Corporation, Northampton, MA, USA software (version 8.0773) was used to assess the statistical difference between current pollutant production and degradation. P values ($P<0.05$) were used to measure all significance of treatment effects.

3. Results and discussion

3.1. Degradation of petroleum hydrocarbon (PHCs) by Electro-Fenton process

An electro-fenton process using a boron-doped diamond (BDD) anode was used to improve the degradation of soil polluted with PHs for about 72 hrs, and degradation efficiencies were measured in terms of reduction rate. As revealed in Fig. 4.2, the removal efficiency of soil polluted with PHs was reached at about 70.6 % after 72 hours of reaction when experiments were conducted in aerated bottles at an initial pH of 3.0 (T2), which indicated that degradation of petroleum hydrocarbons was probably attributed to the BDD electrode being able to produce a high quantity

of •OH, which is accountable for high oxidative degradation of micro-pollutants due to weak adsorption on its surface as soon as power was supplied (Periyasamy et al., 2022). However, compared to biological processes, the reduction rate of electro- fenton process is decreased in the second phase, as shown in Fig. 4.2, which shows that changes in the oxidation environment such as H_2O_2 and pH in electro fenton process (T2) were counteractive to •OH generation reduction during the second half of electro -fenton process. This datum demonstrated that the degradation rate of PHs was high in the initial period, which is highly related to the generation of •OH species as shown in Fig. 4.3 (Xu et al., 2015; Liu et al., 2020).

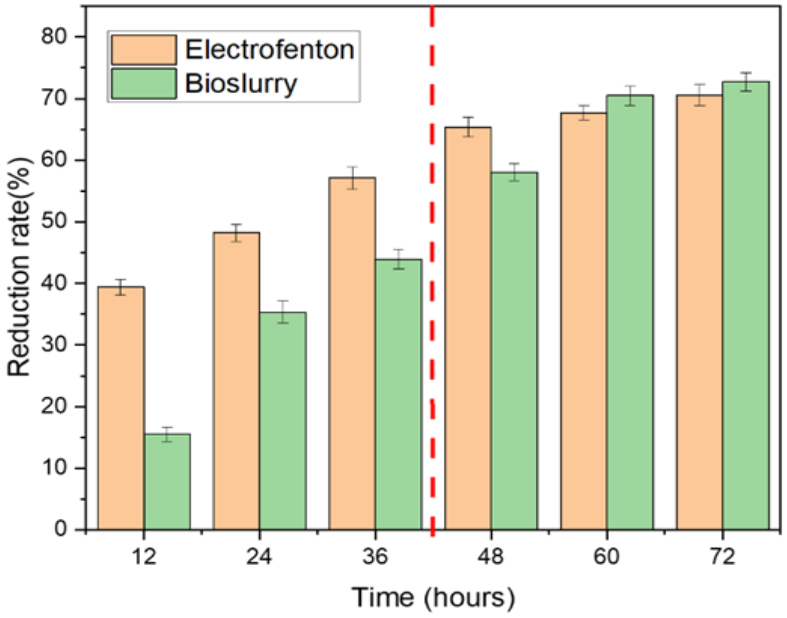


Figure 4-2. TPH degradation by electro-fenton and bioslurry process: Data are means of duplicates, and error bars show standard deviation**.**

3.2. Effect of bioslurry process on the degradation of petroleum hydrocarbon

A bioslurry process experiment to degrade PHCs from polluted soil was done in the aerated bottle containing 500 mL of rhamnolipid biosurfactant and nutrients of 1.5 g KNO_3 and 0.1 g NH_42HPO_4 for about 72 hours (T1). As indicated in Fig**.** 4.2, a PHCs removal rate of almost 72.8% was observed. It demonstrated that heterotrophic bacteria in biosurfactants are anticipated to emulsify hydrophobic pollutants and increase bioavailability by lowering interfacial tension in soil and the aqueous phase (Ambaye et al., 2021). Compared to the electro-fenton process, the reduction process increased in efficiency in the second phase, as shown in Fig. 4.2, demonstrating that

heterotrophic bacteria in a slurry reactor required an adaptation period before they could proliferate logarithmically during bioslurry treatment (Xu et al., 2015; Liu et al., 2020). Results also showed that the bioslurry degraded PHs slowly at first but quickly as a population of heterotrophic bacteria in biosurfactants increased. This increase in bacterial population was positively related to petroleum hydrocarbon degradation, as shown in Fig. 4.3.

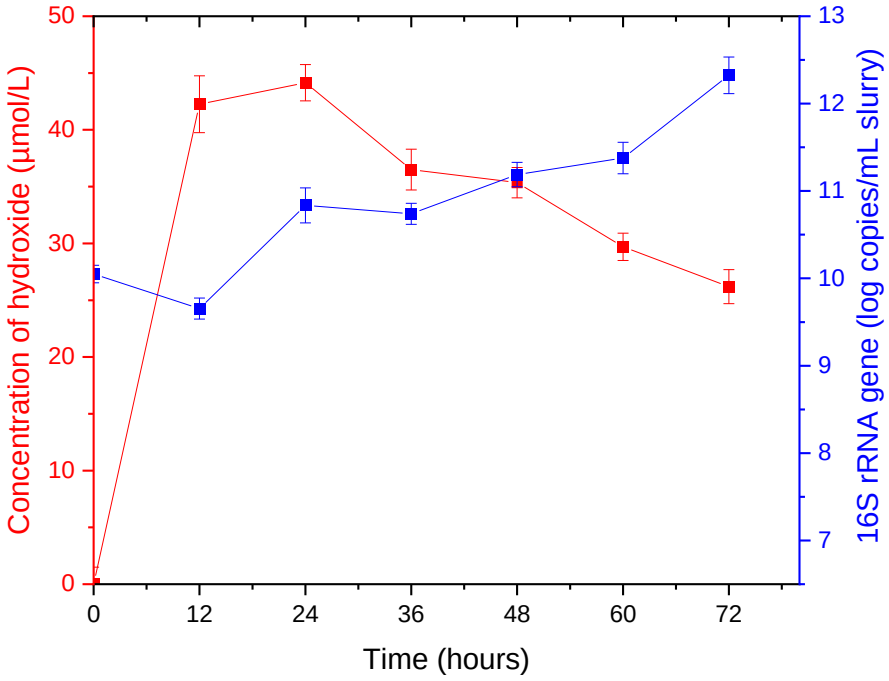


Figure 4-3. •OH concentrations during EF process (T2) and microbial 16S rRNA gene copy number during bioslurry process (T1).

The result also showed that electro-Fenton treatment is characterized by strong oxidizing ability (and thus high degradation rates). In contrast, bioslurry treatment necessitated a lengthy adaptation period before achieving similar results. Maximizing the first half of the electro-Fenton process and minimizing the bioslurry process's adaptation phase was the key to integrating the two processes. Additionally, •OH is a powerful oxidant that can transform organic pollutants into usable substrates for living organisms. Therefore, the combined method of Fenton and biotreatment in

contaminated soil remediation shows better treatment efficiency than the sole method. However, pH and electrolysis time optimization would need to improve the degradation of PHC-polluted soil (Periyasamy et al., 2022).

3.3. Effect of pH on the electro-Fenton degradation process

Electro-Fenton process efficiency depends on pH, temperature, and the generation of free radicals that degrade the pollutant. In addition, the speciation of chemical compounds and the reactions involved in many advanced oxidation processes are influenced by acidity or alkalinity. For the electro-Fenton process, pH (2-4) has been widely observed to play a vital role (Nadais et al., 2018). This research work studied the effect of pH on the degradation of PHCs from soil polluted using the electro-Fenton process. The airflow rate was 10 mL/ minute. A 2.0 V voltage was applied, and 1 g/L of Na2SO4 was used as a supporting electrolyte (T1). Results showed that the initial pH was 3.0 during the electro-Fenton process. However, it rapidly rose to 5.5 in the initial 12 hours. At the end of the experiment, pH reaches a value of 7.0 (Fig. 4.4). A decrease in degradation, as shown in Fig. 4.2, and an increase in pH during the electro-fenton process can be attributed to three factors. (i) Electrolysis produces OH^- and H^+ on the cathode and anode respectively, and H^+ is used to produce H_2O_2, while OH^- is produced concurrently with OH radicals during the fenton-reaction (Quiroz et al., 2014). (ii) The oxidation potential of generated reactive oxygen species (ROS), especially •OH, is strongly influenced by the solution pH, which explains why different solutions have varying degrees of degradation efficiency. (iii) As the solution pH rises, the electrogenerated •OH amount in an alkaline solution decrease as well as in its reactivity (redox potential) (Periyasamy et al., 2022).

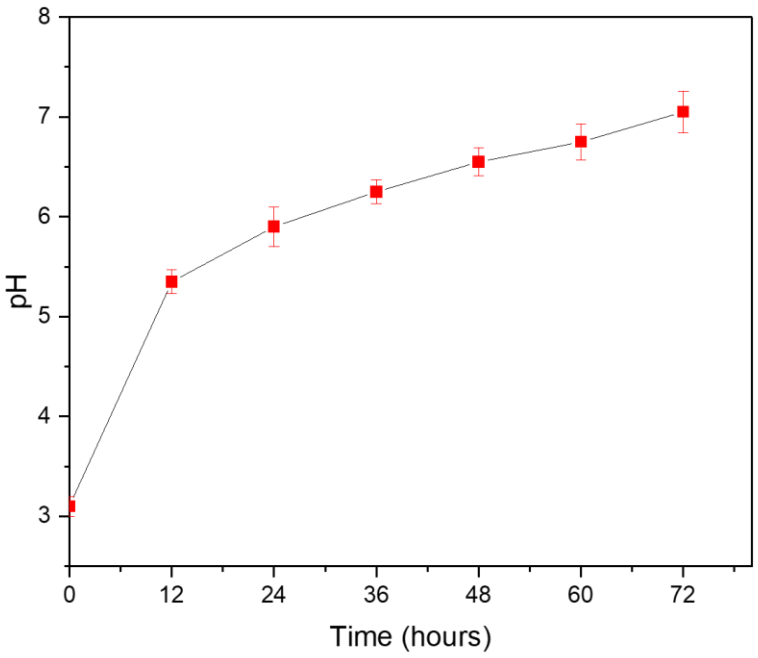


Figure 4-4. Changes in pH during the operation of the electro-fenton Process

Furthermore, this study showed that a lower pH, i.e., 3, promotes oxidation-reduction reactions and favours the decomposition of H_2O_2. A pH range of 5 to 9, ideal for bacterial development, is required during bioslurry processes following the electro-Fenton process for degrading PHCs. These findings are consistent with those reported by Wang et al. (2010). They found the highest rates of contamination degradation by electro-Fenton treatment at pH values between 2 and 4. According to this study, when the electro-Fenton process was completed, the slurry pH attained a value adequate for the bioslurry process (pH 5–7). As a result, if the biological process was used after the electro-Fenton procedure, it was not essential to alter pH.

3.4. Evaluation of integrated electro-fenton and bioslurry treatment for PHCs degradation

Biotreatment and the electro-fenton process are effective remediation techniques for soil polluted with petroleum hydrocarbons. However, the electro-fenton process caused secondary pollution in the soil environment, and biotreatment is typically incapable of effectively removing very harmful contaminants; this prompted the development of an integrated approach to fenton and biotreatment methods for remediation of petroleum hydrocarbon-polluted soil, which demonstrated greater treatment efficacy than either method alone (Huang et al., 2017). As a result, four scenarios (T4-T7) were developed in this study by selecting the highest rate time interval in the electro- fenton process (48 h), as shown in Fig. 4.2, to investigate the combination approach of electro-fenton with

different periods (12, 24, 36, and 48 h) with bioslurry for the degradation of soil polluted with petroleum hydrocarbons. As shown in Fig. 4.5, after 72 hours of incubation, the removal efficiency of petroleum hydrocarbons reached 92.6, 70.12, 64.02, and 61.03 %, respectively, demonstrating that organic pollutants can be chemically broken down into more biodegradable intermediates via electro fenton process, resulting in lower concentrations (Ambaye and Hagos, 2020). More over, when compared to individual treatments of electro-fenton (T1) and biolsurry (T2), the combined approach increased degradation by 21.02 and 22.78 %, respectively, and this result is in line with Xu et al. (2015). Using the electro-fenton process as a pretreatment for the bioslurry process, they claimed that the pretreatment process using electro-fenton increased the reduction rate from 50 % (individual bioslurry and electro-fenton) to 91% (integrated approach), and they found that pyrene removal increased significantly in the hybrid system. Therefore, an integrated approach is an effective way to remediate polluted soil.

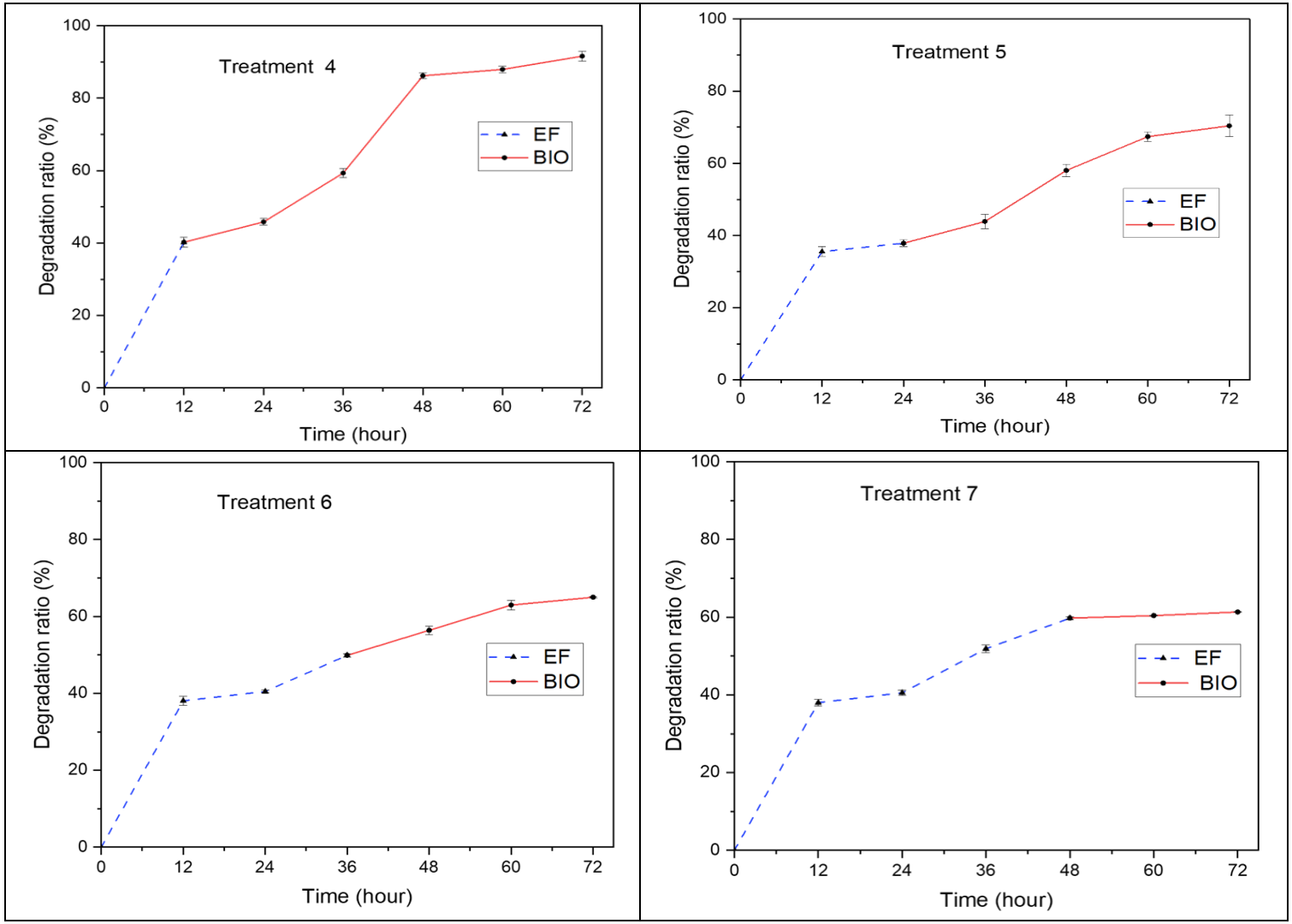


Figure 4-5. TPH degradation ratio between electro fenton and bioslurry process and effect of incubation time (T4: 12h, T5: 24h, T6: 36h, T7: 48): Data are means of duplicates, and error bars show standard deviations.

The results also demonstrated that T4 showed maximum performance in comparison with the other treatments (Fig. 4.5). After 72 hours, the total removal efficiency touched 91.3 %, and as time increased, the PHs-degradation rate enhanced. However, it gradually declines with time (Xu et al., 2015). This indicates that some competing processes may have occurred, resulting in the opposite of the predicted effect. During the experiment, organic matter in soil and intermediates would have acted as competitors. For this integrated approach, the best time to use the electro-fenton process had to take into account changes in the organic matter composition of soil polluted with petroleum hydrocarbons.

3.5. Effect of bacterial population on best treatment selection for EF-Bioslurry process

Biodegradation of oil-contaminated soil is highly dependent on viable microorganisms (Ambaye et al., 2022). This experiment investigated the dynamics of the bacterial populations due to the application of the electro-fenton process as a pretreatment during the combination of EF and bioslurry. Results showed that the number of bacteria grew faster after inoculation in electro-fenton with the bioslurry process until T7, which is 36 hours of electro-fenton and 36 hours of bioslurry, and then gradually declined at the end of the experiment with different incubations of the electro-fenton process. Furthermore, the total bacterial population's 16S rRNA gene copy number changes over time in the combined electro-fenton and bioslurry process, as shown in Fig. 4.6, and this demonstrated that more petroleum hydrocarbons were dissolved into the aqueous phase during the integration process of electro-fenton with the bioslurry process, making hydrocarbons more bioavailable and that the bacterial population expanded more rapidly following a combined process of electro-fenton and bioslurry process (Usman et al., 2016).

Research findings also showed that prolonged exposure to the electro-fenton process may have resulted in the formation of substances readily usable by bacteria. However, competition between petroleum hydrocarbons and organic matter in soil and intermediates may have decreased the removal of petroleum hydrocarbons if the electro-fenton process had been applied for an extended period. While in bioslurry stimulated with biosurfactant-treated soil, hydrocarbons may have been depleted because of microbial consumption. Similar findings were also stated by Xu et al. (2015) in their research aiming to apply the direct electro-fenton method before biotreatment to stimulate the degradation process of pyrene. Biosurfactant application in PAH-polluted soil is anticipated to

emulsify hydrophobic pollutants and enhance their bioavailability by reducing the interfacial tension between soil media and the aqueous phase. In contrast, the electro-fenton application may generate substances that bacteria can readily utilize (Whang et al., 2008).

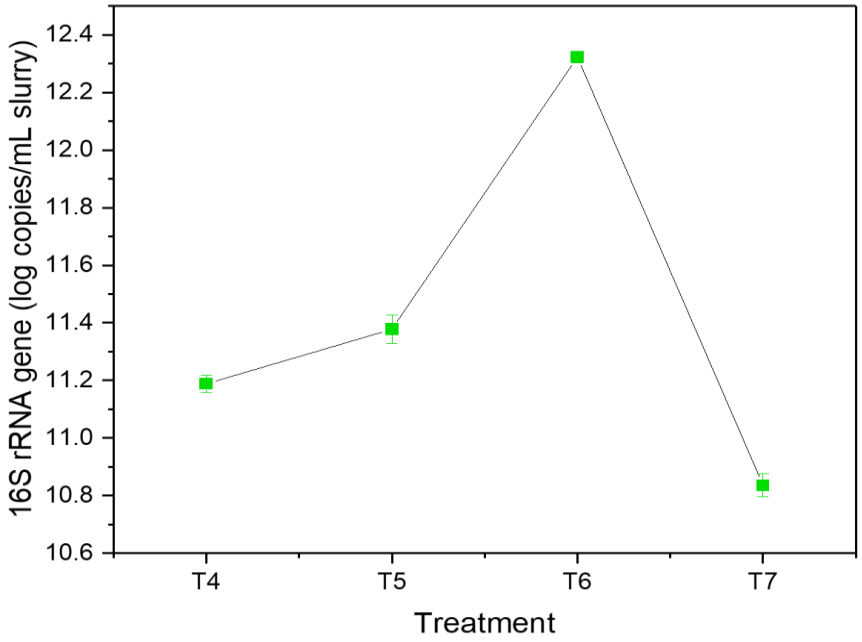


Figure 4-6. Total heterotrophic bacterial population16S rRNA gene copy number changes in the EF-biolsurry process**.**

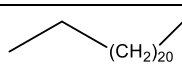
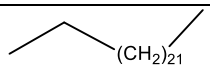
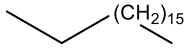
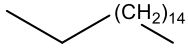
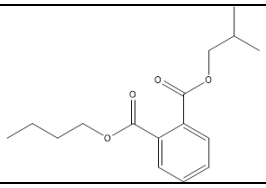
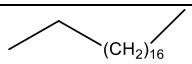
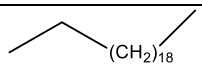
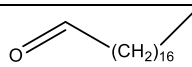
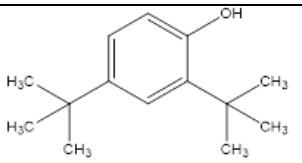
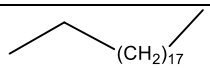
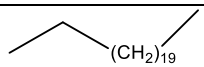
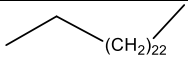
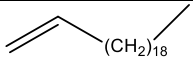
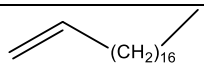
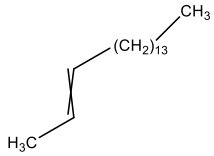
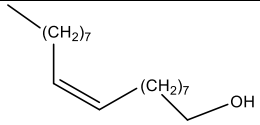
Overall, the number of bacteria able to degrade PAHs gradually decreased during an experiment, suggesting that PAH concentrations in the soil must have fallen. Simultaneously, prolonged soil application in the electro-fenton process may have lowered petroleum hydrocarbon removal due to competition between petroleum hydrocarbons and organic matter in soil and intermediates. Bacteria that can break down the PAHs have increased over time, indicating that native soil microflora has become acclimated to oil-contaminated soil's stressful conditions and are now making use of complex hydrocarbons to grow.

3.6. Effect of intermediates on the selection of best treatment for EF-Bioslurry process

Pretreatment with the electro-fenton process yielded some low-molecular-weight substances with high bioavailability. When petroleum hydrocarbons undergo the electro-fenton process, OH can quickly ring-cleave them, resulting in low-molecular-weight by-products better suited for

biodegradation. Organic pollutants decompose into aldehydes, alcohols, acids, carbon dioxide, and water due to either chemical oxidation or biological decomposition (Aboudalle et al., 2018), as shown in Table 4.2, which lists the most prominent intermediates identified by GC-MS in this investigation. Most byproducts of the electro-Fenton reaction are aliphatic and aromatic hydrocarbons, and exact species of intermediates were detected at varying concentrations throughout the experiment. Furthermore, the oxidation process increased the proportion of aliphatic to aromatic hydrocarbon byproducts. This showed that aliphatic and aromatic hydrocarbon intermediates, produced when rings were cleaved and hydroxylated, can be further chemically degraded (Pérez-Pantoja et al., 2019).

Table 4-2. Main fragmentation and percentages of fragmentation (aliphatic hydrocarbons and aromatic hydrocarbons

S. No.	Detected main intermediates	Retention time (minutes)
1.	 tricosane	2.17
2.	 tetracosane	4.06
3.	 octadecane	4.64
4.	 heptadecane	5.54
5.	 butyl isobutyl phthalate	5.55
6.	hexadecane	5.71
7.	 nonadecane	6.31
8.	 henicosane	6.42
9.	 Octadecanal	7.39
10.	 2,4-di-*tert*-butylphenol	8.03
11.	 icosane	8.18
12.	 docosane	8.39
13.	 pentacosane	9.38
14.	 henicos-1-ene	9.56
15.	 nonadec-1-ene	10.37
16.	 heptadec-2-ene	12.85
17	 (*Z*)-octadec-9-en-1-ol	20.63

The percentages of aliphatic-aromatic hydrocarbon intermediates at 12, 24, 36, and 48 h were 3.63± 0.1, 4.48± 0.1, 5.78 ± 0.2, and 7.50± 0.1, respectively.

Table 4.2 also includes a percentage of the peak area occupied by intermediates of low molecular weight aliphatic and aromatic hydrocarbons. Following 12 hours of application of the electro-fenton process, the rate of degradation of these intermediates was slightly higher than 3%. As expected, increasing the electro-fenton processing time to 48 hours increased the yield by 8%. However, results of petroleum hydrocarbon degradation rates during the integration of electro-fenton with bioslurry treatment showed that the bioavailability of substrates produced initially within 12 h of the electro-fenton process was adequate for the bioslurry phase. Moreover, since the electro-Fenton process improved petroleum hydrocarbon removal by 26% during the electro-fenton process phase, the overall reduction rate was 31% during the entire integration of electro-fenton with the bioslurry process. The findings also revealed that there was no need to extend the application time of the electro-fenton process. This demonstrates that organic pollutants' bioavailability and bacteria's activity were increased by employing an electro-fenton process as a pretreatment before the biotreatment process. In addition, elect fenton process-based further degradation of pollutants can generate low-molecular-weight byproducts that function in the co-metabolism of substrates during the biotreatment phase.

In biological treatment, however, bacteria may favor using more readily metabolizable intermediates with a lower molecular weight and higher bioavailability. Because bacteria used high bioavailability intermediates at the start of the biological process, complete degradation of the pollutant was not possible through biological treatment due to competition between the intermediates (Lu et al., 2010; Abbas et al., 2022; Qian et al., 2022). Because of this, as can be seen in Fig. 4.5, the biological treatment process peaked in Treatment 4 and then declined in Treatments 6 and 7. Overall, the degradation of petroleum hydrocarbons was aided by an electro-fenton application time of 12 hours. In addition, applying the electro-fenton process for 12 hours reduced competition between intermediates, leading to improved biodegradation. However, more study is needed to determine the best way to use the combined electro-fenton and biological treatment with a short incubation period on a commercial scale.

3.7. Effect of SOM composition on the selection of best treatment for EF-Bioslurry process

Electrochemical reactions are potent, non-selective oxidants tools that affect many types of organic compounds. It's worth noting that SOM is a significant contaminant target during the anodic oxidation of polluted soils. The presence of SOM has been shown to negatively affect contaminant removal, positively impact indigenous microbe's survival during anodic oxidation, and significantly alter SOM's content, composition, and structure (Wang et al., 2012; Nidheesh et al., 2019). In this research, the changes of three humic fractions (humic acid, HA; fulvic acid, FA; and humin) in electro-fenton

treatment were evaluated for their effect on the degradation of soil polluted with PHs to select the best scenario for the integrated process of electro-fenton and the bioslurry process, as shown in Fig. 4.7. As a result of the electro-fenton process, total SOM content dropped from 3.25 to 1.61% after 48 hours. The FA fraction's content decreased from 0.6% at the beginning to 1.6% at 12 hours and then dropped to 0.4% at 48 hours. It is only enhanced by 0.1% in electro-fenton from 0 to 12 hours.

Fig. 4.7 showed that HA content decreased from 1.4% to 0.3%, while humin content dropped from 1.1% to 0.4%. An essential factor in the electro-fenton process that led to the reduction of SOM was the non-selective nature of OH radicals (Gao et al., 2020). The anodic oxidation process only managed to oxidize about 1.9% of soil organic matter, despite competition for OH between petroleum hydrocarbons and organic matter. As a result, OH, degraded a large portion of the organic material in the soil. SOM has undergone changes in content and structure, which can significantly affect its capacity to absorb and release organic contaminants such as petroleum hydrocarbons (Gao et al., 2020).

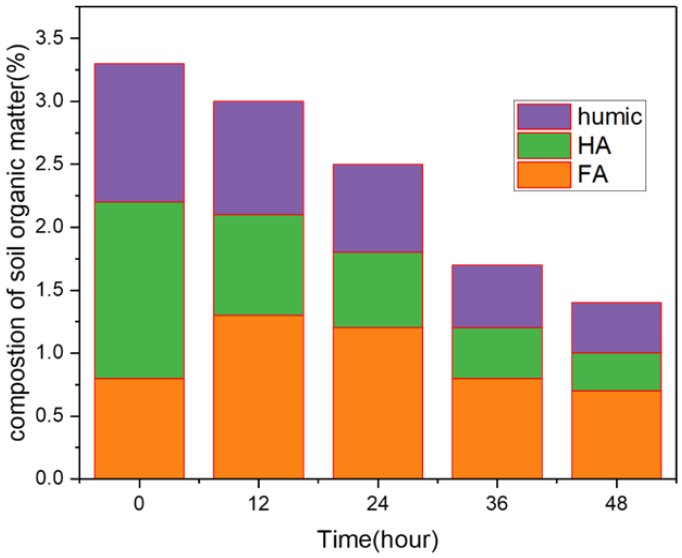


Figure 4-7. Changes in SOM after different times of EF oxidation. Time 0 means raw soil

In addition, this study showed that longer periods of anodic oxidation resulted in higher levels of FA and lower levels of HA and humin, suggesting that the oxidation process altered the structure of HA and converted at least some of it into FA. FA has a smaller molecular size than HA and is more effective at desorbing petroleum hydrocarbons than HA (Velázquez-Vázquez et al., 2022). As a result, more petroleum hydrocarbons in the soil will desorb into the liquid phase, facilitating their removal. Soil organic matter content and composition are affected by anodic oxidation, affecting petroleum hydrocarbons' absorption and desorption. Surfactants formed during chemical oxidation

processes can also aid in the desorption of petroleum hydrocarbons (Zhang et al., 2019). Soil organic matter fractions like FA and HA have been shown to play an essential role in the adsorption and degradation of petroleum hydrocarbons (Chen et al., 2019).

On the other hand, after 12 hours, degradation rates slowed to the point where less HA could be converted to FA, and FA content began to decline. As we've seen, bacteria preferentially utilize FA as a carbon source, contributing to a more efficient bioslurry process. Hence, this result shows that using anodic oxidation as pretreatment for about 12 hours followed by bioslurry for about 60 hours enhanced the overall degradation of soil polluted with petroleum hydrocarbons during the hybrid system, as shown in Fig. 4.5 treatment 4.

4. Environmental implications of BDD anode in electro-fenton process

It has been established that anodic oxidation can efficiently remove numerous organic contaminants in a short period. It can be easily adapted to function as a component of distributed wastewater treatment systems, which is another central selling point. Not only is it simple to automate, but it also takes up little space in design and doesn't require the use of any harmful chemicals (Jiang et al., 2021). While many studies have documented the effectiveness of BDD electrodes as anodes for pollutant removal (De Luna and Bensalah, 2022; Ltaïef et al., 2018; Olvera-Vargas et al., 2021), few have discussed the potential drawbacks of anodic oxidation, such as the alteration of SOM in its content, composition, and environmental effect. Since SOM has been known to play a significant role in the sequestration of hydrophobic organic compounds in soil, the influence of chemical oxidation on SOM is a critical issue related to technology (Wang et al., 2012).

The fate and transport of organic contaminants would be affected by shifts in the composition of SOM. Humin is the soil fraction most responsible for producing organic pollutants that cannot be extracted (Yang et al., 2010). In this study, anodic oxidation using a BDD electrode changed the sorption properties of soil by modifying soil organic matter and reducing humin content from 1.1 to 0.4 %, as shown in Fig. 4.7. This demonstrates that the binding state of contaminants in soil could be altered by lowering humin content and increasing production of surfactant-like compounds under optimal conditions (Wang et al., 2012). Furthermore, the bioavailability of poly aromatic hydrocarbons in humic acid was found to be greater than that of humin (Chen et al. 2017, Zhang et al. 2009), suggesting that lowering the total solvent extractable matter affinity in humin would increase the bioavailability of petroleum hydrocarbons after anodic oxidation by BDD. Because of this effect, biodegradation can proceed well after chemical oxidation.

Multiple findings (Mousset et al., 2014; Ferrag-Siagh et al., 2013; Ganzenko et al., 2020; Mansour et al., 2012) have found that using BDD as an anodic oxidation process increases the biodegradation of

soil polluted with petroleum hydrocarbons. Even though BDD electrodes are excellent at maximizing oxidation efficiency, their widespread application is still debatable. Further study is required to reduce electrode costs, scale up fabrication while maintaining high physical stability, and find a solution to separating BDD film from the substrate surface and cracking issues. The process may also be hard to use on a large scale because soil polluted with PHs in the real world has many different properties, such as low bioavailability of organic contaminants and the presence of other radical scavengers (Nidheesh et al., 2019).

The electro-Fenton process generally is based on an electron transfer between hydrogen peroxide (H_2O_2) and a homogeneous metal catalyst. Therefore, it is a good and promising alternative for soil treatment. However, the EF method still faces some problems that must be addressed. For example, in the EF process, the production of H_2O_2 is slow because oxygen has low solubility in water. In addition, the current efficiency under reduced pH (pH 2) is also low, and a large volume of iron sludge generation (Cheng et al., 2022).

On the other hand, combining the electro-Fenton process with other advanced oxidation methods has led to better results in removing contaminants. For example, the Ultrasonic (US) mechanism decomposes pollutants based on the creation of cavities or microbubbles resulting from acoustic cavitation in water. These cavities give rise to hydroxyl radicals and hydrogen radicals. Eventually, the combination of the electro-Fenton with the ultrasonic process, called sono-electro-Fenton, is reported to lead to an increase in cleaning the electrode surface, adaptability to the environment, and ultimately increase in the higher percentage of pollutants removal due to the chemical and physical reaction created (Babuponnusami and Muthukumar, 2012).

5. Conclusions

This study evaluated the performance of an integrated electro-Fenton and biodegradation process for removing soil polluted with petroleum-derived aromatic hydrocarbons (PHCs). As revealed from the total petroleum hydrocarbon reduction rate analysis, PHC was mineralized to a certain extent, and the removal ratio reached 71%. A change in oxidation conditions, such as radical •OH and pH concentrations in the electro-Fenton process, was identified as counteractive to •OH generation, leading to a low PHC removal rate. After being combined with biological treatment, the removal ratio of PHCs was elevated to 94 %. As indicated by the results, the pretreatment of PHC pollutants increased the pH value from 3.0 to a suitable value for biodegradation. The addition of biosurfactants increased bioavailability and resulted in PHCs with a lower molecular weight. It also produced intermediates and a high flavunic acid content, a function of soil organic matter. It helps to improve

the removal of polluted soil. Finally, combining electro-Fenton and biological treatment stimulated with biosurfactant was effective and feasible in removing PHCs from polluted soil. However, more research is needed to optimize key parameters for large-scale application.

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CHAPTER 5.

INSIGHTS INTO RHAMNOLIPID AMENDMENT TOWARDS ENHANCING MICROBIAL ELECTROCHEMICAL TREATMENT OF PETROLEUM HYDROCARBON CONTAMINATED SOIL

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Abstract

Environmental pollution by hydrophobic hydrocarbons is increasing, notably nowadays due to a large amount of industrial activity. Microbial electrochemical technologies (MET) are promising bio-based systems that can degrade hydrocarbon pollutants and produce bioelectricity simultaneously. However, MET faces some issues in terms of soil remediation, including low mass transfer, low bioavailability of hydrocarbons, limited activity of beneficial bacteria, and inefficient electron transport. Herein, the effect of adding rhamnolipid biosurfactants on the bioremediation of PHC-contaminated soil was studied. To enhance the degradation of PHCs and electron transfer, 10 mg/L, 50 mg/L, and 100 mg/L of rhamnolipid biosurfactants were added to the anolyte of MET. The results showed that rhamnolipids at a dose of 100 mg/L facilitated the degradation of PHCs (72%) and promoted the highest bioelectricity generation (9.5 Am^{-2}) in 20 days. The biosurfactants induced a unique microbial enrichment associated with electrochemically active bacteria such as *Geobacter*, *Desulfovibrio, Klebsiella,* and *Comamonas* on the anode surface, as well as *Pseudomonas, Acinetobacter,* and *Franconibacter* in soil MET, indicating the occurrence of a metabolic pathway in microbes working with the anode and soil bioelectrochemical remediation system. According to cyclic voltammetry analysis, redox peaks appeared, showing a minor shift in redox MET-biosurfactant compared to the bare MET system. Furthermore, the phytotoxicity of polluted soil to *L. sativum* seeds after and before MET remediation shows a decrease in phytotoxicity of 77.5% and 5% for the MET-biosurfactant system and MET alone, respectively. With MET as a tool, this study confirmed for the first time that novel long-chain RL produced from non-*Pseudomonas* bacteria could remarkably facilitate the degradation of petroleum hydrocarbons via extracellular electron transfer, which provides novel insights to understand the mechanisms of RL regulating petroleum hydrocarbon degradation.

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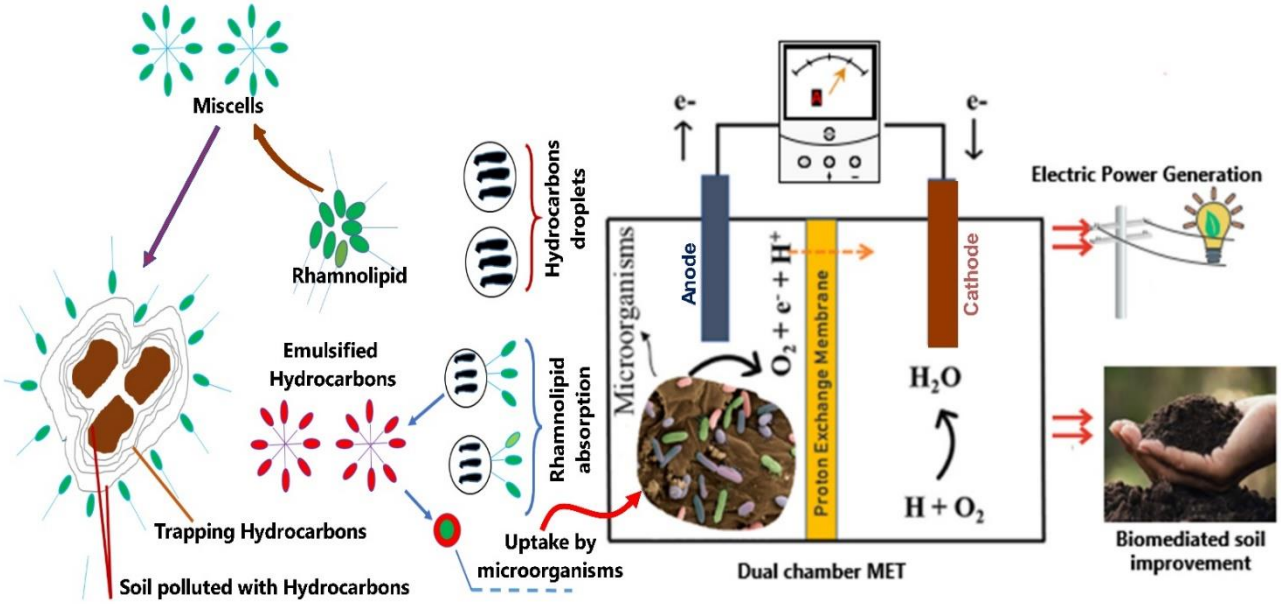
Keywords: Biosurfactants; bioelectrochemical system; circular economy; Illumina; remediation; current density

Graphical abstract



1. Introduction

Oil exploration and processing have increased dramatically over recent decades. Oil-derived fuels are still used in global transportation, and the manufacture of plastics consumes a significant quantity of hydrocarbons. As a result, one of the large-scale industrial branches is the transportation and processing of crude oil and its derivatives (Ambaye et al., 2022), which in turn provokes huge environmental contamination by petroleum hydrocarbon chemicals. Ecological disasters erupted in many regions around the world as a result of large discharges of hydrocarbon contaminants, one of the most common risky consequences, alerting us to the importance of such an environmental issue (Collivignarelli et al., 2018; Vaccari et al., 2020). In terms of soil decontamination, several approaches are applied, including chemical oxidation, thermal desorption, incineration, and soil flushing, with some pros and cons for each technology, but the majority of them require the use of high energy and produce secondary pollution (Daniel et al., 2022; Ghanbari et al., 2020). In contrast, bioremediation, which includes biostimulation and bioaugmentation, is widely acknowledged as a green technology for remediating PAH-contaminated soils with evident benefits. Bacteria decompose PAHs in either aerobic or anaerobic circumstances (Sayara and Sánchez, 2020). On the other hand, PAHs tend to concentrate in soils rather than dissolve in water or suspended in air, making them a prevalent contaminant in anaerobic environments like sediments and soils, implying that anaerobic biodegradation should be a major factor in PAH degradation in soils. Since bacteria can oxidize organic molecules under anoxic circumstances, anaerobic biodegradation of organic contaminants is an important process in nature (Fragkou et al., 2021). However, the availability of electron receptors such as metal oxides, sulfate, or nitrate, is a critical factor limiting the efficiency of the process. As a result, even though numerous bacteria may degrade PAHs under anaerobic circumstances, only a handful can efficiently perform PAH degradation in nature. Insufficient supplies of suitable electron receptors are linked to increased electron buildup and lower breakdown rates (Ambaye et al., 2021).

Microbial electrochemical treatment (MET) is one of the most recent green solutions created in the last few decades (Hassani et al., 2022). They are promising ways of improving environmental bioremediation. Bacteria with extracellular electron transport capabilities are used in MET (Borello et al., 2021). These microbes thrive in anaerobic conditions and catabolite organic compounds, including various pollutants, generating, and releasing electrons outside the cell. These are then carried to ultimate acceptors via various mechanisms, resulting in electricity generation and treatment of pollutants (Hamelers et al., 2010). However, MET's performance in a soil remediation system is influenced by several factors. One of the most important mechanisms affecting the efficiency and performance of MET is the extracellular electron transfer to the anode. Exoelectrogenic bacteria can

transfer electrons to a conducting surface, such as the anode. Transfer of electrons can be accomplished using conductive pili, nanowires, or mediated by naturally secreted or artificially added redox mediators. Microorganisms extract energy from their surroundings and transfer the electrons to the anode to generate current (Bajracharya et al., 2016). It is not only the species' redox potentials that influence the efficiency of these electron transfer mechanisms; the oxidative metabolism of microorganisms also plays a role in releasing electrons in the process (Espinoza-Tofalos et al., 2020).

Various ways for extracellular electron transfer augmentation have been established so far, which have greatly enhanced MET performance, such as electrode nano-engineering, bacterial metabolism of membrane engineering, and chemical treatment of bacteria (Kato, 2015). Surfactant-based chemical therapy of bacteria has recently received a lot of interest. Exogenous addition of surfactants effectively promoted power generation and bioremediation in MET (Li et al., 2018). Furthermore, Tween-80, polyethyleneimine, and EDTA have also been utilized as practical additions to improve bacterial extracellular electron transfer in MET (Li et al., 2018, Liu et al., 2012).

Chemical surfactants, instead, are usually toxic to bacteria, preventing biofilm development and reducing bacteria viability (Gomaa et al., 2021). Thus, adding a chemical surfactant to a MET could affect the process, and the operation parameters are needed to be carefully optimized. Short-chain RLs produced by microorganisms *Pseudomonas* from renewable feedstocks are less harmful and effective in bioremediation processes. Because of their high foaming, low toxicity, and biodegradability. They are more effective in improving the performance of extracellular electron transfer in bioelectrochemical systems for energy generation (Davey et al., 2003; Zheng et al., 2015). For instance, Wen et al. (2010) demonstrated that when 0 to 80 mg/L short-chain rhamnolipids were introduced to an air-cathode, single-chambered MET system, the maximum power density, and open-circuit voltage increased from 22 W/m^3 to 275 W/m^3 and 483 mV to 878 mV, respectively. It was mainly due to hydrophobic and hydrophilic groups found in rhamnolipids, which led to the microorganisms' ability to cling to hydrophilic anodes and produce anodic biofilms. When used at the appropriate dose, they can act as a bridge to increase the adsorption of microorganisms onto anode surfaces. Consequently, rhamnolipids revealed potential capacities to promote anodic microorganisms to adsorb and transmit electrons. Reports regarding the study of bioremediation techniques and the use of RL produced by suggesting studying the glycolipids from the well-known pathogenic Pseudomonas aeruginosa species (predominately, Rha-Rha-C10-C10) to enhance the electrochemical remediation process (Chebbi et al., 2017). However, there are no studies observed that combine bioremediation and the use of novel long-chain RLs produced by non-*Pseudomonas*

aeruginosa, such as long-chain RLs (e.g., B. *thailendensis E264)* as a model to enhance the electrochemical remediation process through extracellular electron transfer (Chebbi et al., 2021).

The main objective of this study is to evaluate for the first time the effects of adding novel long-chain RL, i.e., biosurfactant concentration in the synergetic application of biosurfactants, bioremediation, and electrochemical remediation for purposes of degradation of soil polluted with petroleum hydrocarbons. It also focused on phytotoxicity and bacterial community evolution in response to the enhancement of electro-remediation of soil polluted with petroleum hydrocarbons and electricity generation.

2. Materials and methods

2.1. Soil preparation and biosurfactant addition

The soil contaminated with crude oil used in the experiment was collected from a polluted site in northern Italy. The soil sample is taken 5 to 10 cm below ground level, sealed, and transported to the lab. After being partially air-dried, the soil is sieved to a diameter of less than 2 mm. The main properties of the initial soil samples were analyzed for various physicochemical parameters using the standard method of ALPHA 5310 and are summarized in Table 5.1 (Napoli et al., 2017). The broths containing long chain rhamnolipids used in the MET reactor were previously produced by a safe bacterium *B. thailandensis* E264 at a large-scale fermenter (10 L) using winey residues, a sole carbon, and energy source and it has yielded 1070 mg/L after extraction, reduced the surface tension of water from 71 mN/m to 34.5 mN/m and had a CMC value of 500 mg/ L and three congeners with the predominance of Rha-Rha-C14-C14 (MS[M-H = 761]) has been described to have two identical genes containing copies of RL synthesis genes (rhlA,rhlB,rhlC) present at different sites in its genome (Chebbi et al., 2022).

Table 5-1. Main Physico-chemical parameters of PHs contaminated soil

Parameter	Value
pH	7.6 ± 0.1
Organic C(g/kg)	12 ± 1.1
Total N (g/kg)	0.25 ± 0.3
C/N (g/kg)	48 ± 0.3
Sand (g/kg)	801 ± 0.5

Silt (g/kg)	113 ± 0.4
Clay (g/kg)	87 ± 0.2
Conductivity (µS/Cm)	113.2 ± 0.1
Moisture (%)	8.14 ± 1.2
Total petroleum hydrocarbon (TPH)	4500 mg/kg

Note: Mean values with a standard deviation

2.2. Media composition and microorganisms

The inoculum for the experiment - made up of anaerobic sludge taken from a municipal wastewater treatment had been acclimated for one month at 30 °C. The defined mineral medium for bioelectrochemical experiments is prepared to add the following: $MgSO_4$: 0.25 g/L; Na_2HPO_4: 8.24 g/L; NH_4Cl: 1.0 g/L; NaH_2PO_4: 5.08 g/L; and NaCl: 0.5 g/L; 12.5 mL of Wolfe vitamin solution and 12.5 mL of Wolfe trace mineral solution (Adelaja, 2015).

2.3. Operation and configuration of the reactor

A double-chambered MET was constructed (Fig. 5.1) and is made up of two identical Plexiglass bottles detached by a cationic exchange membrane with an internal diameter of 7 cm (total volume of 250 mL, working volume of 200 mL) (20 cm^2, CMI-7000 Membranes, Netherlands). A metallic clip holds the glass bottles together, with two rubber gaskets between them to keep the reactor airtight. The electrode used for the anode is made of granular graphite. Whereas titanium net/mesh was used for the cathode (length = 6 cm, width = 5 cm). Both electrodes are electrically connected with titanium wires covered with a heat-shrinkable tube. Assay: Ag/AgCl (RE-1S; Biologic, Grenoble, France) is used as a reference electrode. Anolyte medium is poured into the anode chamber. At pH 7.0, a sterile phosphate buffer solution (0.5 M PBS), $Na_2HPO_4 \cdot 12H_2O$: 7.56 g/L; KH_2PO4: 1.5 g/L; 0.31 g/L: NH_4Cl; 0.13 g/L: KCl was added to the cathode chamber (Rabaey et al., 2005).

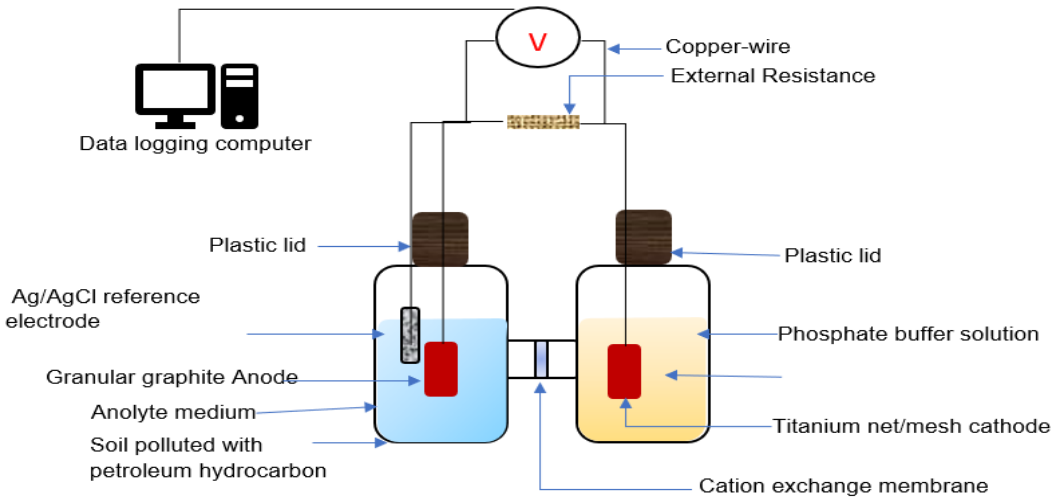


Figure 5-1. Graphical representation of a dual-chambered-MET reactor.

The MET was sterilized by autoclaving for 30 min at 121°C, then aseptically adding a sterile anolyte to the anode chamber. A semi-batch mode was used in all the experiments in this study. The anode chambers were purged with 100% N_2 for 15 minutes before the MET operation started to maintain anaerobic conditions. In all Experiments, the electrodes are connected to a single resistor (R = 1000). According to the experimental design shown in Table 5.2, the MET system, which contains the hydrocarbon-contaminated soil sample, is operated in a long-term fed-batch mode at 20 °C for 20 days. (i.e., four cycles) in two set of experiments. The anolyte medium was made up of 25 g/L (1:10) diesel oil-polluted soil and mineral medium. The effect of biosurfactant dose on the degradation of petroleum hydrocarbon in the MET system anode was investigated. Three different biosurfactant concentrations in the anolyte feed solutions were used at 10, 50, and 100 mg/L selected based on previous in other studies (Cheng et al.,2018). In addition, a negative control is also envisaged, with the same conditions as biotic systems, but without biosurfactants in the medium.

Table 5-2. Experimental design of the bioremediation tests

Treatment	Anode	Cathode	RL	Inoculum
Cycle 1	25 g/L soil polluted with diesel oil Inoculum (sludge) Mineral medium	Catholyte solution (buffer)	10 mg/ L in the anodic chamber	Supplemented as a source of carbon 1 g/L

Cycle 2	25 g/L soil polluted with diesel oil Inoculum (sludge) Mineral medium	Catholyte solution (buffer)	50 mg/ L in the anodic chamber	Supplemented as a source of carbon 1 g/L
Cycle 3	25 g/L soil polluted with diesel oil Inoculum (sludge) Mineral medium	Catholyte solution (buffer)	100 mg/ L in the anodic chamber	Supplemented as a source of carbon 1 g/L
Cycle 4 (Negative control)	25 g/L soil polluted with diesel oil Inoculum (sludge) Mineral medium	Catholyte solution (buffer)	No biosurfactant	Supplemented as a source of carbon 1 g/L

2.4. Analytical methods.

A modified EPA Method 8015D was used to determine TPH content in soil samples. To eliminate moisture from the soil sample, 2 g of the soil and mild anhydrous Na_2SO_4 were combined with it. After that, the soil was mixed with 20 mL of hexane and sonicated for 30 minutes to extract the material. The supernatant was obtained after centrifuging the slurry for 5 minutes at 8000 rpm. Then the extracts were mixed and dehydrated with anhydrous Na_2SO_4. After that, the operation was repeated. The liquid extracts are stored in sealed vials, and GC-FID analysis was performed as described (Palma et al., 2019). The TPH degradation rate was calculated as follows: Eq. (1).

$$Degradation\ of\ TPH\ (\%) = (Initial\ TPH - Final\ TPH) / Initial\ TPH \times 100 \qquad \text{Eq. 1}$$

2.5. Electrochemical analysis and power generation

Bioelectrochemical activity is controlled and measured with the Autolab potentiostat-galvanostat (PGSTAT302N). A potential of 0.5 mV is applied to the anode and held constant vs. (Ag/AgCl). To allow comparison between different systems, current density (A m^{-2}) is calculated by dividing the current intensity (I) by the nominal anodic surface area (4 cm^2). Current I (A) was calculated using Ohm's law: equation V=RI, where R is the external resistance (Ω) and V is the measured voltage (V).

2.6. Cyclic Voltammetry analysis

Using cyclic voltammetry and a Potentiostat-Galvanostat, the bioelectrochemical behavior of soil MECs is investigated. The scanned potential was kept between -600 to +200 millivolts (vs. Ag/AgCl reference electrode) at a scanning rate of 10 millivolts/second. The working electrode was the anode, the counter electrode was the cathode, and a reference electrode was kept **silver/silver chloride** (Ag/AgCl) (RE-1S; Biologic, Grenoble, France, 3 M potassium chloride (KCl), +196 millivolts vs. standard H_2 electrode at 25°C) in a sealed chamber. The bioelectrochemical cell was held at 25°C (Adelaja, 2015).

2.7. Kinetics of biodegradation

The kinetic study is necessary for (a) calculating bioremediation speed, (b) appreciating the biodegradation process, and (c) creating a cost-effective cleanup approach for a PAH-contaminated environment. The kinetics of soil bioremediation are essential because they reveal the pollutant concentration at any given time and allow the prediction of the level that will be present at a later period (Oleszczuk and Baran, 2003). First-order kinetics is commonly used to explain PAH biodegradability, as shown in Eq. (2):

$$C=C_0e^{-kt} \qquad \text{Eq. 2}$$

where C is the residual petroleum hydrocarbons (PHs) content in the soil at time t (mg kg^{-1}); C_0 is the initial petroleum hydrocarbons (PHs) content in the soil (mg kg^{-1}); k is the constant rate biodegradation (day^{-1}); t is time (day). From the plot slope In C vs. t, the biodegradation rate constant (k) can be derived.

2.8. Microbial community analysis

DNA extraction was performed from soil and anodic biofilm samples using Fast-DNA™ SPIN Kit using MP Biomedicals Solon, OH instruction, and protocol. The Illumina-MiSeq® platform was used for sequencing the 16S rRNA V5-V6 hypervariable region, as previously reported (Tartari et al., 2021). UPARSE pipeline was used, where forward and reverse reads were merged, and default parameters were done with quality filter and perfect overlapping. Singleton sequences were performed, where sequences appearing only once in the whole dataset were removed from each sample. UPARSE method for generating clusters (OTUs) was performed on the whole data set. Sequences at 97% identified and defined a representative sequence for each cluster. Classification of

the sequences representative of each OTU at different taxonomic ranks was obtained using the Ribosomal Database Project database. (RDP; http://rdp.cme.msu.edu/).

2.9. Germination index measurement

To determine the germination index (GI), *Lepidium sativum* L. soil samples were grown on the raw and treated soil samples. The experiment was performed by taking 5 g of dry soil in Petri dishes, and the appropriate amount of deionized water was added to keep it moisturized. After about 24 hours, these Petri dishes were covered with Whatman filter paper # 1. In each Petri dish, 10 undamaged and healthy *Lepidium sativum* seeds were placed. After that, all the seeded Petri dishes were incubated at 27 °C for 72 hours in the darkroom. After that, germinated seeds (G) were counted. All treatments were measured, including control relative root length (L). All the seed germination tests were carried out in triplicate. As described by Avona et al., 2022, (GI) (%) was calculated as follows: GI (%) = (G*L)/(Gc*Lc) x100 Eq. 3.

3. Statistical Analysis

Origin Lab Corporation, Northampton, MA, USA (version 8.0773) software was used to assess the statistical difference between current pollutant production and degradation. P values ($P<0.05$) were used to measure all significance of treatment effects.

4. Results and discussion

4.1. Effect of biosurfactant addition on the generation of electricity from MET

The addition of biosurfactant for the degradation of petroleum hydrocarbons during the double-chamber MET operation over 20 days leads to enhanced generation current density as compared to control, except for the high biosurfactant concentration (100 mg/L) as shown in Fig. 5.2. The current density reached a maximum of approximately 9.5 Am^{-2}, and 1 Am^{-2} for MET treated with 100 mg/L biosurfactants and MET treated without biosurfactants, respectively. But lower current density production was observed in all experiments for MET treated without the addition of biosurfactants (control) via an external circuit containing a single resistor (R = 1000). This demonstrated that the anode chamber's current generation performance has significantly improved, implying an increase in the number of bacteria capable of using biosurfactant extracellular redox mediators (Cheng et al.,2018).

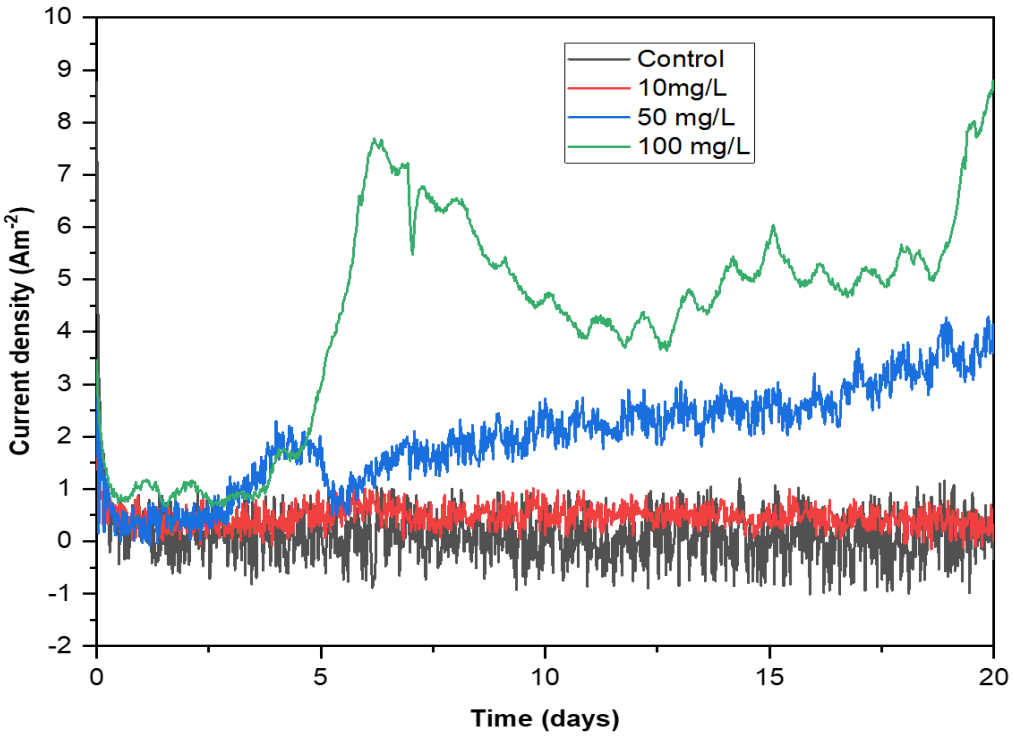


Figure 5-2. Current densities generated in MET during 20 days.

Current output profiles exhibited qualitative relationships in the degradation of hydrocarbons after an initial lag phase. Due to the microbe's acclimatization, and increased electrochemical activities, current progressively rose with modest current variation in experiments with 50 and 100 mg/L doses of biosurfactant representing the heterogeneous nature of the hydrocarbon compounds and unequal mass transfer within the polluted soil. Similar findings were also reported during operational and unequal mass transfer within the polluted soil (li et al., 2018; Zhang et al., 2017). The peak current density emerged around day 4, which may be attributed to the use of a biosurfactant near the working electrode by the microbial community. The disparity between high microbial petroleum hydrocarbon needs and slow mass transfer to the working electrode could explain the drop-in current densities (Xiao et al., 2017). According to Lu et al. (2014), if diffusion did not provide quick enough hydrocarbon transfer in the petroleum hydrocarbon-polluted soil of a bioelectrochemical system, a reduction in pollutant levels on the anode would result in current output reduction. In general, adding biosurfactants could be an excellent way to improve MET's ability to make electricity generation. As a result, further research and investigation are needed in optimizing the dose required in soil MET remediation systems.

4.2. Effect of biosurfactant addition on the degradation of petroleum hydrocarbons

Biosurfactant concentration is usually a critical factor for the removal of PHC compounds from soil. To evaluate the performance of rhamnolipids in removing TPH from the contaminated soil, 10, 50, and 100 mg/L biosurfactant concentrations were applied as an anolyte to the MET. As illustrated in Fig. 5.3 (green color), 72.5 % of petroleum hydrocarbons were degraded after 20 days in the soil for the rector of MET with a 100 mg/L dose of biosurfactant compared to the control without biosurfactant. This prevailed that after sterilizing the soil, the efficiency of petroleum hydrocarbon degradation was considerably reduced, indicating that exoelectrogen bacteria are the dominant process for petroleum hydrocarbon degradation (Idris et al., 2022).

The first kinetic biodegradation rates (k) were 0.0118, 0.0136, and 0.0643 d^{-1} for soil amended with 10 mg/L, 50 mg/L, and 100 mg/L application of biosurfactant, respectively Fig. 5.3 red color). Biosurfactant with a dose of 100 mg/L enhanced the bioavailability and degradation of petroleum hydrocarbon in the MET soil remediation system. As previously observed first-order rate constant k values 0.043 d^{-1} was reported in total organic carbon (TOC) biodegradation and petroleum hydrocarbon for electric generation by sediment MFCs with biosurfactant as electron donor, which showed the complete transformation of petroleum hydrocarbons after 28 days of treatment (Pacwa-Płociniczak et al., 2014). Another study by Xu et al. (2015) found that the biotransformation of petroleum hydrocarbons was little or significantly less in the treatment with no added biosurfactants in bioelectrochemical system treatment. The anode successfully captured the electrons derived from hydrocarbon oxidation with the help of graphite, demonstrating that the biosurfactant-assisted bioelectrochemical systems facilitated the kinetic degradation of petroleum hydrocarbons while also promoting bioelectricity production. Although the MET system's K value appears to be lower than other values reported in the literature, the addition of biosurfactants improves the biodegradation and bioavailability of petroleum hydrocarbon-contaminated soil and necessitates further investigation into the microbial characteristics, soil characteristics, and composition of petroleum hydrocarbons to enhance the value of kinetic degradation (Gidudu and Chirwa, 2020).

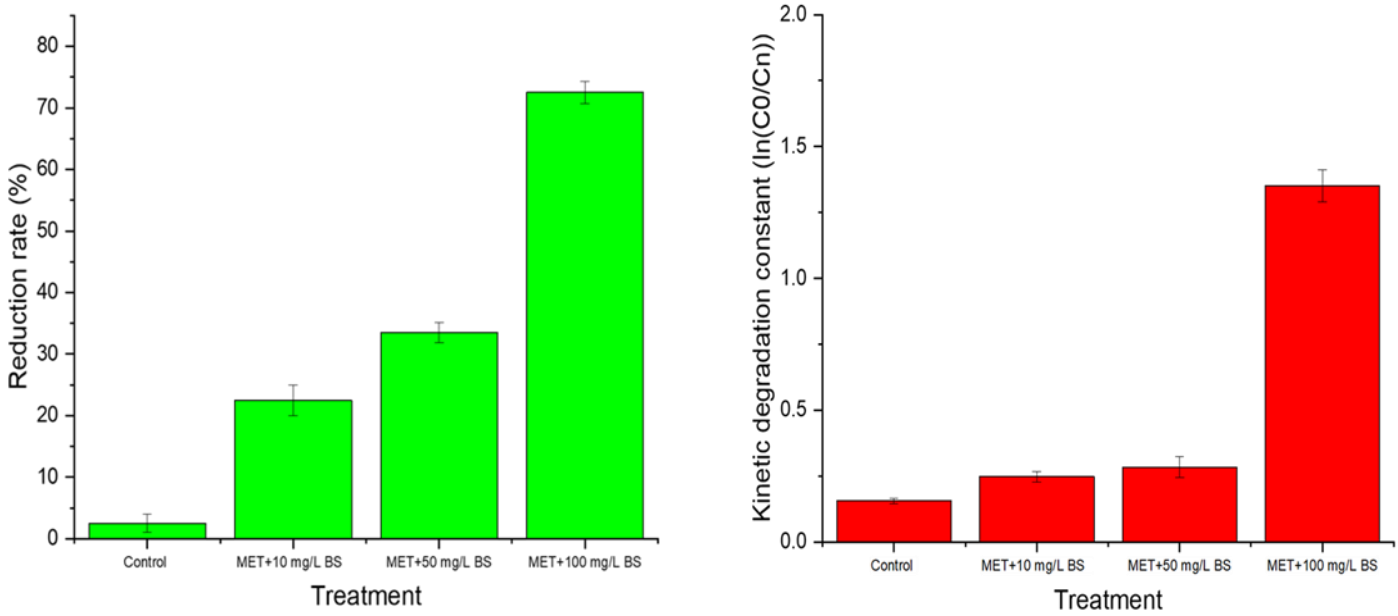


Figure 5-3. Total petroleum hydrocarbon reduction rate (green color) and kinetic degradation rate (red color) in MET and the addition of different amounts of biosurfactant. Data are means of duplicates, and error bars show standard deviations.

4.3. Extracellular electron transfer analysis in the MET

To evaluate the extracellular electron transfer process between bacteria and the electrode, the cyclic voltammograms (CV) of the MET's anode chamber were studied during incubation on day 20 (Fig. 5.4). As petroleum hydrocarbon degradation by microbes progressed in the MET, cyclic voltammograms for all experiments treated with biosurfactant were shown to have a significant oxidation/reduction peak potential due to increased microbial electrochemical oxidation processes at the anode (Zheng et al., 2015). As indicated in Fig. 5.4, MET with biosurfactant amendment had a greater shift in redox potential than MET alone. It could imply that adding biosurfactants has a beneficial effect by increasing the bioavailability and mobility of petroleum hydrocarbons within the soil matrix. However, it can also act as a redox electron shuttle in mediated electron transfer to the anode (Zheng et al., 2015). This can lead to increased electricity generation and degradation. This is supported by the results of the microbial community (Figure 5.5), which show the presence of the electroactive community with direct electron transfer was more prevalent than the community with mediated electron transfer in the addition of 100 mg/L biosurfactants.

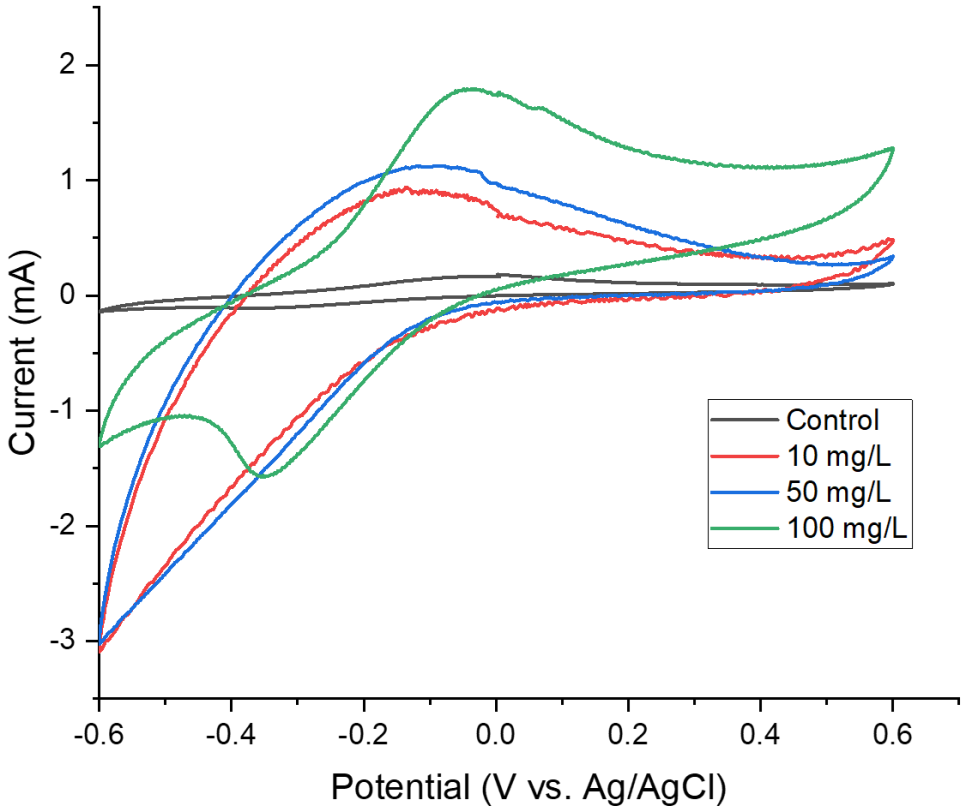


Figure 5-4. The cyclic voltammetry curve for soil with petroleum hydrocarbon in MET with or without biosurfactant at day 20 at a sweep rate of 10 mV/s.

These anodic CV curves also show that adding a biosurfactant to MET can improve the electrochemical performance and removal of petroleum hydrocarbons due to the anode chamber contains more electrochemically active bacteria. The same result was also reported by Karlapudi et al. (2017) and Carmona-Martínez et al. (2013) during the degradation of petroleum hydrocarbon in the double chamber of bioelectrochemical system operation. Generally, the electrical current generated during bioelectrochemical system operation can be further used as a power source for remote sensors and an indicator of petroleum hydrocarbon degradation as well as, and CV revealed the involvement of extracellular redox mediator-mediated electron transfer.

4.4. Effect of biosurfactants on microbial communities' composition in anodic biofilms and soil

The content and microbial community on the anode surface and soil significantly impact bioelectrochemical system performance. Therefore, analyzing the dynamic features of the microbial community's composition and structure is critical for enhancing the bioelectrochemical system's

ability to generate energy and degrade organic matter. Next-generation sequencing is a molecular ecology technique that does not require microbial cultures. This approach has been applied to determine microbial communities' organization and composition in polluted environments and to accelerate the breakdown of pollutants (Yu et al., 2017).

Adding rhamnolipid biosurfactant to the anolyte at a concentration of 100 mg/L enhanced an MFT's current density considerably, as seen above. The control bacterial community (without biosurfactant), MET + 10 mg/L, MET + 50 mg/L, and MET + 100 mg/L, were studied using 16 S rRNA sequencing after MEC operation for 20 days, as shown in Fig. 5.5. The compositions of soil bacteria genera in treatments and control were comparable. However, as shown in Fig. 5.6, the MET reactor fed with 100 mg/L has a higher abundance of exoelectrogen bacteria like *Geobacter, desulfovibrio, Pseudomonas spp, Klebsiella spp, Acinetobacter spp, Comamonas spp,* and this result is consistent with prior studies (Ailijiang et al., 2016; Boonchan et al., 1998). Therefore, the finding of this study indicates that the amount of rhamnolipid in a solution significantly impacts the bacterial population on the anodic surface.

As compared to MET + 50 mg/L, the proportion of Geobacter in MET + 100 mg/L increased by 65 %. *Geobacter* was the most common genera producing electricity in MET, and its dominance on the anode surface may have hampered the competitiveness of other electrogenic bacteria (Bond et al., 2003; Corbella et al., 2015; Wang et al., 2016). The result shows a significant prevalence of Geobacter enriched with 100 mg/L rhamnolipid biosurfactants, indicating higher exoelectrogens anodic bacteria proportion. That would be consistent with a maximum current density of 9.5 Am^{-2}. Exoelectrogen abundance differences could imply rhamnolipid effects on bacteria and bacterial compositions, which could affect the bioelectrochemical system's current density output (Chen et al., 2022).

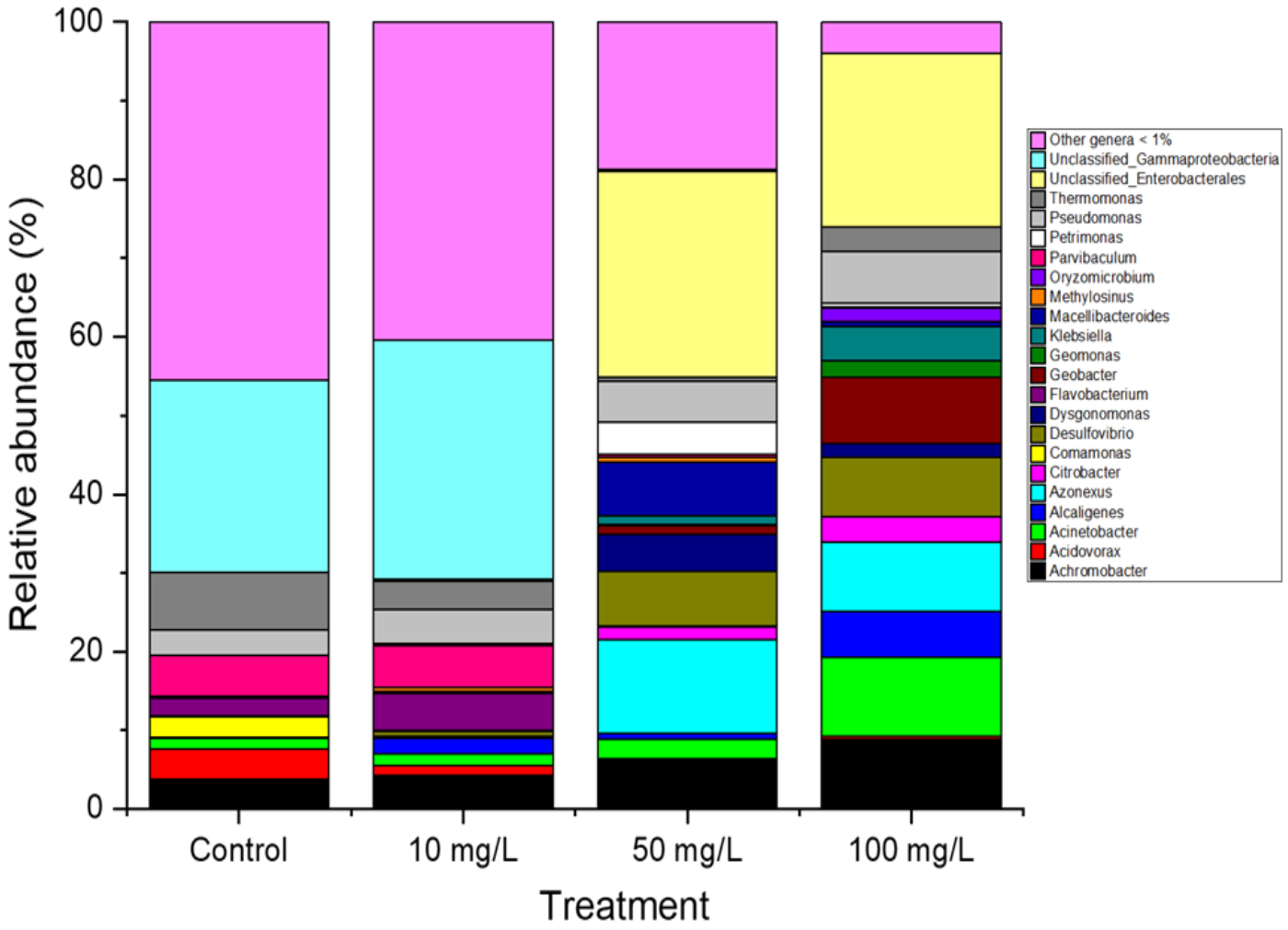


Figure 5-5. Relative abundance and community composition of bacteria in the anode surface of MET within various concentrations of biosurfactant treatments.

The bacterial composition of soil bacterial communities was also studied to validate the enrichment of bacterial communities by adding a biosurfactant (Fig.5.6). Compared to the other treatments, adding 100 mg/L rhamnolipids boosted the enrichment of *Pseudomonas, Acinetobacter, Franconibacter, Immundisolibacter, and Parvibaculum* in the soil microbial community. The genus *Pseudomonas* had the highest average abundance, at 36.7 %. Therefore, these bacteria were identified as promising oil degraders, and it was discovered that the abundances of *Pseudomonas, Acinetobacter, Franconibacter, Immundisolibacter, and Parvibaculum* were positively connected with hydrocarbon degradation (Ailijiang et al., 2016; Mathis et al., 2008; Kiely et al., 2011).

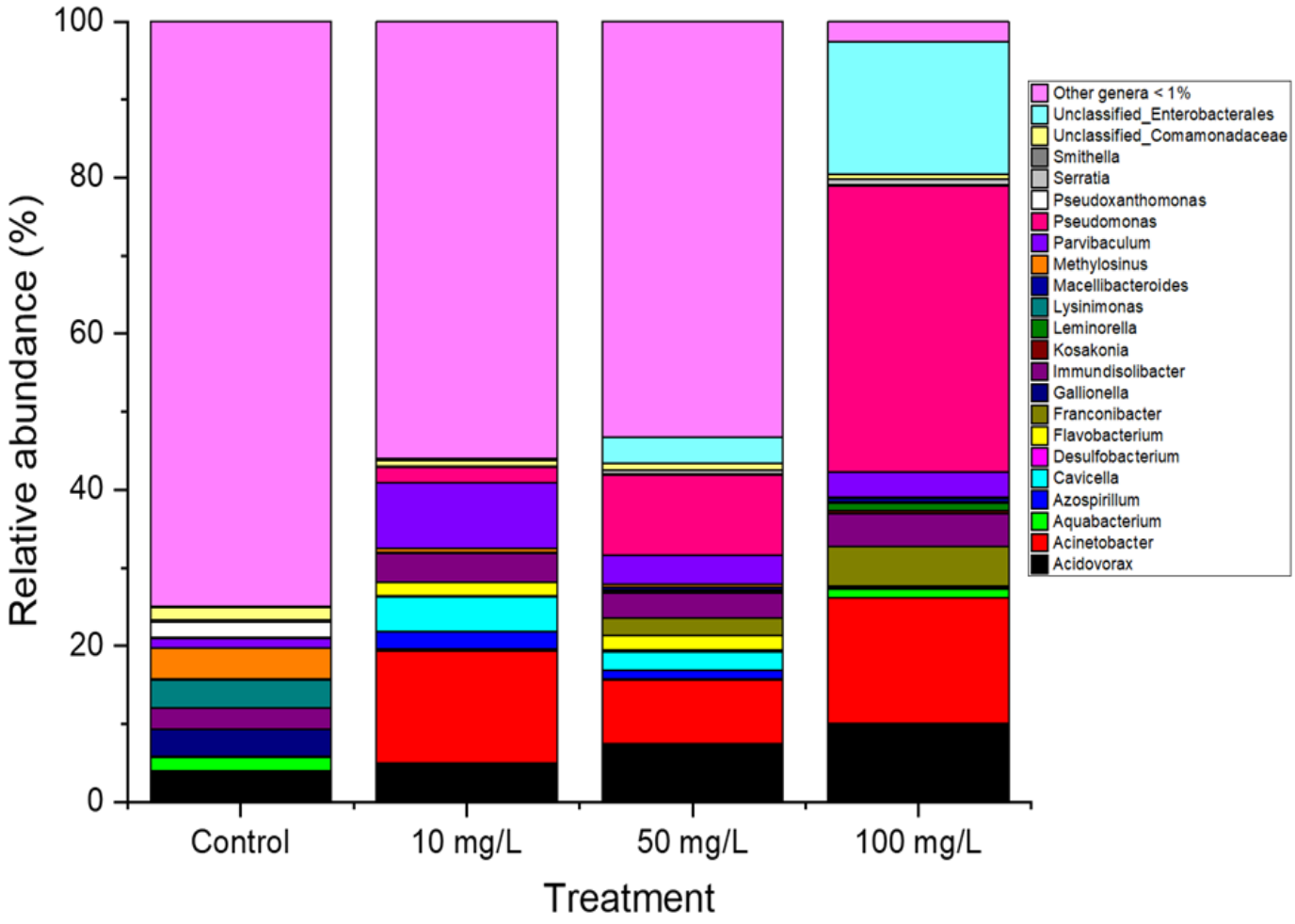


Figure 5-6. Relative abundance and community composition of bacteria in the soil of MET within various concentrations of biosurfactant treatments.

However, this result contradicts a previous finding by Zhang et al. (2017). They found that adding biosurfactant above 40 mg/L inhibits bacterial metabolism and integrity which may result in lower microorganism electroactivity. It also necessitates optimizing a proper biosurfactant concentration to be used in the bioelectrochemical system ahead of time. As a result, the bioelectrochemical system maintained the stability of microbes at its anode surface, which is favorable for generating electricity and degradation of organics. That was accomplished by encouraging and discouraging the growth of various soil bacteria. Meanwhile, it improved the biotope for most bacteria present in soil by using electric stimulus linked to its operation, resulting in greater community variety and abundance. When bioelectrochemical processes were present, this could be the key reason for the improvement in the removal of PAHs from soil (Yu et al., 2017).

4.5. Phytotoxicity determination of the polluted soil after and before MET treatment

Soil can only be disposed of in the environment or reused for alternative agricultural applications if contamination levels and other harmful organic intermediate products are under the regulatory agencies' permissible concentration limits (Jayasinghe et al., 2022). It guarantees that it is environmentally friendly and offers no immediate threat to ecosystems or human health, which is the intent of any good remediation technique (Köninger et al., 2022). Degrading petroleum hydrocarbon from polluted soil via microbial processes frequently leads to incomplete mineralization. It results in degraded products with unknown toxicological and chemical properties, which can be even more harmful than the parent pollutant. GI value > 100 % indicates a plant–stimulating effect, GI > 80% suggests that the material present is not phytotoxic and GI ranging between 50 and 80 % means moderate phytotoxicity. In comparison, GI < 50 % indicates a high phytotoxicity effect (Bożym et al., 2021). Fig. 5.7 shows the average percentage germination index (GI) of *Lepidium sativum* L. seed growth in the soil before and after the MET operational time.

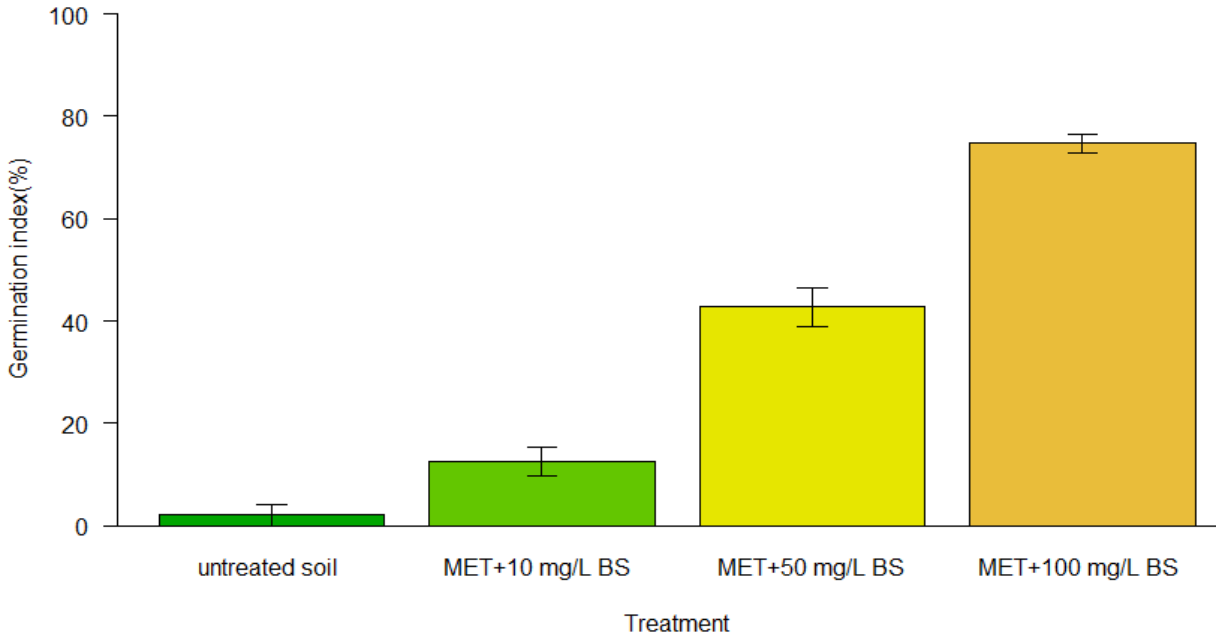


Figure 5-7. Germination index of Lepidium sativum L. seed growth in the soil before and after the MET operational time. Data are means of duplicates, and error bars show standard deviations.

The root length of the L. sativum control sample was 63.1 4 mm on average. The average root length in soil polluted with petroleum hydrocarbons that were not treated was 6.5 mm. Growing *L. sativum* on MET with 100 mg/L biosurfactants produced the longest roots (mean 75 mm). For *L. sativum*, the GI values from the soil MET treatment are as follows: MET + 10 mg/L of biosurfactant 12 %, MET+

50 mg/L of biosurfactant 43 %, and MET + 100 mg/L of biosurfactant 73 %, respectively. That means that the soil from MET treatment is moderately phytotoxic to L. *sativum* seed. A possible explanation for moderate phytotoxicity could be the treatment's poor performance at lower concentrations of biosurfactant. That could be related to a substantial quantity of petroleum hydrocarbon and the formation of high molecular weight intermediate products. These products may have a highly toxic effect. They may also have significant root length differences between the controls (deionized water and soil), which influenced the GI value and necessitated further treatment. This research showed that, under the same environmental conditions, the MET system surpasses natural attenuation in bioremediating petroleum hydrocarbons from polluted soil quickly and effectively.

5. Conclusions

This study found that incorporating RL into the anode of MET resulted in highly efficient electricity generation for the enhancement of petroleum hydrocarbon degradation and the reduction of phytotoxicity. The use of RL in MET significantly impacts the composition of exoelectrogens that transport electrons in MET and revealed that, with RL assistance, electro-active microbes of *Geobacter* and *Desulfovibrio* were remarkably enriched on anode biofilm. In addition, redox peaks have been observed after adding a biosurfactant as an anolyte. The redox system, particularly direct electron transfers as well as biofilm adhering to the electrode surface, was responsible for the observed peaks. The obtained findings indicate that adding RL to the anode of MET enhanced electricity generation and petroleum hydrocarbon degradation, and the RL produced by the *Burkholderia thailandensis* strain has excellent bioremediation-friendly properties, but it needs to optimize the dose of the RL to avoid the toxic effect of biosurfactants on the exoelectrogens bacteria and should be investigated further to advance this technology.

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CHAPTER 6.

EFFECT OF RICE-BASED BIOCHAR ON PETROLEUM HYDROCARBONS DEGRADATION AND ELECTRICITY GENERATION IN MICROBIAL ELECTROCHEMICAL REMEDIATION SYSTEMS

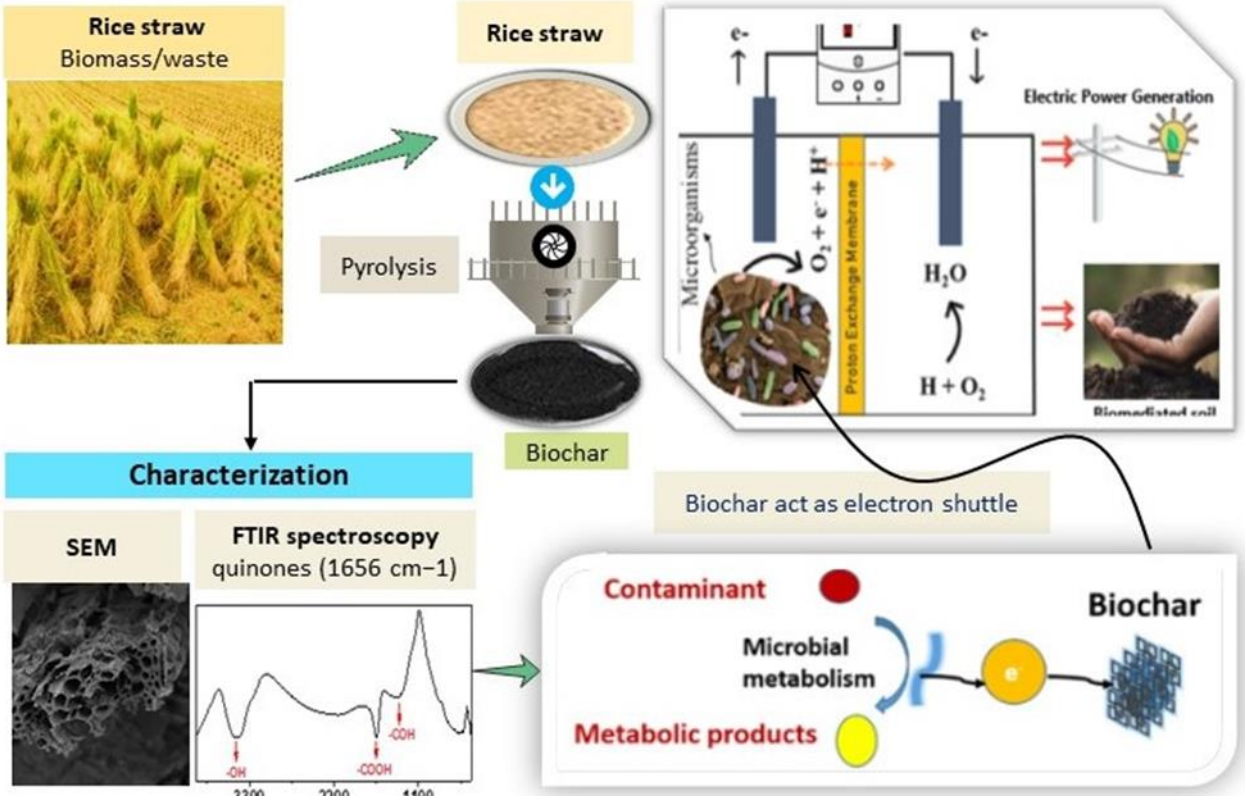
This chapter is under review in the Elsevier Journal of Environmental Chemical Engineering .

Abstract

Petroleum and petroleum product exploration, transportation, and processing negatively impact soil ecosystems and cause severe damage. Hence, a study was conducted to evaluate the performance of microbial electrochemical treatment (MET) in terms of biodegradation and electricity generation with petroleum hydrocarbon-polluted soil by adding rice straw biochar (BC) at different doses of 4, 8, and 12 % w/w. After the addition of an optimal dose of biochar (8% w/w), the maximum current density was 3.5 A/m^2, and the highest petroleum hydrocarbon reduction rate of 87.8 % was achieved in 20 days. Biochar also enriched the petroleum hydrocarbon degradation microbes in MET. *Desulfuromonas* was discovered to be the most prevalent genus among the microbial population detected in response to petroleum hydrocarbon treatment. The electrochemical investigation also shows that the redox-active quinone in biochar is responsible for extracellular electron transfer. Overall, the findings demonstrated the potential for adding biochar to MET for effective petroleum hydrocarbon degradation and energy production.

Keywords: Biochar, Bioelectrochemical system, Electricity generation, Extracellular electron transfer, Petroleum hydrocarbon degradation, Microbial community

Graphical abstract



1. Introduction

Petroleum is one of the most important energy sources for a country's economic and social development. However, petroleum and petroleum products exploration, transportation, and processing negatively impact soil ecosystems and cause severe damage (Ambaye et al., 2022). One of the most common causes of soil pollution is the unintentional release of crude oil into the environment. Even at low concentrations, these pollutants pose a significant hazard to human health and the environment (Ambaye et al., 2022). Bioremediation is a low-cost, environmentally friendly remediation process often used to treat soils contaminated with petroleum hydrocarbons (Vaccari et al., 2020). On the other hand, bioremediation has several drawbacks, including poor abundance and diversity and slow growth rates of indigenous degrading bacteria, resulting in restricted petroleum hydrocarbon bioavailability in the soil (Ambaye et al., 2022). Therefore, effective technologies are necessary to improve the efficacy of soil bioremediation.

The system employing microorganisms to catalyze oxidation/reduction reactions using donor/accept electrons from an electrode is known as a bioelectrochemical system (BES). It has been demonstrated that BESs simultaneously facilitate the degradation of petroleum hydrocarbons and bioelectricity generation (Li et al., 2019). However, the biodegradation efficiency and current density generation in BES are constrained by the significant internal resistance of soils and electron migration in soils contaminated with petroleum hydrocarbons. To reduce the system's internal resistance and enhance ion and substrate movement, attempts have been made in this area by adding a variety of materials to the soil matrix (Li et al., 2022). For instance, adding silica colloid, graphene oxide, and carbon fiber to the soil matrix of the anodic chamber in BES enhances the electricity generation and degradation of organic pollutants compared to the unamended controls (Domínguez-Garay et al., 2013, Camedda et al., 2019; Li et al., 2016). However, the mobility and bioavailability of adsorbed pollutants in the soil matrix are severely constrained, and the effective distance of pollutant removal is often confined to a few centimeters around the electrode surface, which may present a difficulty for bioremediation. The effective radius of stimulation for bioremediation is a problem that poses severe difficulties in the use of BES in practice (Cai et al., 2020).

A stable carbon-rich residue known as biochar is produced when biomass is thermally decomposed. Biochar has a vast surface area, a highly porous structure, and a high ability to exchange ions (Ambaye et al., 2021). Advantageous applications of biochar include soil fertility enhancement, carbon sequestration, and the remediation of pollutants (Ambaye et al., 2021a). In addition, biochar's electro-active qualities, including its electrical conductivity and redox activity, have recently attracted

strong interest because of their potential to mediate electron transfer in various chemical and biological processes, such as BES (Cai et al., 2021). For instance, Chen et al. (2014) performed an experiment using defined co-cultures of *G. sulfurreducens* and *G. metallireducens* in a medium with fumarate as an electron acceptor and ethanol as an electron donor. They discovered that biochar might speed up the flow of electrons from *G. metallireducens* to *G. sulfurreducens* and encourage their growth. Moreover, they claimed that biochar facilitates interspecies electron exchange via a conduction-based process in which electrons travel through the biochar from electron-donating to electron-accepting cells. In another study by Yu et al. (2015), it was reported that biochar surface redox-active moieties and electric conductivity could play a significant role in the enhanced microbial transformation of pentachlorophenol. It has been discovered that the primary factors governing the electro-active characteristics of biochar are the feedstock type and pyrolysis temperature (Nastro et al., 2021). Additionally, biochar has been incorporated into the soil matrix to improve BES's ability to transmit electrons. For example, Chen et al. (2016) supplemented BES soil with pyrolyzed biochar at 400-900 °C to increase BES power generation by 2-10 times. Li et al. (2019) also reported similar results, showing that soil supplemented with biochar (600 °C) boosted power generation and the decomposition of petroleum hydrocarbons.

However, the increased number of surface oxygenated groups, such as redox-active hydroquinone or quinone moieties, is linked to the features of the biochar-enhancing effect. The properties of biochar have been demonstrated to depend on the different starting feedstocks and pyrolysis temperatures (e.g., elemental composition, specific surface area, aromaticity, yield, porosity, ash, and chemical properties). As a result, biochar obtained by varying these parameters is used to treat different forms of soil contamination. However, further research is needed to understand how it facilitates extracellular electron transport in varied contexts. Moreover, there is no information on how the dose of biochar affects the microbial activity and degradation of PHs in BES (Zhao et al., 2022). Surprisingly, very few recent studies have examined whether biochar is capable of causing the biodegradation of PHs in BES, which would likely offer new insight to clarify the role of biochar-in the enhanced biodegradation through extracellular electron transfer. Due to their low solubility, most petroleum hydrocarbon pollutants remain adherent to soil particles; thus, the microbial community involved in the bio-oxidation process is unknown and requires further investigation (Cai et al., 2021).

As a result, this work investigated the viability and related mechanisms of rice straw-based biochar to improve extracellular electron transfer, using typical soil polluted with petroleum hydrocarbons as a model pollutant. The ideal dose of biochar amendment for enhancing extracellular electron transport was determined by changes in current density, the degradation efficiency of PHs, and cyclic

voltammetry (CV). To understand how the dose of biochar affects extracellular electron transfer during the degradation of PH, the microbial community on the anodic surface and slurry sample are also studied.

2. Materials and methods

2.1. Soil and biochar preparation

The soil contaminated with crude oil used in the experiment was collected from a polluted site in northern Italy. The soil sample was taken from 5 to 10 cm below ground level, sealed, and transported to the laboratory. After being partially air-dried, the soil was sieved to a diameter of less than 2 mm. After being partially air-dried, the soil was sieved to a diameter of less than 2 mm. The main physico-chemical parameters of the initial soil samples were determined using the standard ALPHA 5310 method and are summarized in Table 6.1 (Napoli et al., 2017).

Table 6-1. Main physico-chemical parameters of PHs contaminated soil

Parameter	**Value**
pH	7.6 ± 0.1
Organic C(g/kg_{dw})	12 ± 1.1
Total N (g/kg_{dw})	0.25 ± 0.3
C/N(g/kg_{dw})	48 ± 0.3
Sand (g/kg_{dw})	801 ± 0.5
Silt (g/kg_{dw})	113 ± 0.4
Clay (g/kg_{dw})	87 ± 0.2
Conductivity (µS/cm)	113.2 ± 0.1
Moisture (% w/w)	8.14 ± 1.2
Total petroleum hydrocarbon (TPH) (mg/kg_{dw})	4500 mg/kg

Note: Mean values with a standard deviation

Rice straw collected from rice fields in a village in the Pavia province, in the Lombardy region of Italy, was used to produce biochar. The rice straw was cleaned in deionized water before being dried for 24 hours at 90°C in an oven. The material was then diced into 3–4 cm long pieces and pyrolyzed in a cylindrical quartz tube at 600 °C for 2 hours with a 1.5 L min^{-1} N_2 flow. Biochar (BC) produced at these pyrolysis temperatures has been demonstrated to promote the degradation of organic pollutants (Sánchez-Monedero et al., 2019; Li et al., 2019). Finally, biochar was milled and sieved using a 100-mesh (0.15 mm) sifter after cooling to room temperature (Cai et al., 2021). According to

the pyrolysis temperature, the resulting biochar was designated as BC 600 °C, and the basic physical and chemical properties of BC600 °C are summarized in Appendix I1.

A four-probe powder resistivity meter was used to determine soils' electrical conductivity (EC) in a mixture of distilled water and soil at a 2g: 10mL ratio. Scanning electron microscopy (SEM) images and quantitative energy dispersive X-ray spectroscopy (EDS) mapping are depicted in supplementary Fig. S1 from a Hitachi S-4800 microscope, which was used for this study (Hitachi, Japan). Energy dispersive spectroscopy (EDX; X-max 50 mm^2 probe by Oxford Instrument, Abingdon, UK) connected to a Carl Zeiss EVO MA10 scanning electron microscope (Oberkochen, Germany) was used to determine the elemental composition of rice straw biochar after a sample was dried at 45 °C for 6 hours in an oven. An infrared spectrometer (Bruker VERTEX 70, Germany) was used to identify the biochar's functional groups (Cai et al., 2020). These pyrolysis temperatures have been shown to promote the degradation of organic pollutants and act as redox mediators in biochar production (Li et al., 2019).

2.2. Media composition and microorganisms

The inoculum for the experiment - made up of anaerobic sludge taken from a municipal wastewater treatment had been acclimated for one month at 30 °C. The defined mineral medium for bioelectrochemical experiments was prepared by adding the following compounds: $MgSO_4$: 0.25 g L^{-1}; Na_2HPO_4: 8.24 g L^{-1}; NH_4Cl: 1.0 g L^{-1}; NaH_2PO_4: 5.08 g L^{-1}; and NaCl: 0.5 g L^{-1} 12.5 mL of Wolfe vitamin solution and 12.5 mL of Wolfe trace mineral solution was also added (Adelaja, 2015).

2.3. Operation and configuration of the reactor

A double-chambered BES, microbial electrochemical technologies (MET), was used based on previous studies, as shown in Fig. 6.1 (Adelaja, 2015), and was made up of two identical Plexiglass bottles detached by a cationic exchange membrane with an internal diameter of 7 cm (total volume of 250 mL, working volume of 200 mL) (20 cm^2, CMI-7000 Membranes, Netherlands). A metallic clip holds the glass bottles together, with two rubber gaskets between them used to keep the reactor airtight. A granular graphite electrode was used as an anode, whereas titanium net/mesh was used as a cathode (length = 6 cm, width = 5 cm). Both electrodes were electrically connected with titanium wires covered with a heat-shrinkable tube. Assay: Ag/AgCl (RE-1S; Biologic, Grenoble, France) was used as a reference electrode. The anolyte medium was poured into the anode chamber. At pH 7.0, a sterile phosphate buffer solution (0.5 M PBS) made of, $Na_2HPO_4 \cdot 12H_2O$, 7.56 g L^{-1}; KH_2PO4, 1.5 g L^{-1}; 0.31 g L^{-1} NH_4Cl; 0.13 g L^{-1} KCl was added to the cathode chamber (Rabaey et al., 2005).

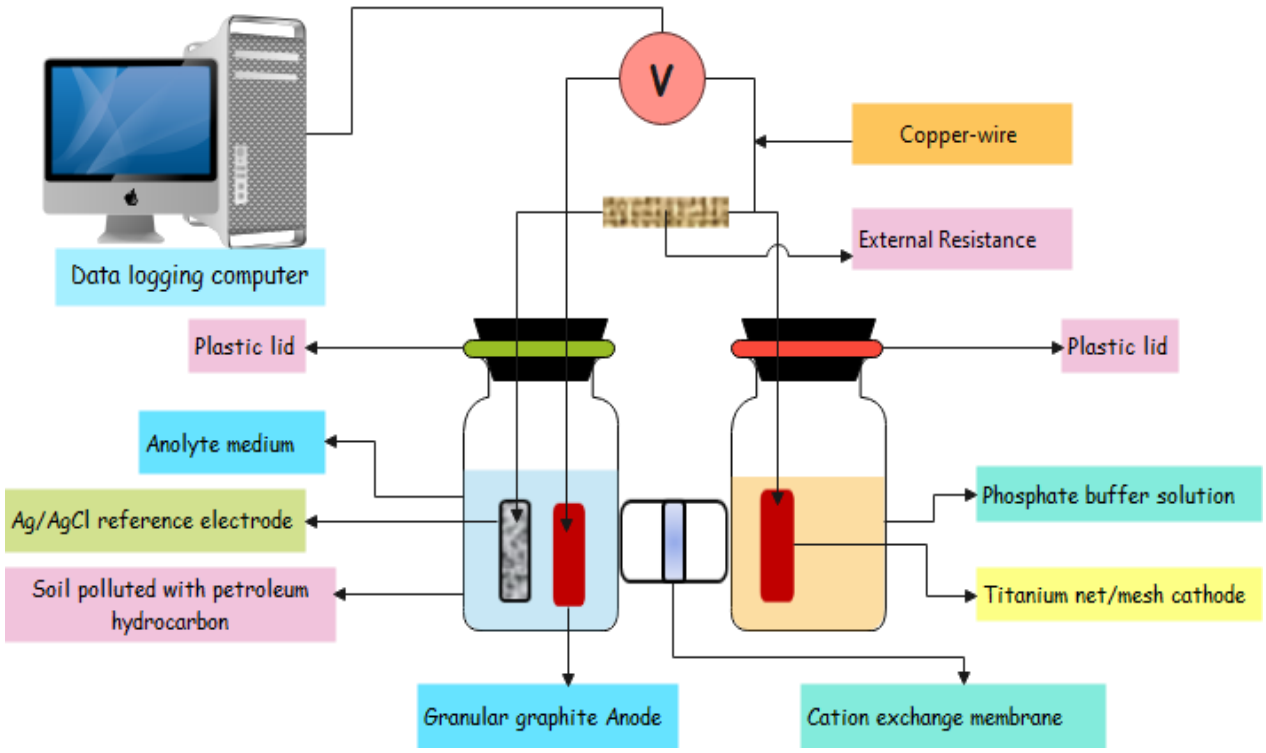


Figure 6-1. Graphical representation of a dual-chambered-MET reactor

The MET was sterilized by autoclaving for 30 minutes at 121°C, then aseptically adding a sterile anolyte to the anode chamber. A semi-batch mode was used in all the experiments in this study. The anode chambers were purged with 100% N_2 for 15 minutes before the MET operation started to maintain anaerobic conditions. In all experiments, the electrodes were connected to a single resistor (R = 1000 Ω). According to the experimental design shown in Table 6.2, the MET system, which contains the hydrocarbon-contaminated soil sample was operated in a long-term fed-batch mode at 20 °C for 20 days. (i.e., four cycles) in one set of experiments.

The effect of biochar dose on petroleum hydrocarbon degradation in the anode of the MFC system was investigated using three different dosages in the anolyte feed solutions, (8, 10, and 12 %) were selected based on previous studies (Cai et al., 2021). In addition to the abiotic control, another negative control was also envisaged, with the same conditions of the biotic systems, but without biochar in the medium. Moreover, a negative control was envisaged, with the same conditions as biotic systems, but without biosurfactants in the medium.

Table 6-2. Experimental design of the bioremediation tests

Treatment	Anode	Cathode	Biochar (BC)	Inoculum
Cycle 1	• 25 g/L soil polluted with diesel oil • 24 g soil + 1 g biochar • inoculum (sludge) • mineral medium	Catholyte solution (buffer)	4 % in the anodic chamber (1g)	Supplemented as a source of carbon 1 g/L
Cycle 2	• 25 g/L soil polluted with diesel oil • 23 g soil + 2 g biochar • inoculum (sludge) • mineral medium	Catholyte solution (buffer)	8 % in the anodic chamber (2 g)	Supplemented as a source of carbon 1 g/L
Cycle 3	• 25 g/L soil polluted with diesel oil • 22 g soil + 3 g biochar • inoculum (sludge) • mineral medium	Catholyte solution (buffer)	12% in the anodic chamber (3g)	Supplemented as a source of carbon 1 g/L
Cycle 4 (Negative control)	• 25mg/L soil polluted with diesel oil • inoculum (sludge) • mineral medium	Catholyte solution (buffer)	No biochar	Supplemented as a source of carbon 1 g/L

2.4. Analytical methods.

A modified EPA Method 8015D was used to determine TPH content in soil samples. To eliminate moisture from the soil sample, 2g of the soil and mild anhydrous Na_2SO_4 were combined with it. After that, the soil was mixed with 20 mL of hexane and sonicated for 30 minutes to extract the material. The supernatant was obtained after centrifuging the slurry for 5 minutes at 8000 rpm. Then the extracts were mixed and dehydrated with anhydrous Na_2SO_4. After that, the operation was repeated. The liquid extracts were stored in sealed vials, and GC-FID analysis was performed as described (Palma et al., 2019).

2.5. Electrochemical analysis and power generation

The bioelectrochemical activity was controlled and measured with the Autolab potentiostat-galvanostat (PGSTAT302N). A potential of 0.5 mV is applied to the anode and held constant vs. (Ag/AgCl). To allow comparison between different systems, current density (A m^{-2}) is calculated by dividing the current intensity (I) by the nominal anodic surface area (4 cm^2). Current I (A) was calculated using Ohm's law: equation V=RI, where R is the external resistance (Ω) and V is the measured voltage (V).

2.6. Cyclic Voltammetry analysis

The bioelectrochemical behavior of soil MET was investigated using cyclic voltammetry and a Potentiostat-Galvanostat. The scanned potential was kept between -600 to +200 mV (vs. Ag/AgCl reference electrode) at a scanning rate of 10 mV/second. The working electrode was the anode, the counter electrode was the cathode, and a reference electrode in silver/silver chloride (Ag/AgCl) was used (RE-1S; Biologic, Grenoble, France, 3M potassium chloride (KCl), +196 mV vs. standard H_2 electrode at 25°C) in a sealed chamber. The bioelectrochemical cell was held at 25°C (Adelaja, 2015).

2.7. Microbial community analysis

DNA extraction was performed from soil and anodic biofilm samples using Fast-DNA™ SPIN Kit using MP Biomedicals Solon, OH instruction, and protocol. The Illumina-MiSeq® platform was used for sequencing the 16S rRNA V5-V6 hypervariable region, as previously reported (Tartari et al., 2021). UPARSE pipeline was used, where forward and reverse reads were merged, and default parameters were done with quality filter and perfect overlapping. Singleton sequences (i.e. sequences appearing only once in the whole dataset) were removed, from each sample. UPARSE method for generating clusters (OTUs) was performed on the whole data set. Sequences at 97% identified and defined a representative sequence for each cluster. Classification of the sequences representative of each OTU at different taxonomic ranks was obtained using the Ribosomal Database Project database. (RDP; http://rdp.cme.msu.edu/).

3. Results and discussion

3.1. Effects of biochar addition on electricity generation in the MET

The MET reactors amended with 4%, 8%, and 12% of biochar in the anodic chamber were found to have a current density of 1.5, 3.5, and 0.5 A/m^2, respectively, as shown in Fig. 6.2. However, MET reactors without biochar addition reached a current density of 0.2 Am^2, which was 94.3 % lower than that of MET reactor amended with 8.0% biochar in the anode. This finding suggests that a favorable impact of biochar on the anode to generate electricity due to additional electro-active sites for electron

transport may be provided by conductive components in the soil matrix (Li et al., 2019). Although conductivity is necessary, most of the extracellular electron transfer in biochar is caused by electro-active groups on its surface, which can reversibly transfer electrons via Geobacter processes (Prado et al., 2019). The chemical moieties of BC600 °C were identified using FTIR spectroscopy. As shown in Fig. S2, the FTIR spectra showed the presence of –OH groups (band at 3462 cm-^{1}), C=O stretching of quinones (1656 cm^{-1}), and –COH bonds (1445 cm^{-1}). The presence of quinone groups suggests that biochar serves as an electron redox mediator (Chen et al., 2018).

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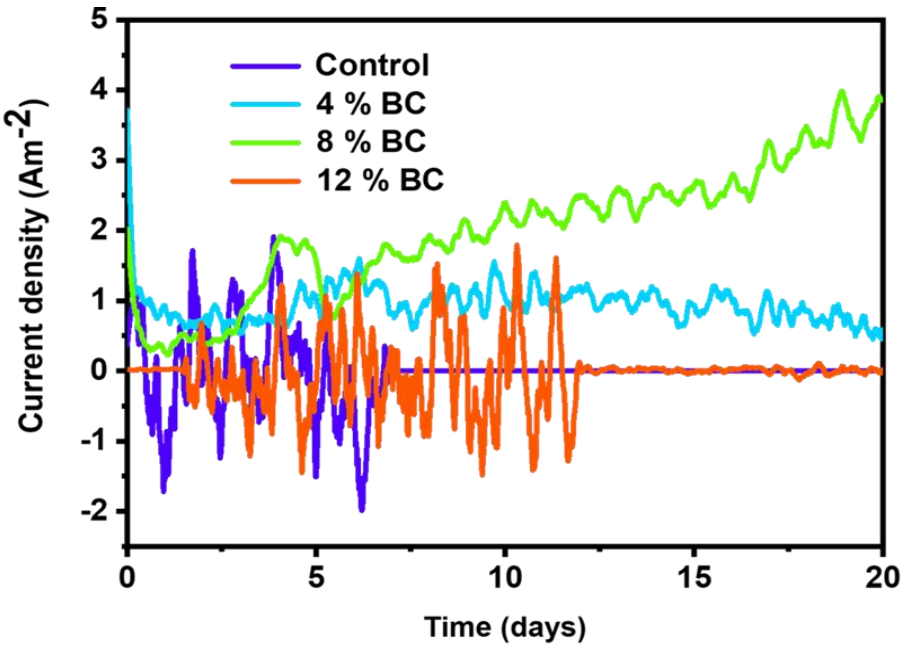

Figure 6-2. Electricity generation with various doses of biochar**.**

Adding a biochar dose of about 12 % to the MET reactor leads to a decrease in its electricity generation, as shown in Fig. 6.2, suggesting the weak activity of electro-active bacteria, which could inhibit bacterial metabolism and integrity in lower electroactivity of the microorganism. It follows, therefore, that microbial activity and kinetics may be inhibited by the addition of a high amount of biochar in the MET treatment of soil polluted with petroleum hydrocarbons. Ayyappan et al. (2018) reported that when monitoring organic pollutant degradation and energy production using coconut shells in bioelectrochemical systems, adding biochar increases the decolorization and degradation of organic pollutants to a certain level. However, the study concluded that adding biochar beyond a specific limit does not contribute to further improving the degradability. Instead, it constrains the growth of microorganisms and makes the biomass inhibited, which agrees with the present study.

This research finding also showed that the current density steadily rose in all MET on day 4 (Fig. 6.2), showing the bacterial microbial community's adaptability to the electrochemical process. The maximum peak current densities developed around day 18 with the reactor, possibly because of microbial use of electron donors near the working electrode. The subsequent decrease in current densities could be due to the slower mass transfer to the working electrode. Lu et al. (2014) reported the same outcome. If diffusion in petroleum hydrocarbon-contaminated soil was unable to transfer hydrocarbons quickly enough, a drop in pollutant concentration on the anode would result in a reduction in the current generation. This study's findings also demonstrated that the application of a biochar dose affects its efficiency in bioelectrochemical systems. However, the topic needs further research and investigation concerning the optimization of the dose of biochar to advance this approach for soil remediation.

3.2. Effect of biochar on the degradation of petroleum hydrocarbon

PHs in soil samples collected from four MET treatments were monitored after incubating for 20 days. The degradation efficiency for biochar amendment in four treatments of control, 4BC, 8BC, and 12 BC, was different for petroleum hydrocarbons (as shown in Fig. 6.3). Treatment control reduced the PHs content after the 20-day MET treatment, with a removal efficiency of 18.5%. However, amendment of the soil with biochar application rates of 4 % (w/w) and 8% (w/w) enhanced the removal efficiency of petroleum hydrocarbons by 56.2 and 87.8%, respectively. The same result was also reported by Kong et al. (2018) in their research by using biochar at an application rate of 5 % (w/w) made from sawdust, and wheat straw had a positive effect on the remediation of soil contaminated with polycyclic aromatic hydrocarbons (PAHs). In comparison to the control treatment (without biochar), they found that adding biochar increased the removal effectiveness by about 55.7%. Additionally, it was discovered that reactors supplemented with biochar prepared from vegetable waste are at least twice as effective at degrading soil polluted with diesel oil compared to the control treatment without supplementation of biochar fuel as non-amended soils (Aziz et al., 2020). Another study Wang et al. (2017), showed that the rate of petroleum hydrocarbon degradation increased by 46.9% adding biochar prepared from bulrush straw biochar compared to the control. However, the researchers claimed that raw bulrush straw was substantially less effective than biochar at removing TPH. This might be because biochar has improved soil properties such as nutrient status, and water-holding capacity and its high surface area, and biochar can support microbial growth more effectively than the precursor material.

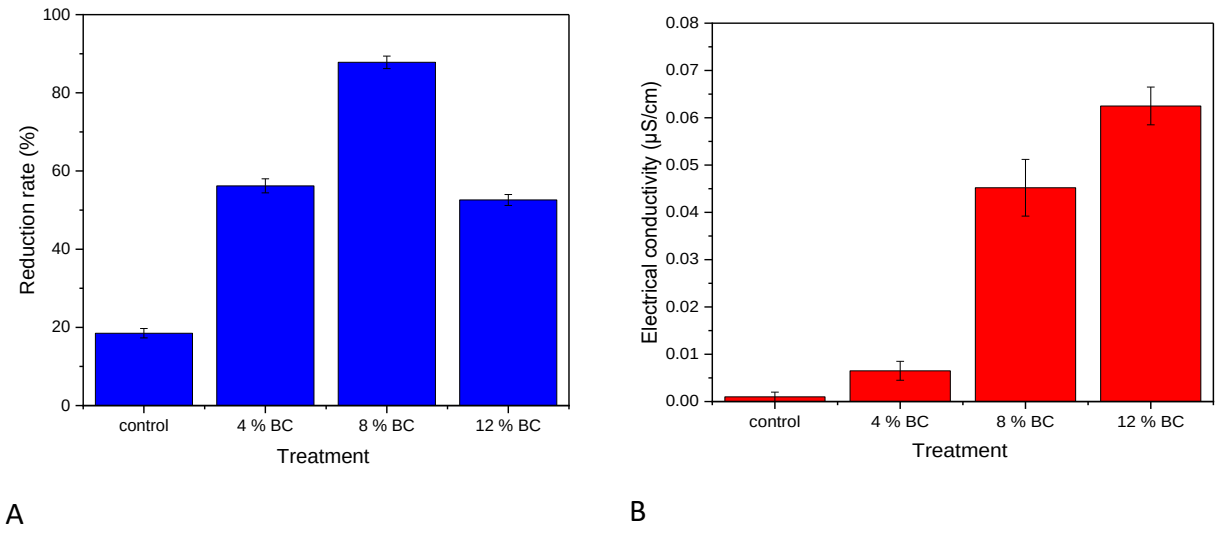

Figure 6-3. Degradation rate (A), the electrical conductivity of soil and soil-biochar mixtures (B), and the addition of various biochar doses are all shown in MET. Data are averages of replicates and standard deviations are displayed in error bars.

This research finding also indicated that increasing the dose of the biochar to 12 %(w/w) leads to a decrease in its degradation, even though raising the biochar content could lead to an improvement in soil EC. The ECs of soil with varying doses of BC600 (0 %, 4 %, 8 %t, and 12 %) were 0.03, 0.0065, 0.0452, and 0.0625 µS/cm respectively, as shown in Fig. 3b**;** this outcome is also consistent with Li et al. (2016), reported that adding of biochar to soil leads to increase in soil cation exchange capacity (CEC). However, Song et al. (2017) reported that EC in the biochar treatment was slightly higher than in non-amended soil after 24 weeks (15.5–16.1 cmol/kg) and these leads decreased their bioavailability and toxicity rather than enchaining the removal of PAHs. Due to biochar-nutrient or biochar-only enriched soil has also been found to have a significantly greater capacity to hold water than soil that has not been amended with either biochar or nutrients (Wang et al., 2018). As a result of applying biochar to the soil, water holding capacity increased because soil porosity increased, bulk density decreased, and evapotranspiration increased (Gul et al., 2015). Other factors that affected the degradation of petroleum hydrocarbons were bulk density and porosity. Wang et al. (2018) in their research to degrade soil polluted with petroleum-hydrocarbons using biochar and nutrients as stimulants showed that soil that had been treated with nutrients and biochar had bulk density and porosity higher and lower than the raw soil, respectively. Another study by Han et al. (2016) also shows that biochar pores have a diameter of less than 1 µm, which is not suitable for bacterial growth. Hence, more research and investigation are needed on the correlation of biochar properties such as

water holding capacity, soil porosity, bulk density, and on the degradation of PH and microbial population.

3.3. Role of biochar on the extracellular electron transfer analysis in the MET

A common electrochemical tool for the characterization of electron transfer between microorganisms or biofilms and electrodes is cyclic voltammetry (CV), which uses direct redox signal and electrochemical reaction detection. On day 20, CVs were carried out at various doses of biochar to look into the development of the biofilm and assess the electrochemical activity of the electrode materials (Quejigo et al., 2018). The electrochemical behavior of biochar amended with (4, 8, and 12% w/w) in response to the degradation of soil contaminated with petroleum hydrocarbons was determined using the CV, as shown in Fig. 6.4. By creating hydrogen or by causing the unfolding/oxidation of adsorbed proteins in oxidizing condition, the application of particular potentials might disturb biofilms. However, the analyses were only performed at a scan rate of 10 mV/s from 0.6 V to -0.6 V vs. Ag/AgCl (De Nicolás et al., 2022).

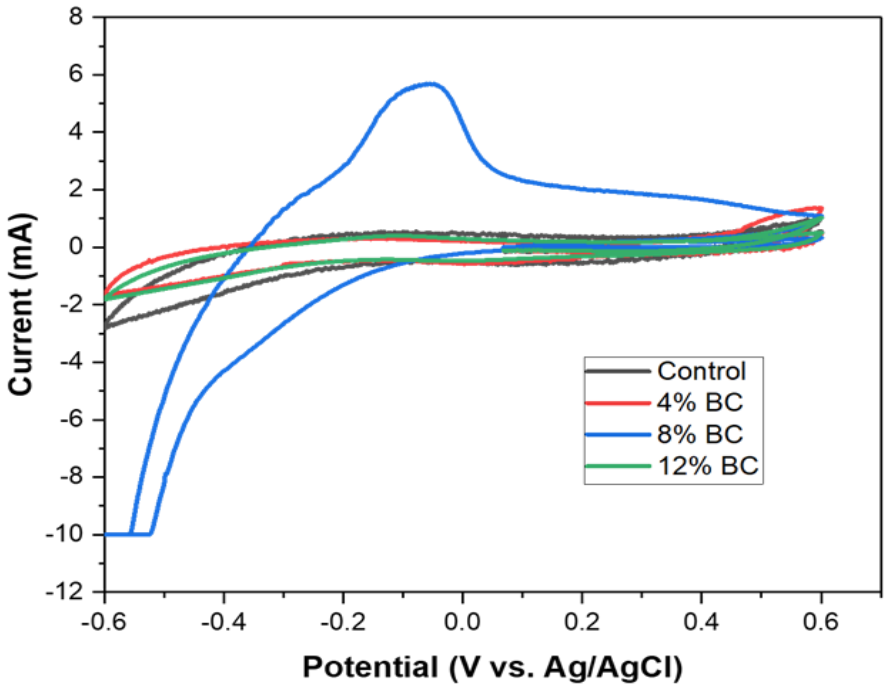

Figure 6-4. The cyclic voltammetry curve for soil with petroleum hydrocarbon in MET only and soil amendment with biochar (4,8,12 %) at day 20 at a sweep rate of 10 mVs-1

The CV analysis of MET for the control, 4BC, and 12 BC did not reveal any oxidation/reduction peaks within this dose of biochar, suggesting that the biochar within this dose is either poorly

conductive or was initially colonized by a mediator-based electro-active community. Unfortunately, soluble redox mediators cannot be detected when a single granule is analyzed in an independent electrochemical cell, and this result is consistent with previous research (De Nicolás et al., 2022).

The oxidation-reduction peaks and current output density were, however, dramatically boosted by the addition of 8% biochar to the analytic chamber of the BES. This might be explained by the biochar harboring quinone groups which can increase extracellular electron transfer by *Geobacter*, and other electroactive populations and this result are consistent with published data (Cai et al., 2020). Furthermore, long-distance electron transfer between microbes and pentachlorophenol is known to occur in biochar harboring quinone groups, and the oxidation and reduction peaks in the biochar are signs that redox-active species are present (Cai et al., 2020).

Considering this, CV data showed that the efficiency of biochar on BES depends on the type of feedstock, the dose of biochar, and the temperature at which it was produced during the pyrolysis process. It also showed that the extracellular redox mediator-mediated electron transfer pathway was involved in the transfer of electrons.

3.4. Impact of biochar dose on microbial community in soil remediation using BES system

Recent studies showed that soil physicochemical properties improve when biochar is added, and this enhancement in the properties of the soil is due to the ability of biochar to stimulate microbial activity in soils and leads to increased biodegradation efficiency (Gul et al., 2015, Kong et al., 2018, Aziz et al., 2020).

In this work, during the breakdown of PHs incubated in MET procedures, the microbial community structures in soils supplemented with biochar were examined to see if biochar encouraged significant population changes. In theory, the sustained electrons released by the electro-active biofilm on the anode using EET cause the MET to generate electricity. Therefore, investigating the impact of different biochar concentrations on the sequencing of the microbial community, particularly on the enrichment of usually electro-active bacteria, was essential to uncovering the underlying cause of the remarkable performance of biochar-assisted MET.

As shown in Fig. 6.5, *Geobacter* could pass electrons either directly through nanowire structures or indirectly via mediators, where its abundance was enriched to about 6.8% in MET + 4% BC compared to the treatment without biochar (1.5%). This is in agreement with Zhao et al. (2022), who demonstrated that Geobacter was capable of passing electrons either indirectly via mediators or through nanowire structures. Furthermore, another recent study claimed that in methanogenic consortia, the presence of biochar had enhanced the relative abundance of both *Geobacter* and pertinent *syntrophic bacteria* (Wang et al., 2022).

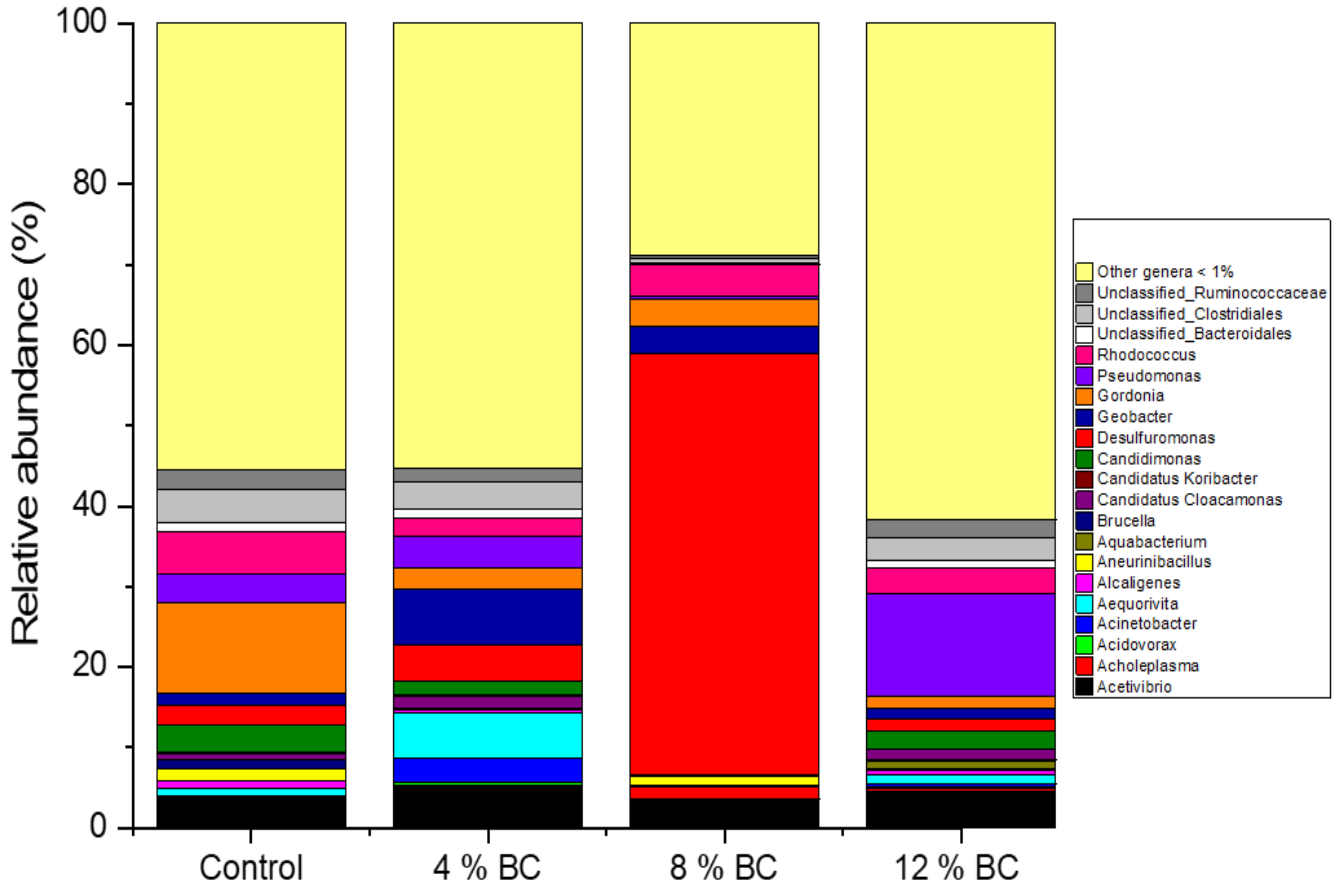

Figure 6-5. Relative abundance and community composition of bacteria in the anode surface of MET within various concentrations of biochar treatments

Interestingly, after adding 8% BC to MET, another type of electro-active bacterium, *Desulfuromonas*, rose from 2.50% to 52.32%. Recently, it was shown that the electro-active *Desulfuromonas* was capable of bidirectional electron transport using electrodes (Katuri et al., 2022). The expansion of this genus suggested that the biochar's favorable development conditions for electro-active bacteria, which can both accept and donate electrons from other redox species, such as electrodes and other electro-active bacteria, were present. With the addition of biochar, such an improvement helped electro-active bacteria produce better EET (Katuri et al., 2022).

Desulfuromonas was also the most prevalent in the treatment of an addition of 8% BC in MET, with a relative abundance of 52.32% compared to *Geobacter's* mere 3.47%, indicating that the two competed in the early stages of biofilm formation. However, *Desulfuromonas* became predominately enriched as the biofilm matured, which consistently suggests that it generates more electricity than *Geobacter* due to the reduction of CO_2 after complete degradation (Holmes et al., 2014). Contrarily,

the relative abundances of *Desulfuromon* and *Geobacter* in the MET + 12% BC treatment are 1.5 and 1.4%, respectively. This finding suggests that increasing the dose of biochar causes a decrease in *Geobacter* abundance because the electrons from the biochar do not flow to the organism for CO_2 reduction. Instead, the electrode or other electro-active microorganisms would absorb these electrons. *Geobacter* became the dominant family of anode biofilms in all treatments, even though biochar stimulated them, substantially supporting the outstanding electricity generation in this treatment (Logan et al., 2019).

The bacteria belonging to genera that are known for their biodegradative capabilities were also measured for the soil at various doses of biochar (4, 8, and 12% BC) to further study the role of biochar in the MET degradation of soil contaminated with petroleum hydrocarbons. The results showed that the relative abundances of both anaerobic electrogenic bacteria and aerobic ones such as *Gordonia*, *Rhodococcus*, and *Acinetobacter* increased by 8% BC, ranging from 3.43 to 16.39, 2.6 to 7.88, and 3.06 to 5.28%, respectively (Fig. 6.6). Additionally, in comparison to the control treatment, the addition of biochar increased the abundance of *Gordonia*, and the improvement was dose-dependent. This result is consistent with earlier studies and showed that bacteria strains of the genera *Rhodococcus* and *Gordonia* are identified as those that degrade hydrocarbons (Kubota et al., 2008; Koma et al., 2007; Lv et al., 2022).

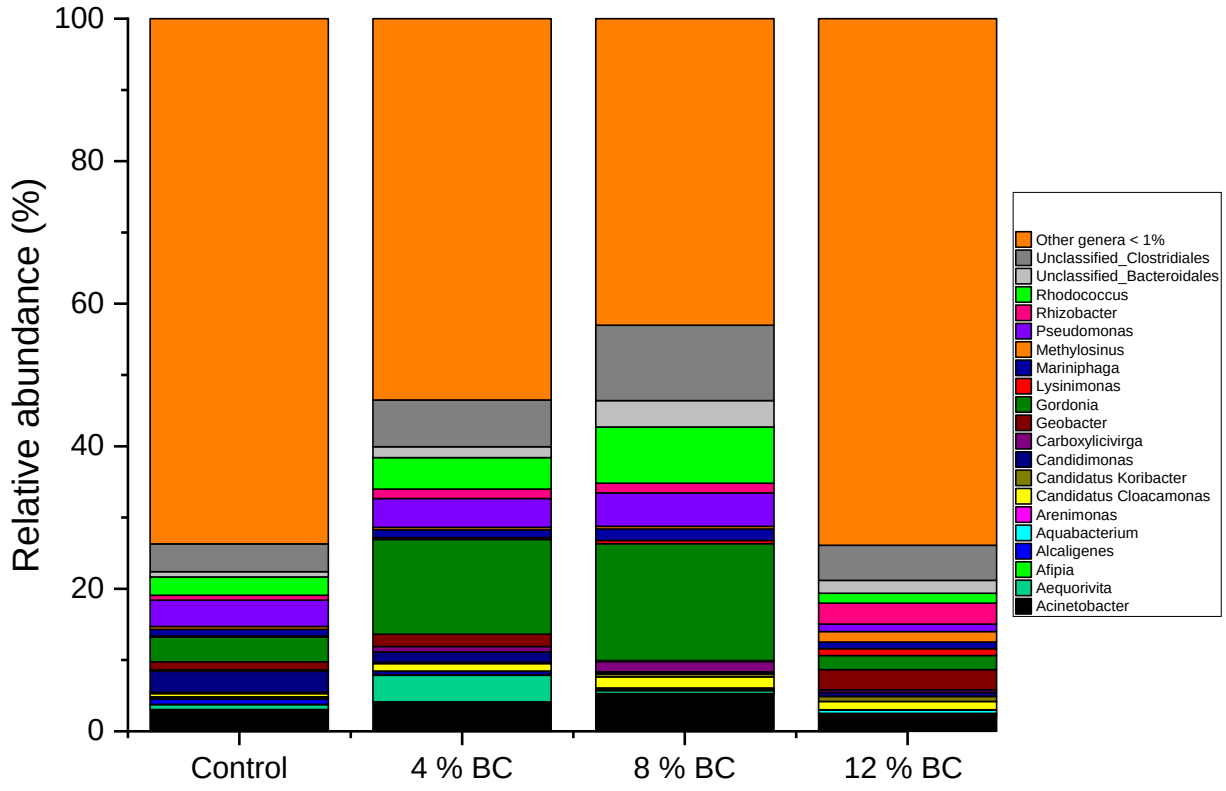

Figure 6-6. Relative abundance and community composition of bacteria in the soil of MET within various concentrations of biochar treatments.

In line with the spatial distribution patterns of PHs degradation, this finding showed a general tendency for electrically active bacteria to decline with increasing doses of biochar, and adding biochar into an anode chamber could stimulate electricity generation, which also addressed the significance of redox-active EEC rather than EC of biochar to stimulate a bioelectrochemical reaction. Hence, to fully understand the crucial function that biochar plays in EET improvement, additional studies and investigations into the dose of biochar, pyrolysis temperature, and feedstock type, as well as more comprehensive data at lower species levels, are required.

4. Economic and environmental benefits of using biochar as a catalyst in MET

Biochar can also be a low-cost catalyst in microbial electrochemical technologies (MET). It is a new technology that can remove organic contaminants from soil and generate electricity simultaneously. (Huggins et al., 2016). Protons and electrons are produced by exoelectrogenic bacteria that oxidize fuel on the anode of MET. Soil remediation is currently a promising application of MET, in which electrons travel through an external circuit to reach a cathode and generate a current. However, its widespread use has been restricted due to high system-wide costs associated with the MET (Chakraborty et al., 2021). Table 6.3 compares our study's findings on power density and prices with other biochar studies. Our study's power density was comparable to and even slightly higher than other carbonaceous studies, demonstrating that biochar made from rice straw biochar has efficient electrochemical performance. Table 6.4 shows that the biochar in our study has a lower price (0.6 $ g^{-1}) than other types of charcoal. Although biochar catalysts are relatively inexpensive compared to other MET catalyst options, their low cost provides a benchmark for large-scale biochar-based catalyst applications. A wide range of uses for rice straw is possible, and this material has much room to grow.

Table 6-3. Performance comparison of the biochar of this research with other carbonaceous materials from past investigations

Amendment used	**#Maxium power density (W m$^{-2)}$**	**Catalyst cost ($ g^{-1})**	**References**
CuZn	0.0751	0.09	Das et al. (2020)
Water hyacinth biochar	0.0123	Not reported	Wang et al. (2015)
Graphitic biochar	0.1467	0.0004	Huggins et al. (2015)
Vulcan carbon	0.1566	1.0	Huggins et al. (2015)
AC fiber felt	0.315	0.04	Deng et al. (2010)
KOH treat**s** activated carbon nano-fiber	0.061	9.63	Ghasemi et al. (2011)
Activated carbon	0.31	Not reported	Zhang et al. (2014a)
Rice straw biochar	**1.75**	**0.31**	**Present work**

A recent meta-analysis of biochar's life cycle environmental and economic performance compared to activated carbon shows that the cost of using biochar as an adsorbent is lower or equal for organic pollutants compared to activated carbon (Alhashimi and Aktas, 2017). However, the varying capacities and efficiencies of biochar materials are not adequately reflected by average prices. With prices for biochar ranging from $200 to 1800 $/t and activated carbon from $1100 to $5,000 /t, this is evident (Jayakumar et al., 2021; Thompson et al., 2016; Alhashimi and Aktas, 2017). For environmental and economic reasons, biochar will perform better if its properties are tailored to the intended use. To design such a long-lasting soil remediation system, it is critical to thoroughly examine the contaminant properties, contaminant removal mechanisms, and conditions of the remediation system. Generally, economic comparisons between biochar and other carbonaceous materials are lacking in the literature because of a lack of standard production conditions and an understanding of contaminant removal mechanisms and need further research and investigation.

Table 6-4. Cost analysis for biochar production

Sl. No.	Component	Unit	Rate US (dollar)	Qty.	Amount US (dollar)
1	Organic raw material (rice straw)	g	0.05	1	0.05
2	Biochar production from rice straw				
2a	The power consumption in muffle furnace operation is 2 hours (power rating 3 kW). Assuming only 5% of the energy used for pyrolysis = power rating (3 kW) × hours operated (2 h) × unit cost of electricity (0.24/kWh) × fraction of power utilized (20%)	kWh	0.24	0.3	0.07
2b	Nitrogen gas is used. Assuming only 0.3 L /hour % is used for pyrolysis. The cost of 99.9% Nitrogen gas is 0.5 euro/1L. Hence, the cost of nitrogen gas is used = hours operated× cost per liter (1 L) × qty. used per hour (0.3 L)	liter	0.05	0.3	0.015
2c	Post-treatment with deionized water (procured from the lab)	litre	0.01	2	0.02
2d	Power consumption in the oven drying the treated biochar in a 1.0 kW rated hot air oven. Assuming only 5% of the energy used in drying. Hence cost of electrical energy = power rating (1 kW) × hours operated (2 h) × unit cost of electricity (0.24/kWh) × fraction of power utilized (5%)	kWh	0.24	0.1	0.02
	Cost of 1 g biochar production				**0.31**
	Cost per 8 grams of biochar				**2.5**

Considering the environmental impact of biochar as a catalyst material is just as important as evaluating its economic performance in comparison to other carbonaceous materials. Compared to activated carbon (AC), biochar required less energy and had a lower Global Warming Potential (GWP) for the removal of organic pollutants from soil (Alhashimi and Aktas, 2017). The activation process is energy-intensive, and AC yields are low, especially when made from renewable feedstock, so the average energy demand for biochar production was significantly lower than that of AC

production, at 6.1 MJ/kg than AC, at 97 MJ/kg. As a result, the average global warming potential (GWP) of biochar production was negative, at 0.9 kg CO_2-eq/kg, while the GWP of AC was 6.6 kg CO_2-eq/kg (Boateng et al., 2015; Stavropoulos and Zabaniotou, 2009; Wang et al., 2020).

Much more research is needed to improve the production process and evaluate the benefits of using biochar as a MET in soil remediation over its entire life cycle, despite its cost and life cycle advantages, considering that feedstock materials have no economic value and that enhances energy production. Biochar properties vary widely, so pyrolysis parameters must be adjusted to produce chars with high surface area and conductivity for MFCs and to have surface functional groups if biochar is to be used as an amendment material. Higher surface area and electrical conductivity correlate with higher carbonization temperatures (Downie et al., 2009). Increased lignin volatilization and the transformation from low-density disordered carbon to high-density aromatic crystalline carbon structures are to blame for this (Keiluweit et al., 2010). In terms of environmental stability, it is clear that biochar's aromatic structure makes it exceptionally environmentally stable and has been suggested to remain chemically unchanged over millennia (Nguyen et al., 2010). Moreover, the majority of current research is done on a laboratory scale. This significantly impacts the final properties of biochar (Keiluweit et al., 2010). More exhaustive research is needed to understand how biochar behaves in practical conditions such as field soil remediation. Biochar with different physical and chemical properties have been tested to see if they can remove pollutants from soil and water. Regarding the target pollutants, there is no conclusive evidence that optimal biochar exists. It takes great effort to ensure biochar's precise use and accuracy in terms of preparation conditions, biochar type, application rate, recovery procedures, and application time.

5. Importance of the research

Understanding how biochar affects microbial electrochemical behavior is critical to developing biochar-assisted microelectrochemical platforms that can be used in various applications (Ambaye et al., 2021). When biochar was added to MET, it was found that hydrocarbon degradation and electricity generation increased, and the biofilm dominated by electro-active microbes of *Desulfuromonas* and *Geobacter* on the anode was detected. Among the factors that made this study significant were the following. Because of the syntrophic metabolism characteristics, few studies have observed the enhanced extracellular electron transfer behavior released by PH-degrading bacteria when biochar is used for soil remediation. This study used MET to demonstrate that biochar can enhance the degradation of PHs via extracellular electron transfer, which provides the foundation for understanding the mechanisms of biochar regulating PHs degradation.

Furthermore, even though MET was considered a promising technology for generating electricity from PHs via electro-active microbe enrichment, the lengthy start-up period and high cost of high-efficiency electrode fabrication are two significant obstacles to its commercialization (Wang et al., 2022; Yaqoob et al., 2020). However, modifying anodes with high-conductivity nanomaterials such as polyaniline, poly-N-isopropyl acrylamide, graphene, and nanotube could boost electricity generation, but their current costs make this an impractical option for real-world use (Patwardhan et al., 2021). Furthermore, the results of this study suggested that biochar derived from agricultural waste with low electrical conductivity, but a high redox-based electron exchange capacity could also be used as an anode fabrication anode (Patwardhan et al., 2021). Anthraquinone-2-sulfonate and biochar were found to increase power density generation from azo dye biotransformation by 15% in a recent study, providing an alternative strategy to improve extracellular electron transfer efficiency in MET (Wang et al., 2021). Biochar's ability to promote bioremediation processes by sparking extracellular electron transfer between syntrophic microbes is worthy of further study. This will help us understand how biochar regulates syntrophic microbes' electron transfer mechanisms.

6. Conclusions

Biochar from rice straw showed high redox ability and soil conductivity, enhancing extracellular electron transfer in the soil matrix. This study critically evaluated rice-based biochar as an additive in MET for degrading petroleum hydrocarbons from polluted soil. Adding biochar to the MET's anode resulted in highly efficient electricity generation and PH degradation. *Desulfuromonas* and *Geobacter* were significantly enriched in anode biofilms after biochar addition. However, adding 12 % (w/w) biochar decreased the degradation rate of PHC and reduced the abundance of bacteria in the anodic surface and slurry. The variability of bacterial communities reported in this study demonstrates the need to understand biological interactions in this emerging technology. As an amendment to MET, Biochar provides economic and environmental benefits in terms of reduced energy demand and global warming potential (GWP). However, further research is needed to use this feedstock as a raw material for manufacturing biochar electrodes to increase their performance.

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CHAPTER 7.

LIFE CYCLE ASSESSMENT OF BIOSLURRY AND BIOELECTROCHEMICAL PROCESS FOR SUSTAINABLE REMEDIATION OF SOIL POLLUTED WITH PETROLEUM HYDROCARBON: AN EXPERIMENTAL STUDY

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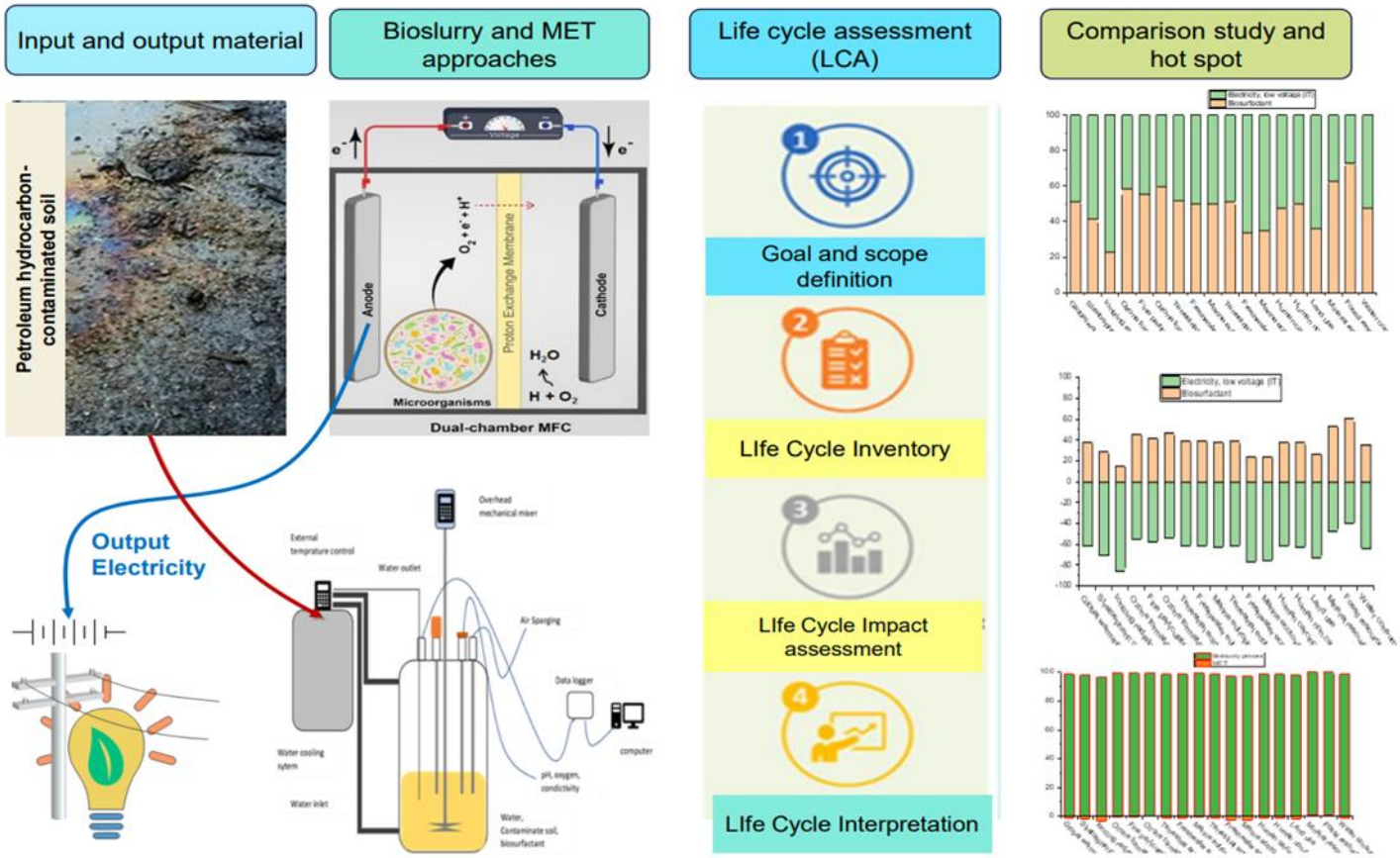
Abstract

Bioremediation technologies have been widely used to clean up sites contaminated with various pollutants. Although these biological-based technologies may present a more sustainable appeal, the impacts they can pose on natural resources or climate change are rarely assessed. Hence in this study, the environmental performances of bioslurry and microbial electrochemical treatment (MET) are compared using a life cycle assessment method of SimaPro 9.1 software, and the primary environmental hotspots of the system and fifteen life cycle impact assessment options are evaluated using the ReCiPe 2016 characterization approach. The findings indicate that, compared to the bioslurry treatment, the MET approach is the most environmentally friendly choice since their value-added byproduct, the production of energy, offsets the environmental loads from the consequences that would have been averted. For up to 100% of the impact category on global warming, environmental loads in both MET and bioslurry processes are dominated by biosurfactant production and electricity use. The system's environmental loads outweigh the impacts of the process by a factor of 1.6 for the global warming potential (GWP) due to the significant environmental gain from MET's energy output. Under every environmental impact category, the bioslurry process results in 100 times more environmental loads than MET. This study indicated that MET is an appropriate and feasible environmental remediation technology to support decision-making on the most suitable and least impactful strategy for cleaning contaminated sites. It also provides scientific references on the remediation of soil contaminated with petroleum hydrocarbons.

Keywords: Bioenergy, bioslurry process, biosurfactant, Environmental impact, Microbial electrochemical treatment, Remediation process, petroleum hydrocarbon

Graphical abstract



1. Introduction

Since the expansion of polluted areas in recent decades, the worldwide concern over soil contaminated by petroleum hydrocarbons has grown (Ambaye et al., 2022). As the world's need for oil has increased rapidly in recent years, vast quantities of petroleum hydrocarbons have been released into the environment through various pathways, including light hydrocarbon volatilization, accidental crude oil leakage, incomplete combustion, and oil-bearing wastewater (Ossai et al., 2020; He et al., 2022). In addition, pollution contains toxic components that change the soil microbiome and reduce plant growth (Haider et al., 2021). Therefore, to protect human health, food safety, and the ecological balance of the environment, it is essential to reduce the pollution caused by petroleum hydrocarbons (Behera et al., 2022).

Biological technology is more cost-effective, environmentally friendly, and efficient than conventional physical and chemical remediation methods, but when the concentration of the pollutant is above the limit, biological methods are less effective and take longer to restore (Qadri et al., 2020; Patel et al., 2020). On the other hand, bioelectrochemical systems, one of the new emerging bioremediation technologies, have a high advantage in their ability to save more energy, as well as their safety, ease of use, low operating costs, cleanliness, environmental friendliness, and long-term viability properties (Abourached et al., 2014; Ambaye et al., 2022).

Microbial electrochemical technologies (MET) are a bioelectrochemical system that uses microbial catalytic (metabolic) activity to convert the chemical energy of organic compounds, such as those found in soil polluted with petroleum hydrocarbons, into electrical energy. The double-chambered MET is a type of MET with an anode and cathode separated by a proton exchange membrane. Electrons and protons are produced in the anode chamber when microorganisms metabolize chemicals (the substrate) through oxidation reactions. The ion-selective permeable membrane (in this case, the soil itself) allows the protons generated by microbial oxidation to pass through into the cathode chamber. Meanwhile, the microorganisms' released electrons are gathered by the anode and flow through an external circuit into the cathode, where they are absorbed and reduced by chemical species with high positive redox potentials, such as oxygen, ferricyanide, and other oxidizing chemical agents (Ambaye et al., 2022; Mathuriya and Sharma, 2009). Hence, the MET's core functionality includes the degradation of soil polluted with petroleum hydrocarbons and the simultaneous production of electricity from organic sources (Pant et al., 2016). However, as a relatively new field of study, MET has yet to overcome several obstacles, including ohmic losses,

non-bioavailability of petroleum hydrocarbons, difficulties in scaling up production, and an incomplete understanding of the microbiological transfer of electrons (Ambaye et al., 2022b).

It is reported that biosurfactants can be applied to MET as amendments to enhance electricity generation and degradation of the hydrocarbons contaminating the soil petroleum by increasing the contaminant's bioavailability to microorganisms and reducing surface tension, mineralization, and solubilization (Mulligan et al., 2001; Yeung and Gu, 2011). However, activities and substances such as the amended biosurfactant, the chemicals used in the mineral salt medium in the anode, the phosphate-buffered saline in the cathode, the use of external power for mixing of anolyte in the anode chamber, the aeration of the cathode chamber, and the byproduct of the anaerobic MET systems may cause other environmental impacts. Thus, methods to be used on a pilot scale for evaluating their environmental impact and long-term sustainability are highly needed. Another technology used for soil degradation is the bioslurry reactor, one of the most important in-situ and ex-situ techniques. Slurry bioreactors have become one of the best options for the bioremediation of soils polluted by recalcitrant pollutants under controlled environmental conditions (Ambaye et al., 2022). However, large amounts of soil must be excavated to use biological retractors on a large scale. In addition, reactor mixers consume energy for construction, operation, and dewatering the process after bioremediation, which has a high impact on the environment.
Moreover, a recent study shows that some investigation or experimentation may be required to achieve optimal operating conditions in a full-scale operation that typically increases treatment costs compared to other bioremediation techniques (Ambaye et al., 2022). In a bioslurry reactor, it was also revealed that adding stimulants such as fertilizers, activated sludge, surfactants, and biosurfactants enhanced the decomposition of soil contaminated with petroleum hydrocarbons. However, these external stimulant techniques involve energy and material exchanges with the environment (Ambaye et al., 2022). Although these intensification strategies enhanced the degradation of petroleum hydrocarbons, the effects on the environment were not investigated. Therefore, it is crucial to evaluate the environmental implications of these intensification techniques before they can be proposed for industrial-scale application.

Life cycle assessment (LCA) is a methodology used to quantify the potential environmental burdens associated with a product, process, or activity by identifying and quantifying the energy and materials used and the emissions released into the environment. It is a powerful method to identify and evaluate opportunities for environmental improvement (Martins et al., 2017). For this reason, LCA seems to be a well-suited approach for assessing the effects of the bioremediation process. LCA can be

consequential or attributional, especially in soil remediation. Attributive LCA evaluates the fundamental residual contamination or the secondary effects of the method life cycle. Consequential LCA is focused on secondary impacts and considers environmental and economic effects following remediation (Visentin et al., 2019).

LCA has been used to investigate the potential impacts of treatments of soil contamination with trichloroethene, mineral oils, chromium, and polycyclic aromatic hydrocarbons. These studies demonstrate that LCA is a valuable management tool for assessing the environmental effects of various contaminants' soil remediation approaches (Song et al., 2018). However, no LCA studies have been conducted to date on the environmental impacts of petroleum hydrocarbon remediation via MET and bioslurry reactors. Hence, the main objective of the study is to evaluate the environmental impact under various conditions involved in the bioremediation of soil polluted with petroleum hydrocarbons using experimental data as input in the LCA.

2. Material and method

This study uses the LCA methodology since it is one of the most commonly used methods for making decisions when evaluating and reporting the environmental performance of the bioremediation process (Chin et al., 2022). To evaluate potential environmental effects, the research should go through the following four stages: ISO 14040: goal and scope definition, life cycle inventory, life cycle impact assessment, and life cycle interpretation (International Organization for Standardization, 2006). SimaPro 9.1 was used to build the LCA model and impact analysis. In addition, the ReCiPe 2016 v1.1 midpoint, the Hierarchist version, was selected as the approach.

2.1. Goal and scope definition

This study compares the environmental performances of bioslurry and microbial electrochemical treatment (MET) for the remediation of soil contaminated with petroleum hydrocarbons. The goal is to find the primary environmental hotspots, and fifteen life cycle impact assessment options as well as electricity generation from treating petroleum hydrocarbon-contaminated soil are considered as tools for evaluation.

Due to the lack of LCA studies on this bioremediation method and the rising prominence of MET and bioslurry technologies in environmental management research (Ambaye et al., 2022), it was decided to focus the assessment on bioslurry and MET. It is now essential to find out if the developed process can be used on a large scale while having little effect on the environment.

The University of Brescia, the University of Milano Bicocca, and the Italian Agency for Development Cooperation commissioned the study. Researchers, practitioners, and policymakers from related

industries are the target audience. Table 7.1 below explains the specific process descriptions for each process.

Table 7-1. Description of the selected BES and bioslurry treatment options

Treatment option (Reference)	**Description**
MET (H-type) (Ambaye et al., 2022)	A double chamber BES microbial electrochemical technology (MET) was used based on previous studies (Ambaye et al., 2022). Moreover, two similar Plexi-glass bottles were detached by a cation exchange membrane (CEM) with an inner diameter of 7 cm, having 250 mL volume with a working volume space of 200 mL was used. Two rubber gaskets with a metal clip holder for glass bottles were used to keep the bioreactor air-tight. The anode (graphite) and cathode (titanium) electrodes (6 cm length, 5 cm width) were utilized. These electrodes were fastened with titanium electric wires wrapped with a heat-shrink tube. Ag/AgCl assay was employed as a reference electrode. Anolyte media was poured into the anode chamber. In the cathode chamber, sterile phosphate buffer (0.5 M PBS) solution having (i) $Na_2HPO_4 \cdot 12H_2O$, 7.56 g L^{-1}; (ii) KH_2PO_4, 1.5 g L^{-1}; (iii) 0.31 g L^{-1} NH_4Cl; (iv) 0.13 g L^{-1} KCl was added, and pH 7.0 was maintained as described by Rabaey et al. (2005). The MET reactor was sterilized by autoclave for 30 minutes at maintaining a temperature of 121°C. Then a sterile anolyte was aseptically added to the anode chamber. All the experiments of this study were carried out in a semi-batch mode. Before the MET process began, the anode chamber was purged with 100% nitrogen for 15 minutes to maintain anaerobic conditions. All experiments connected electrodes to a single resistor (R = 1000 Ω), and the MET system, which contains the PAHs polluted soil sample, was run in a long-term fed-batch mode at 20 °C for 20 days. (i.e., four cycles) in one set of experiments. The effect of the biosurfactant dose on the degradation of petroleum hydrocarbon in the MET system anode was investigated. Three different biosurfactant concentrations in the anolyte feed solutions were used and selected based on previous studies at 10, 50, and 100 mg/L (Ambaye et al., 2022). In addition, a negative control was included with the same conditions as biotic systems but without biosurfactants in the medium. A maximum reduction of petroleum hydrocarbons was found to be up to 72.5%, while the current density reached 9.5 Am^{-2} for the MET reactor having a dose of 100 mg/L within 20 days of the operational period.
Bioslurry reactor (Ambaye et al., 2022a)	Degradation studies were conducted in mesocosm consisting of bioslurry reactors using 100 g of soil polluted with diesel oil suspended in 500 mL of distilled water with a 1:5 soil-to-water ratio. They stimulated with 1.5 g NH_4Cl and 0.1 g KH2PO_4+ 5 g /L of activated sludge + supplemented with crude novel rhamnolipids (275 mL). 1L Erlenmeyer /conical flasks were used as bioreactors with constant mixing by mechanical mixers at 150 rpm to ensure substrate availability and oxygen transfer for about 20 days. The analyses revealed that the combination of activated sludge, nutrients, and biosurfactant showed a 79.4% reduction in TPHs after 20 days in the mesocosm experiment.

The functional unit for this study is related to treated soil polluted with petroleum hydrocarbons using MET (100 g, 0.5 KWh) and bioslurry (100 g, 36.5 KWh). The initial concentration of total petroleum hydrocarbons is 4500 mg/kg. Generally, the functional unit (FU) for soil remediation is not standardized. However, since these are also functional units that are frequently used to enable comparison with relevant studies and future work, the correlation factors used to calculate the impacts of weight (in kg) and energy capacity (in kWh) are also provided (Chin et al., 2022).

Fig. 1 depicts the system boundary of this investigation, which encompasses the operating stage, For both the bioslurry and MET processes, input data like energy resources (i.e., electricity) and output data like sludge waste. Electricity generation is also taken into account throughout the MET process. Since the soil has been recovered and the end-of-life stage of the treatment alternatives has no more influence than the operating stage, it is not included (Yao et al., 2021). Within the system boundaries, the MET's power production is an environmentally advantageous operation since it is anticipated to help prevent impacts. The production of biosurfactants is also included in both treatments because it is a significant input to enhance the degradation of petroleum hydrocarbon and the production of these stimulants have a considerable impact on environmental performance.

Given the lab-scale systems' short time frame and low maintenance requirements, it is anticipated that this study does not involve maintenance tasks. Large-scale maintenance necessitates higher use of chemicals, materials, and electricity. Inoculum cultivation is excluded from the assessment because it includes farms and is outside the purview of the investigation.

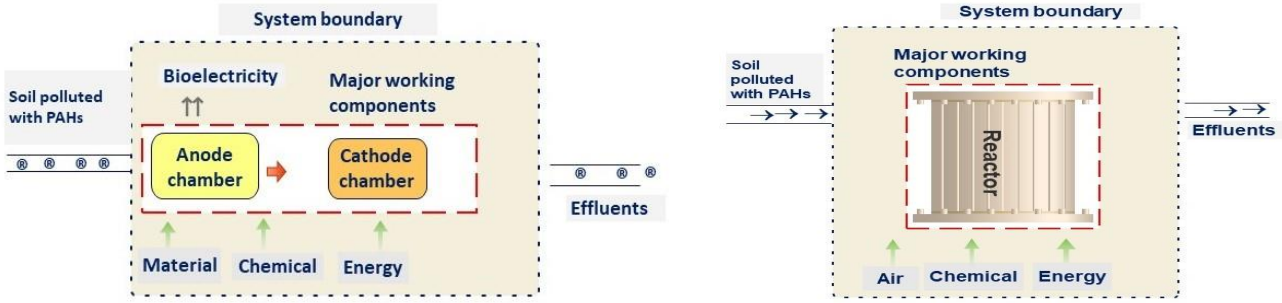


Figure 7-1. System boundary and characteristics of scenarios for MET (A) and bioslurry system (B)

2.2. Life cycle inventory

The inventory of the degradation process was based on primary data collected in laboratory experiments. This includes experiments for biosurfactant production (Ambaye et al., 2022a). The database, Ecoinvent v3.6 (allocation, cut-off by classification), which has background information on materials and energy sources, is connected to the inventory (Terlouw et al., 2021). The complete list of inputs and outputs adopted here is displayed in Table 7. 2, 7.3, and 7. 4.

Table 7-2. Life cycle inventory of the degradation of soil polluted with petroleum hydrocarbon process in the study via MET.

Input/output SimaPro		1kg polluted soil	Data procurement method	Source - LCA process
Inputs from Technosphere		***g or kWh***		
Materials/fuels	Magnesium sulfate {GLO}| market for | Cut-off, S	0.25 ± 0.01	Measured	Ecoinvent
	Sodium phosphate {RER}| market for sodium phosphate | Cut-off, S	3.48 ± 0.01	Measured	Ecoinvent
	Trisodium phosphate {GLO}| market for trisodium phosphate | Cut-off, S	4.76 ± 0.1	Measured	Ecoinvent
	Ammonium chloride {GLO}| market for | Cut-off, S	1.0 ± 0.12	Measured	Ecoinvent
	Sodium phosphate {RER}| market for sodium phosphate | Cut-off, S	5.08 ± 0.01	Measured	Ecoinvent
	Sodium chloride, powder {GLO}| market for | Cut-off, S	0.5 ± 0.13	Measured	Ecoinvent
	Sodium phosphate {RER}| market for sodium phosphate | Cut-off, S	1.27 ± 0.14	Measured	Ecoinvent
	Trisodium phosphate {GLO}| market for trisodium phosphate | Cut-off, S	1.73 ± 0.01	Measured	Ecoinvent
	Ammonium chloride {GLO}| market for | Cut-off, S	0.31 ± 0.02	Measured	Ecoinvent
	Potassium chloride, industrial grade {GLO}| market for potassium chloride, industrial grade | Cut-off, S	10.13 ± 0.01	Measured	Ecoinvent
	KH2PO4, unspecified	1.5± 0.013	Measured	Ecoinvent
	Inputs from Technosphere (Wolfe trace mineral solution)	mg		
	Manganese sulfate {GLO}| market for | Cut-off, S	447 ± 0.01	Measured	Ecoinvent
	Iron sulfate {RER}| market for iron sulfate | Cut-off, S	54.7 ± 0.001	Measured	Ecoinvent
	Cobalt {GLO}| market for | Cut-off, S	24.8 ± 0.01	Measured	Ecoinvent
	Zinc monosulfate {RoW}| market for zinc monosulfate | Cut-off, S	56.2 ± 0.01	Measured	Ecoinvent
	Copper sulfate {GLO}| market for | Cut-off, S	6.4 ± 0.012	Measured	Ecoinvent
	Boric acid, anhydrous, powder {GLO}| market for | Cut-off, S	10 ± 0.0013	Measured	Ecoinvent
	Aluminum sulfate, powder {RER}| market for aluminum sulfate, powder | Cut-off, S	3.61 ± 0.012	Measured	Ecoinvent
	Nickel sulfate {GLO}| market for | Cut-off, S	11.9 ± 0.013	Measured	Ecoinvent
	Molybdenum trioxide {GLO}| market for | Cut-off, S	5.95 ± 0.01	Measured	Ecoinvent
	Inputs from Technosphere (biosurfactants)	mg		
	Biosurfactant	100 ± 0.1	Measured	Ecoinvent
	Inputs from Technosphere (activated sludge)	L		
	Digester sludge {GLO}| digester sludge, Recycled Content cut-off | Cut-off, S	1.0 ± 0.01	Measured	Ecoinvent
Electricity/heat	Electricity, low voltage {IT}| market for | Cut-off, S	0.1 ± 0.014	Measured	Ecoinvent
Outputs to Technosphere				Ecoinvent
Waste treatment	Wastewater, average {Europe without Switzerland} | market for wastewater, average | Cut-off, S	150 ± 0.015	Measured	Ecoinvent

Avoided products	Electricity, low voltage {IT}| market for | Cut-off, S	2.28 ± 0.05	Measured	Ecoinvent

Table 7-3. Life cycle inventory of the degradation of soil polluted with petroleum hydrocarbon process in a study through bioslurry.

Input/output SimaPro		**1 kg polluted soil**	**Data procurement method**	**Source - LCA process**
Inputs from Technosphere		***g or kWh***		
Materials/fuels	Water, deionised {Europe without Switzerland} | market for water, deionised | Cut-off, S	225 ± 0.01	Measured	Ecoinvent
	Ammonium chloride {GLO}| market for | Cut-off, S	1.5 ± 0.012	Measured	Ecoinvent
	KH2PO4, unspecified	0.1 ± 0.01	Measured	Ecoinvent
	Inputs from Technosphere (biosurfactants)	mg		
	Biosurfactant	225 ± 0.01	Measured	Ecoinvent
	Inputs from Technosphere (activated sludge)	L		
	Digester sludge {GLO}| digester sludge, Recycled Content cut-off | Cut-off, S	5.0 ± 0.012	Measured	Ecoinvent
Electricity/heat	Electricity, low voltage {IT}| market for | Cut-off, S	36 ± 0.05	Measured	Ecoinvent
Outputs to Technosphere		*Cm3*		
Waste treatment	Wastewater, average {Europe without Switzerland} | market for wastewater, average | Cut-off, S	400 ± 0.013	Measured	Ecoinvent

Table 7-4. Raw material for biosurfactant production on a laboratory scale and functional unit

Input/output SimaPro		**1 0 mg biosurfactant**	**Data procurement method**	**Source - LCA process**
Inputs from Technosphere		***g or kwh***		
Materials/fuels	Water, deionised {Europe without Switzerland} | market for water, deionised | Cut-off, S	1.0 ± 0.01	Measured	Ecoinvent
	Base oil {GLO}| market for base oil | Cut-off, S	45.0 ± 0.01	Measured	Ecoinvent
	Magnesium sulfate {GLO}| market for | Cut-off, S	0.0977 ± 0.01	Measured	Ecoinvent
	Sodium chloride, powder {GLO}| market for | Cut-off, S	0.1 ± 0.012	Measured	Ecoinvent
	Ammonium sulfate, as N {GLO}| market for | Cut-off, S	0.0212 ± 0.014	Measured	Ecoinvent
	Iron (II) chloride {GLO}| market for | Cut-off, S	0.02 ± 0.016	Measured	Ecoinvent
	Ethyl acetate {GLO}| market for | Cut-off, S	113 ± 0.01	Measured	Ecoinvent
	Methanol {GLO}| market for | Cut-off, S	261 ± 0.05	Measured	Ecoinvent
	Benzoic acid {RER}| market for benzoic acid | Cut-off, S	0.847 ± 0.001	Measured	Ecoinvent
	Sodium hydroxide, without water, in 50% solution state {GLO}| market for | Cut-off, S	0.555 ± 0.001	Measured	Ecoinvent
	KH2PO4, unspecified	0.391 ± 0.012	Measured	Ecoinvent

	Potassium hydroxide {GLO}| market for | Cut-off, S	0.161 ± 0.014	Measured	Ecoinvent
	Glucose {GLO}| market for glucose | Cut-off, S	0.5 ± 0.016	Measured	Ecoinvent
	Ethanol, without water, in 99.7% solution state, from fermentation {GLO}| market for | Cut-off, S	0.789 ± 0.018	Measured	Ecoinvent
Electricity/heat	Electricity, low voltage {IT}| market for | Cut-off, S(KJ)	75.3 ± 0.05	Measured	Ecoinvent

2.3. Life cycle impact assessment

The midpoint categories were selected since they represent direct short- to mid-term effects on the environment that are more easily quantified. This allows for a straightforward comparison between different lab-scale methods and a detailed hotspot analysis. According to the characterization variables with typical reference substances, such as kg CO2-eq for global warming, the evaluation method converts the life cycle inventory data into several impact categories (Huijbregts et al., 2017). The following impact categories were selected for this study: water consumption; fossil resource scarcity; mineral resource scarcity; global warming potential; land use; stratospheric ozone depletion; human non-carcinogenic toxicity; ionizing radiation; human carcinogenic toxicity; ozone formation, marine ecotoxicity; human health; freshwater ecotoxicity; fine particulate matter formation; terrestrial ecotoxicity; ozone formation, marine eutrophication; terrestrial ecosystems; freshwater eutrophication; terrestrial acidification; Because the assessment's inventory comprised a wide range of processes and materials, a significant number of categories were selected, which might have a wide range of potential effects.

2.4. Life cycle interpretation

In both treatment approaches (bioslurry and MET), in all midpoint categories, the environmental loads of each main compartment are assessed. To evaluate the degree of environmental impact and friendliness of the treatment solutions, the net environmental performances of the various options are also evaluated. Finally, the environmental effect experienced by all of an option's key compartments is added together to calculate this option's net environmental performance.

3. Results and discussion

The environmental impacts of the two processes performed in laboratory-scale experiments for all environmental categories are shown in Table 7.5. The result shows a clear benefit of the MET system. The last column shows that for all categories it shows 3% or fewer impacts as compared to the bioslurry system, and it needs hotspot analysis to investigate the main impact contributors

Table 7-5. Environmental impacts of the two processes performed in laboratory scale experiments. The functional unit is 100g of treated of soil.

Impact category	Unit	MET	Bioslurry	Comparison impact category between MET/ bioslurry
Global warming	kg CO_2 eq	$1.1x10^{1}$	$7.0x10^{2}$	2%
Stratospheric ozone depletion	kg CFC11 eq	$1.6x10^{-6}$	$3.8x10^{-4}$	0%
Ionizing radiation	kBq Co-60 eq	$-3.3x10^{-1}$	$3.6x10^{1}$	-1%
Ozone formation, Human health	kg NOx eq	$3.4x10^{-2}$	1.6x10	2%
Fine particulate matter formation	kg PM2.5 eq	$1.9x10^{-2}$	1.0x10	2%
Ozone formation, Terrestrial ecosystems	kg NOx eq	$3.7x10^{-2}$	1.7x10	2%
Terrestrial acidification	kg SO_2 eq	$4.3x10^{-2}$	2.7x10	2%
Freshwater eutrophication	kg P eq	$4.5x10^{-3}$	$2.3x10^{-1}$	2%
Marine eutrophication	kg N eq	$2.3x10^{-4}$	$1.4x10^{-2}$	2%
Terrestrial ecotoxicity	kg 1,4-DCB	$3.9x10^{1}$	$2.2x10^{3}$	2%
Freshwater ecotoxicity	kg 1,4-DCB	$1.8x10^{-1}$	$1.4x10^{1}$	0%
Marine ecotoxicity	kg 1,4-DCB	$2.7x10^{-1}$	$5.6x10^{1}$	0%
Human carcinogenic toxicity	kg 1,4-DCB	$4.0x10^{-1}$	$2.2x10^{1}$	2%
Human non-carcinogenic toxicity	kg 1,4-DCB	$1.2x10^{1}$	$6.5x10^{2}$	2%
Land use	m^2a crop eq	$1.6x10^{-1}$	$1.7x10^{1}$	1%
Mineral resource scarcity	kg Cu eq	$5.7x10^{-2}$	2.2x10	3%
Fossil resource scarcity	kg oil eq	9.0x10	$3.7x10^{2}$	2%
Water consumption	m3	$1.5x10^{-1}$	$1.0x10^{1}$	1%

3.1. Environmental hotspot analysis of the bioslurry reactor

The environmental effects of the bioslurry reactor are shown in Fig. 7.2 for all mid-impact categories. The graphs' positive values denote environmental burdens, and Table 7.5 lists the environmental loads of the bioslurry reactor for each category of assessed impact.

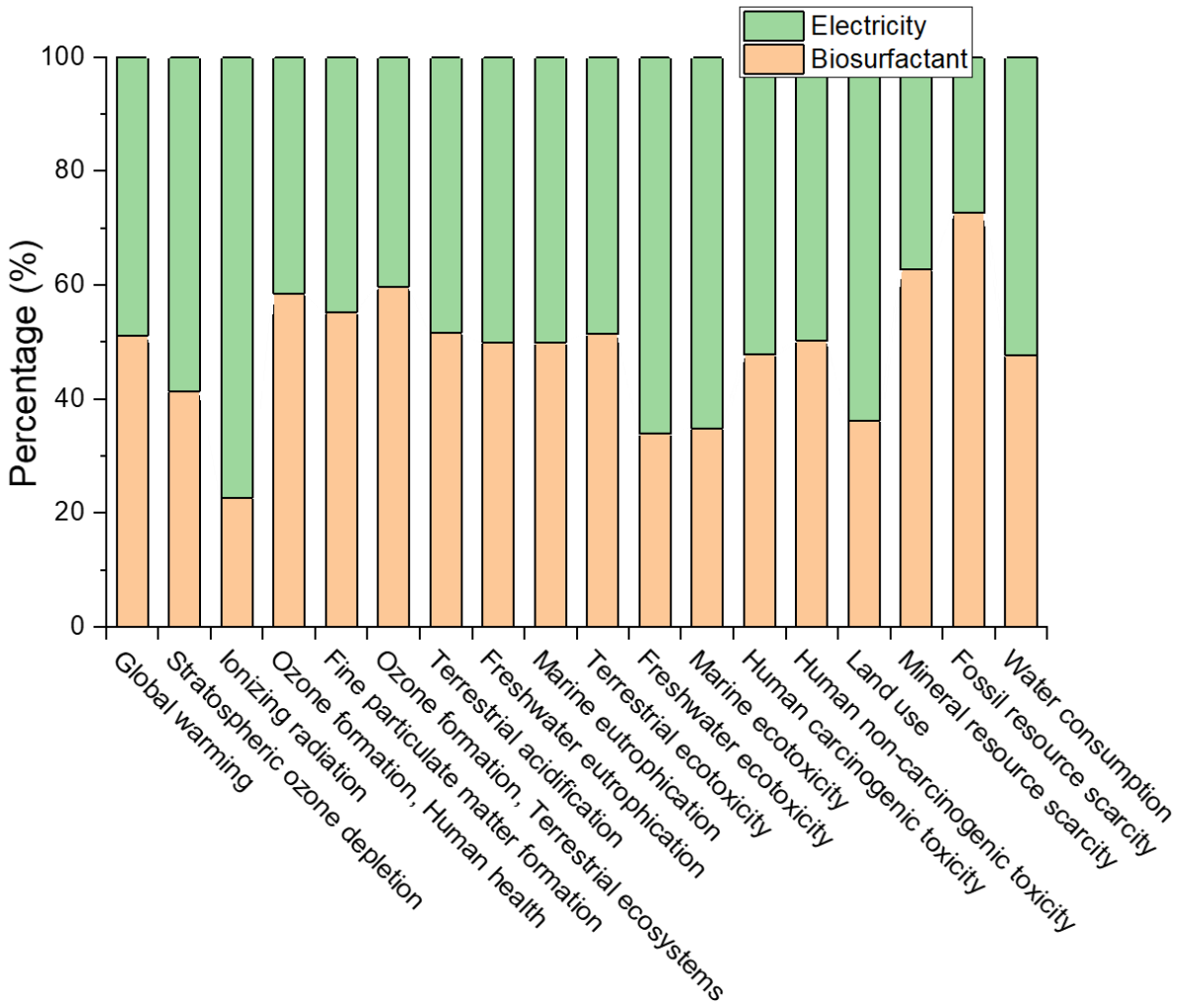


Figure 7-2. Results of the bioslurry process's environmental impact under several midpoint impact categories were expressed in the functional unit (100g of treated of soil). Positive values represent adverse environmental effects.

In all midpoint categories of the bioslurry process, as depicted in figure 7.2, electricity consumption and biosurfactant production hold a sizable share of environmental burdens. For example, in the bioremediation of soil contaminated with petroleum hydrocarbons in the bioslurry reactor, the environmental load related to energy consumption and the stimulant biosurfactant falls under the global warming (GW) category and contributes 312 kg CO2-eq/kg soil. Saranya et al. (2020) discovered that the environmental burdens associated with electricity use pose between 50 and 78% of the overall environmental causes for bioremediation and between 60 and 80% for biosurfactant production (Schonhoff et al., 2022). According to Ni et al. (2020), 72% of the total GW impact category for bioremediation can be attributed to environmental load from electricity usage, and this

result is in line with Busset et al. (2012) reported that aeration, pumping, and heating uses much energy in the bioremediation process.

Fossil-based greenhouse gases (GHGs), sulfur dioxide (SO2), and particulates are released into the atmosphere during the extraction and burning of fossil fuels (coal, natural gas, and oil) to produce electricity, whereas the use of organic chemicals (ethyl acetate, methanol, and oil) during the production of biosurfactant is the leading cause of the high environmental impact for the bioslurry treatment during bioremediation process. Therefore, this research emphasizes the necessity of a self-generating power to replace local fossil fuel use and recycle organic solvents, thereby reducing emissions of particulates and fossil GHG.

3.2. Environmental hotspot analysis of MET

MET demonstrates the capability to produce electricity while degrading petroleum hydrocarbon soil. Therefore, the on-site generated electricity is anticipated to balance their environmental burdens of electricity. Table 7.5 lists the environmental loads of the MET for each category of assessed impact. Generating electricity from bioelectrochemical systems outweighs the impacts of the process by a factor of 1.6 for the Global warming potential (GWP) impact category, which is negligible when compared to the displacement of conventional energy consumption, as illustrated in figure 7.3. However, 40% of the total environmental impact category for the MET related to global warming is caused by using biosurfactants during the MET remediation process.

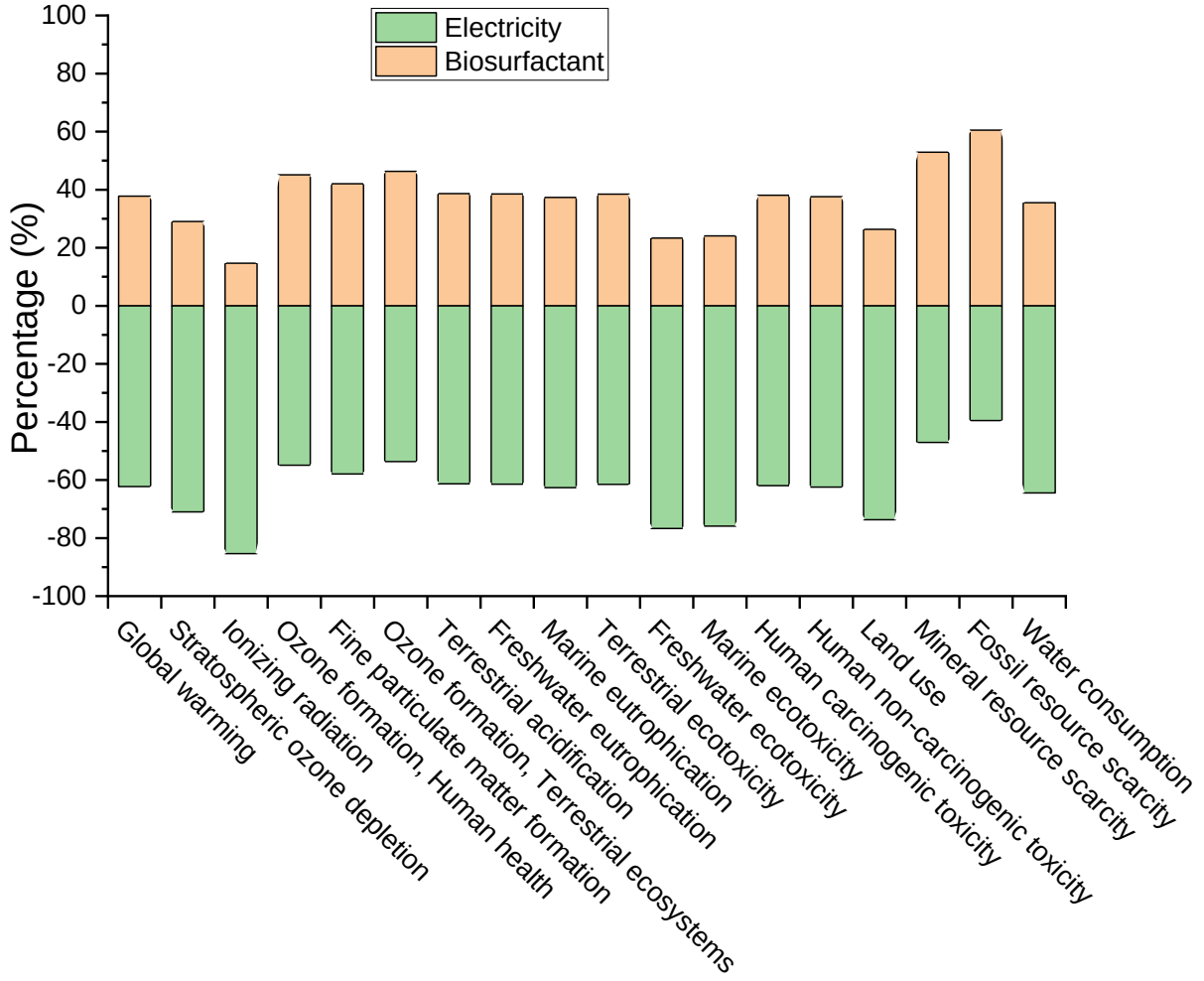


Figure 7-3. MET's environmental impact results under various midpoint impact categories were expressed in functional units (100g of treated of soil). Positive values denote a negative environmental gain, whereas positive values denote an adverse environmental impact.

When treating soil contaminated with petroleum hydrocarbons, according to Ambaye et al. (2022a), the current MET has only been able to perform at a low power density of up to 100 W/m^3, which is only a quarter of the power density that the MET uses. As a result, the MET won't become environmentally friendly until the power density reaches at least 500 W/m^3. Regardless of the low power density of the MET, current research has focused on improving the system's design, electrode, or catalyst material to increase electricity production (Ambaye et al., 2022; Ambaye et al., 2022a).

3.3. Comparison study of bioslurry reactor and MET

The midpoint impact categories for the MET and bioslurry process are shown in Fig. 7.4, along with quantifying and reflecting the midpoint's cause-and-effect chain in terms of real environmental impact. For all criteria considered, the findings indicate that MET is the choice with the least adverse environmental effects. The primary reason for this is that the other compartments' environmental burden is significantly outweighed by the bioelectricity production from MET, which also significantly contributes to avoided impacts. In addition, by substituting alternative fuels for conventional fossil fuels, the energy requirement for the MET process is also reduced, which lowers emissions of particulate matter, sulfur dioxide, and greenhouse gases based on fossil fuels.

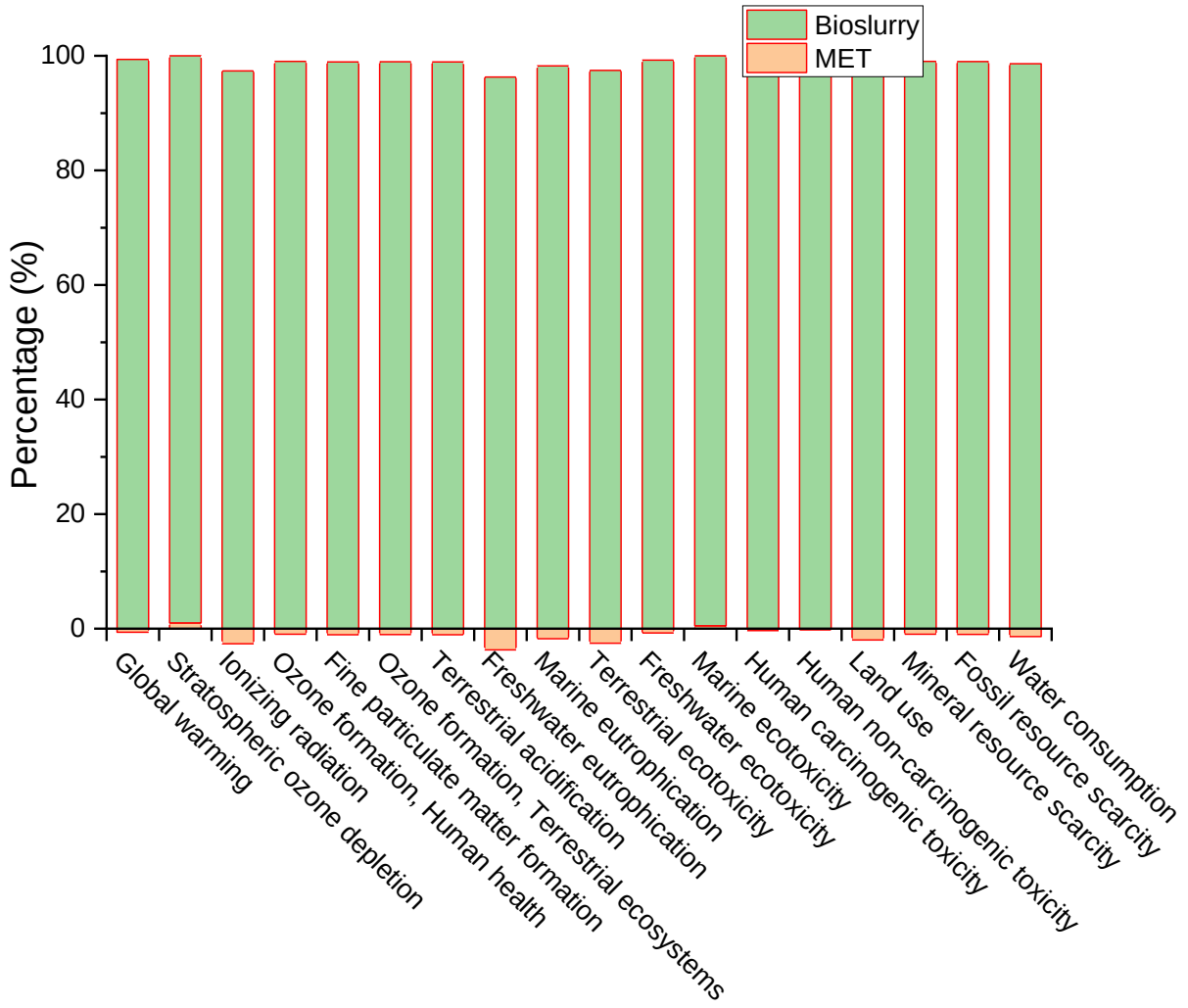


Figure 7-4. Environmental effect data for the MET and bioslurry processes were compared using the functional unit (100g of treated of soil) and various midpoint impact categories. While negative

numbers represent the environmental gain, positive ones indicate the detrimental environmental impact**.**

Under all intermediate damage categories, the bioslurry bioremediation approach has the worst overall environmental performance. This demonstrates that using biosurfactants as a stimulant is problematic because so many chemicals are required for manufacture. This refers explicitly to methanol and ethyl acetate. Additionally, the process is dominated by electricity, which results in significant environmental effects from the extraction and burning of fossil fuels (coal, crude oil, and natural gas), which release particulate and sulfur dioxide into the atmosphere.

The use of Monte Carlo simulation to analyze the environmental load of the two different remediation approaches, as shown in Table 7.6 and Figure 7.5, indicates that bioslurry technology significantly impacted the environment more than the bioelectrochemical system (MET).

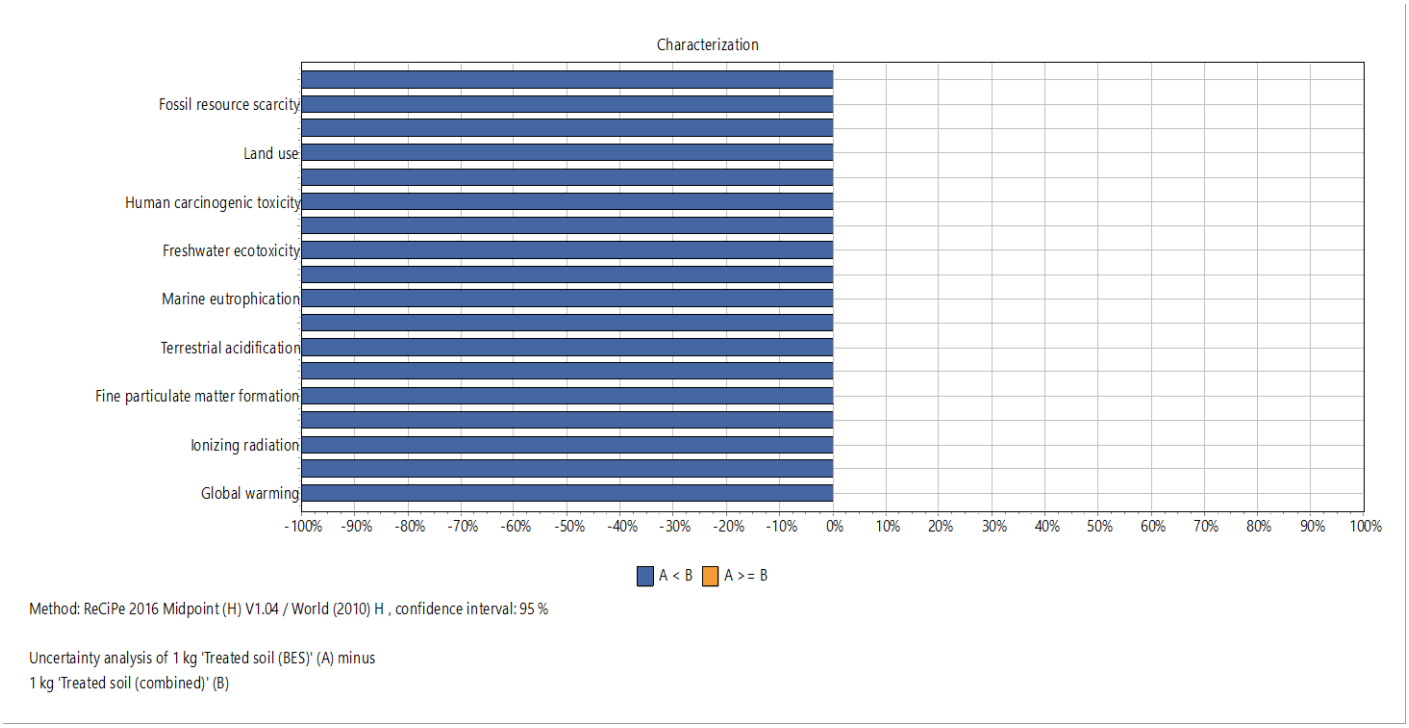


Figure 7-5: Monte Carlo Results on Comparing Characterization of BES(MET) with combined (bioslurry) for remediation of soil polluted with petroleum hydrocarbon on Impact categories

Moreover, according to Figure 5 and Table 7.6, the uncertainty of MET (A) vs. bioslurry (B) via comparative Monte Carlo analysis (1000 runs) has a confidence level of 0.95. Based on relatively low laboratory uncertainties (less than 2% for all categories and both processes), MET is significantly less environmentally damaging than the bioslurry process since the MET process performed better for all of the 1000 runs. The Monte Carlo simulation confirms that using bioslurry for bioremediation

of petroleum hydrocarbon-polluted soil has a greater impact on all impact categories than using MET for remediation of petroleum hydrocarbon-polluted soil. Thus, the use of bioslurry for bioremediation of petroleum hydrocarbon-polluted soil causes a greater environmental load than MET, and the difference is significant due to the source of electricity from the national grid having the highest impact on resources in the damage category. It suggests that applying bioslurry for bioremediation of soil polluted with petroleum hydrocarbons on a pilot scale tends to cause more harm to the environment. Moreover, every unit of product produced using bioslurry depletes the ozone layer. Therefore, it causes more climate change, radiation, and acidification and needs to be replaced with electricity from renewable sources, which generate the least total environmental load.

Table 7-6. Monte Carlo Results on comparative uncertainty analysis of use of MET and bioslurry on Impact categories. A Monte Carlo analysis of 1000 runs was performed based on experimental uncertainties. The BES system is named A, and the combined system is B. The confidence is at 0.95.

Impact category	A >= B	Mean	Median	SD	CV	2.50%	97.50%	Std. err. of mean
Water consumption	0	-4.58	-4.59	0.06	-1.39	-4.73	-4.42	0.013
Terrestrial ecotoxicity	0	-870.70	-873.22	11.37	-1.31	-898.10	-842.35	2.273
Terrestrial acidification	0	-1.17	-1.18	0.02	-1.29	-1.21	-1.14	0.003
Stratospheric ozone depletion	0	0.00	0.00	0.00	-1.54	0.00	0.00	0.000
Ozone formation, Terrestrial ecosystems	0	-0.68	-0.68	0.01	-1.10	-0.70	-0.66	0.001
Ozone formation, Human health	0	-0.65	-0.65	0.01	-1.13	-0.67	-0.63	0.001
Mineral resource scarcity	0	-0.77	-0.77	0.01	-1.03	-0.79	-0.75	0.002
Marine eutrophication	0	-0.01	-0.01	0.00	-1.33	-0.01	-0.01	0.000
Marine ecotoxicity	0	-28.84	-28.93	0.49	-1.70	-30.04	-27.59	0.098
Land use	0	-8.43	-8.46	0.14	-1.68	-8.77	-8.07	0.028
Ionizing radiation	0	-22.74	-22.81	0.45	-1.99	-23.86	-21.58	0.090
Human non-carcinogenic toxicity	0	-257.50	-258.28	3.43	-1.33	-265.79	-248.96	0.685
Human carcinogenic toxicity	0	-8.97	-9.00	0.13	-1.40	-9.27	-8.66	0.025
Global warming	0	-313.94	-314.78	4.10	-1.31	-323.96	-303.72	0.820
Freshwater eutrophication	0	-0.09	-0.09	0.00	-1.34	-0.09	-0.08	0.000
Freshwater ecotoxicity	0	-22.91	-22.98	0.39	-1.72	-23.87	-21.90	0.079
Fossil resource scarcity	0	-163.53	-163.72	1.25	-0.77	-166.60	-160.64	0.251
Fine particulate matter formation	0	-0.42	-0.42	0.01	-1.21	-0.43	-0.41	0.001

In general, Anticipatory LCAs are helpful in predicting the impacts of a given product or technology before marketing. As emphasized by Castro et al. (2022), anticipatory LCAs are not to provide a realistic model but to offer insights into improving environmental performance. Data uncertainty and choices can be explored through scenario analysis, offering the possibility of evaluating the process's viability and proposed changes. In addition, understanding how to close loops in a circular economy approach (Castro, et al., 2022) can be beneficial.

4. Conclusion

Using the LCA methodology, the environmental performances of the MET and bioslurry processes for treating soil contaminated by petroleum hydrocarbons are assessed. Under all considered midpoint impact categories, MET is the least environmentally damaging compared to bioslurry. The most notable environmental hotspots, accounting for the majority of the global warming potential (i.e., 100%), are the electricity consumption for laboratory operations and the production of biosurfactants, which enhance the degradation of soil polluted with hydrocarbons.

The avoided impact of MET reduced the environmental burdens by a factor of 1.6 for the global warming potential (GWP), and these positive environmental effects of electricity production during the MET process were achieved by assuming a replacement of low-voltage electricity from the national electrical grid in Italy. This could prove problematic in real life since the MET's low power density does not equal the properties of the national electrical grid. However, if, in a type of industrial symbiosis, a different production site close to the MET process could make use of the electric energy, it might still substitute another electricity source, which would most likely be the local electrical grid. A pilot plant or industrial case study may provide more data on improving this aspect of the model. Moreover, the environmental impact of biosurfactant production can be decreased by recycling the chemicals.

The Monte Carlo simulation confirms that using bioslurry for bioremediation of petroleum hydrocarbon-polluted soil has a greater impact on all impact categories than using MET for remediation of petroleum hydrocarbon-polluted soil due to the source of electricity from the national grid having the highest impact on resources in the damage category and needing to be replaced with a renewable source to reduce the environmental load.

The results are restricted to the MET and bioslurry processes at the laboratory scale. According to a study, lab-scale pollution is three times worse for the environment than pilot-scale (Chin et al., 2022). Although the pretreatment process in MET and bioslurry reactor remediation systems is not included in the boundary scope of this study, it is recommended to include the pretreatment for a more thorough study because the pretreatment procedure, to some extent, might result in 10% higher environmental

loads (Chin et al., 2022). MET and bioslurry may be used in various places to solve the concerns about climate change and bioremediation, which are currently causing widespread concern. The environmental performance of the MET and bioslurry processes is compared in this study, along with environmental hotspots. Future research will look at the system's viability by evaluating the sensitivity and uncertainty of the electricity mix variation in the bioslurry and MET processes, as well as their economic impact. The findings of the study should help those involved in soil remediation choose the best MET and biological processes to better restore contaminated sites and address climate effects.

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CHAPTER 8

CONCLUSIONS AND PERSPECTIVES

8.1. CONCLUSIONS

The main objective of this thesis was to develop a laboratory-based bioslurry reactor under controlled conditions for bioremediation as well as a bioelectrochemical system for the treatment of soil polluted with petroleum hydrocarbons, coupled with electricity generation that is efficient and effective for various treatment conditions and applicable to all soils contaminated by organic compounds. Therefore, five main studies were conducted to achieve this objective, with detailed discussions reported in chapters 2-7. From each chapter, the following conclusions can be drawn.

The main part of Chapter 2 was aimed at the state-of-the-art understanding of scientific challenges of the most suitable approaches for remediation of PAH-contaminated soils and treatment. The literature reviews identified bioslurry and bioelectrochemical systems are an effective technology for treating soil polluted with petroleum hydrocarbon. This was achieved, firstly, by reviewing the existing literature. Subsequently, specific challenges related to different aspects of the post-use of various technologies for the remediation of soil polluted with petroleum hydrocarbons were selected and addressed in a holistic approach. This way, a comprehensive overview of the scientific challenges and identifying the most suitable approaches for the remediation of PAH-contaminated soils and treatment was provided.

Chapter 3 aims to investigate the presence of external stimulants such as nutrients, activated sludge, synthetic surfactant, and long-chain rhamnolipids on the PAH biodegradation kinetics and on the ability of the microbial community to adapt to environmental changes. From the results, it was concluded that the presence of activated sludge, nutrients, and biosurfactant revealed the lowest half-life time ($t_{1/2}$ = 4.1 days), the highest biodegradation rate constants (k = 0.17 day^{-1}), and the reaction rate data from each bioremediation method was fitted with a first-order reaction rate model. Furthermore, the microbiological community analysis results revealed that petroleum-degrading bacteria such as *Arthrobacter, Pseudomonas,* and *Bacillus* significantly increased their relative abundance after adding activated sludge nutrients and biosurfactants to the bioslurry reactor. The findings of this study can be applied to the investigation of the soil slurry bioreactor for real contaminated soil remediation on a pilot scale. However, it is necessary to consider the characteristics and composition of the soil and the presence of indigenous microorganisms. The mechanistic approach used in this investigation can be seen as a generic method. It can be used to study and better understand the slurry bioreactor treatment for any soil, different pollutants, and microbial communities.

In Chapter 4, the effect of advanced oxidation process as pretreatment for the biological treatment of removing soil polluted with PHs. It was observed that using an advanced oxidation process as pretreatment leads to a 40% increase in the degradation of PHCs and the production of more biodegradable byproducts compared to individual electro-Fenton and biological treatments. The result also showed that anodic oxidation using a boron-doped diamond (BDD) electrode changed the sorption properties of soil by modifying soil organic matter and reducing humin content from 1.1 to 0.4 %. Finally, combining electro-Fenton and biological treatment, the present work has shown that electro-Fenton pretreatment significantly enhances biodegradability. An increase in biodegradability proves the formation of more biodegradable intermediates that a biological process could further degrade.

Chapter 5 focused on the degradation of soil polluted with petroleum hydrocarbon in the anode of dual-chamber MET using various doses of a novel long-chain rhamnolipid (RL) produced by low-cost carbon winery waste through non-pathogenic Burkholderia thailandensis E264 strains. For studies on PAHs degradation, the best overall performing rhamnolipid was 100 mg/L, supplemented with activated sludge, which gave a PAH reduction rate of 72.5% and a maximum current density of 9.5 Am^{-2}. This suggests that the selected best-performing doses of rhamnolipid may offer good prospects for degradation in MET for treating petroleum hydrocarbons on a pilot scale. Furthermore, the phytotoxicity of polluted soil, based on *L. sativum* seeds, for post-treatment samples was four-fold higher than the pretreatment samples suggesting a lower phytotoxicity effect. With MET as a technique, this study confirmed for the first time that novel long-chain rhamnolipid produced from non-Pseudomonas bacteria could remarkably facilitate the degradation of petroleum hydrocarbon via extracellular electron transfer, which provides novel insights to understand the mechanisms of RL regulating petroleum hydrocarbon degradation.

In the work described in Chapter 6, the performance of a lab-scale using rice straw-based biochar to improve extracellular electron transfer, using typical soil polluted with petroleum hydrocarbons as a model pollutant. The study demonstrated that the degradation and electrochemical performance of MET were enhanced significantly when rice straw-based biochar with a dose of 2 g was used as a catalyst in the anodic chamber. This suggests that using rice straw-based biochar in MET as an electron-donating catalyst instead of an expensive and non-renewable metal catalyst is possible. However, adding rice straw-based biochar above the optimal dose leads to inhibit the bacteria's microbial activity and decreases the degradation and electrochemical performance of MET.

In Chapter 7, a life cycle assessment was carried out to evaluate the environmental impact under various conditions involved in the bioremediation of soil polluted with petroleum hydrocarbons through laboratory-scale experiments and SimaPro 9.1 software to select the most appropriate treatment strategy to help decision-makers and stakeholders. Among the two processes, MET is the most environmentally friendly option under all midpoint environmental impact categories, while the bioslurry reactor shows the least environmentally unfriendly process under all midpoint environmental impact categories. The electricity consumption for the operation and utilization of biosurfactants to enhance the degradation of soil polluted with hydrocarbons is the most remarkable environmental hotspot, comprising the largest portion of damage (i.e., 100%) to the global warming impact category. However, the low power density performance of the MET becomes an obstacle to substituting local energy consumption. The environmental impact during the production of biosurfactants can be decreased by recycling the chemicals during the process. The findings are limited to the laboratory scale size of the assessed MET and bioslurry reactor.

Overall, it can be concluded that the MET and bioslurry reactor systems were developed and evaluated for removing petroleum hydrocarbons from contaminated soil under various operational and environmental situations. If these systems are to be used for bioremediation and compete with other conventional technologies, understanding how robust of these designed bioslurry reactors and MET are to various operating circumstances is crucial for determining their technical viability. The findings of the investigations discussed in this thesis suggest that MET technology may be used to effectively treat soil contaminated with petroleum hydrocarbons while also recovering energy. The treatment of petroleum hydrocarbons in contaminated subterranean settings or industrial effluents might be done using MET technology as an independent system instead of alternative bioremediation or combined with existing infrastructures like monitoring wells. It is necessary to conduct experiments at the pilot scale to determine which configuration would be most suitable for industrial-scale applications.

8.2. PERSPECTIVES

For use on a broad scale, bioslurry reactors and MET still have a long way to go. However, several prospective areas for developing bioslurry and MET systems in the treatment of petroleum hydrocarbon-contaminated soil could be researched in the future based on the conclusions of this thesis.

8.2.1 Perspectives and scientific challenges for bioslurry reactor and its integration with advanced oxidation process.

Results obtained during experimental studies showed that the biodegradation efficiency of PHCs was higher in a short period in those treatments where rhamnolipid was added as compared to those where rhamnolipid was not applied. Goveas et al. (2022) used biosurfactants to mediate bioremediation and found similar results. However, the biodegradation efficiency of PH appears to vary depending on a wide variety of other factors. For example, Szulc et al. (2014) reported that the efficiency of PHCs degradation increased when biosurfactants were added to diesel oil-contaminated media. In biodegradation, the solubilization of PHCs occurs in biosurfactants, which mediates their subsequent transfer into the aqueous phase, which may lead to a more rapid degradation process. However, hydrophobic compounds may become entrapped in the micelle core or interact with micelles, reducing their bioavailability (Chebbi et al., 2022). Hence, this shows that adding biosurfactants to the biodegradation rate in the initial treatment stage needs more research to study the long-term effects of adding rhamnolipids compared to other stimulants.

Age of contaminated soil is a significant factor that could affect the efficacy of biosurfactant-mediated processes. In this experiment, rhamnolipids were tested to see how they fared in newly polluted soil. Many studies conducted using freshly contaminated soil microcosms have yielded results that contradict the conclusions drawn in this manuscript, suggesting that the presence of rhamnolipids significantly improved biodegradation efficiency. For example, Whang et al. (2009) found that adding rhamnolipids accelerated the biodegradation of diesel hydrocarbons. In addition, they reported that biopiles treated with rhamnolipids decreased TPH from an initial value of 6981 mg/kg of dry soil to 211 mg/kg of dry soil after 88 days, while the TPH value for the control biopile was at 3689 mg/kg of dry soil. In another study, Cameotra and Singh (2008) showed that soil freshly spiked with crude oil sludge inoculated with a microbial consortium biodegradation efficiency was increased from 52

to 73%; however, with a microbial rhamnolipids inoculation, biodegradation efficiency reached 73.3% after 8 weeks.

The discrepancy in rhamnolipids' effect on hydrocarbon removal efficiency may be due to variations in the amount of organic matter in the soil. A research by Liu et al. (2012) found that soils with a low organic matter (SOM) content had a higher biodegradation efficiency, while soils with a high SOM content benefited from the introduction of rhamnolipids, which stimulated the TPH removal. Due to the low organic matter content of the soil used in the experiments, these findings are consistent with those presented in this manuscript. Furthermore, the impact of aging processes on the bioavailability of hydrocarbons and their efficacy was also elucidated by Liu et al. (2012). Although rhamnolipid addition did not result in significant differences in TPH removal within the scope of this study, this strategy may be feasible for soils with a higher organic matter content than soils contaminated with aged hydrocarbons.

Researchers worldwide are working on developing efficient degradation of PHCs and reducing their toxicity to plants and humans. Our study is somewhat related to this aspect. The use of advanced molecular monitoring tools like 16S rRNA gene sequencing helped for identifying PHCs degraders and microbes such as *Arthrobacter,* and *Pseudomonas*. Improving bioremediation efficiency by applying microbes has become a crescent study field in microbial biotechnology. Therefore, our study has a great future in microbial-mediated biosurfactant production and advances in PHCs biodegradation. Our research revealed that the combined use of activated sludge, nutrients, and biosurfactants in bioslurry potentially enhanced the PHCs-degradation and encouraged the feasibility of biological treatment on a large scale for soil contaminated by PHs. However, some core studies on the age of PHCs, soil contamination, the bioavailability of hydrocarbons, changes in abundance and specific community dynamics, the effect of soil physico-chemical and biological properties on biosurfactant-mediated other processes, including many other related factors to soil organic matter, and the evaluation of contradictory findings must be included in future studies.

Another perspective to consider in the future is integrating bioreactor-stimulated biosurfactants with other approaches since bio-treatment systems have emerged as eco-friendly techniques for treating PHCs and removing them from the soil. Biosurfactant application is preferred because it has greater environmental feasibility, is a renewable and eco-friendly resource, and is non-toxic. However, some challenges have been reported due to hydrophobic organic compounds in the soil. Soil washing, a non-destructive separation method, is more effective when combined with other technologies (Xiao

et al., 2019). Moreover, the introduction of advanced oxidation in the slurry reactor system will likely increase the degradation efficiency of organic compounds by transforming the target pollutants into more favorable forms for microbial degradation. It should also be more cost-effective and practically feasible (Noor et al., 2022). It has been reported that integrated technologies like soil washing and advanced oxidation process followed by treatment of biotreatment in bioreactor systems enhance the mass transfer rate of hydrophobic compounds and achieve efficient degradation of target pollutants. Utilizing a bioreactor system as a post-soil washing and advanced oxidation treatment could be a valuable alternative that is also reported to be far more accessible than controlling them in the field, but it needs further research and investigation in the future to apply on a large scale (Srivastava et al., 2022).

Finally, to demonstrate the effectiveness and sustainability of soil remediation using biosurfactants, scale-up investigations are required, along with estimates of economic costs. For instance, recent research shows that the gap between costs of bio- and chemical-surfactants production is considerable, as available data indicate that the potential monetary value of biosurfactants is between 1 and 60 €/kg compared with a production cost of 0.10 to 0.30 €/kg (Xi et al., 2021) depending on the degree of purity and product specifications required by the desired application. Therefore, it is worth considering the massive production of synthetic surfactants and their lower average price (1–2 €/kg surfactant). However, the cost of biosurfactants is currently higher than chemical surfactants (Xi et al., 2021) and is not a cost-competitive substitute in the marketplace. Furthermore, there is enormous scope to reduce its production cost due to abundant raw materials, optimized fermentation, and extraction process. Moreover, innovations in surfactant chemistry, biotechnology, and remedial engineering technology are necessary to spur the application of biosurfactants and other surfactant-based techniques in the future. However, bio-treatments of petroleum-contaminated soil using biosurfactants have made significant strides in recent years, and these factors are crucial to the success of this endeavor.

The bioslurry process currently used is an environmentally friendly strategy to decontaminate soil; however, insufficient mineralization rates were observed during the treatment of the biolsurry reactor due to the recalcitrance of some degradation by-products. This is the reason why this research (Chapter 3) investigated the use of an advanced oxidation process in combination with pre-treatment using BDD as an anodic oxidation process, which increased the biodegradation of soil polluted with petroleum hydrocarbons. Even though BDD electrodes are excellent at maximizing oxidation efficiency, their widespread application is still debatable. Further study is required to reduce electrode

costs, scale up fabrication while maintaining high physical stability, and find a solution to separating BDD film from the substrate surface and cracking issues. Furthermore, the process may also be hard to use on a large scale because soil polluted with PHCs in the real world has many different properties, such as low bioavailability of organic contaminants and the presence of other radical scavengers (Nidheesh et al., 2019).

8.2.2 Perspectives and scientific challenges on the MET performance amended with biosurfactant and biochar.

Despite our findings showing that employing unique long chain rhamnolipids (RL) as an amendment in the anode electrode improves MET performance, some difficulties were not addressed in this study and should be investigated further, addressing the aspects below. Biosurfactants may be hindered by electron transfer pathways, particularly electron shuttles like biofilm adhesion, the electroactive community on electrodes, petroleum hydrocarbons, biofilm composition, and architecture. Moreover, RLs have broadly confirmed interesting features, e.g., the ability to reduce surface and interfacial tensions of solutions, the formation of microemulsions, and stability over a wide range of salinities, temperatures, and pH values (Chebbi et al., 2022). Most of these findings have been obtained using RLs extracted from *Pseudomonas aeruginosa* (i.e., predominantly Rha-Rha-C10-C10), an opportunistic pathogen in humans, animals, and plants. Some large-scale broth applications containing RL producers are generally weakened for safety-related concerns (Chebbi et al., 2021). As a result, non-pathogenic RL producers, like some *Burkholderia spp.,* are increasingly becoming the focus of cutting-edge methods with lower yields and safe capacities (Chebbi et al., 2021). This makes biosurfactant-producing RLs using non-pathogenic producers an ideal target for novel circular economy approaches in biotechnology, in which agricultural waste is converted into a value-added product. Further research is needed to optimize these parameters to avoid negative phenomena such as toxic effects on the electroactive community when used on a large scale.

As previously stated, adding rhamnolipids improved the current generation after 5 days of MET operation. Furthermore, due to internal MET resistance, rhamnolipid concentration substantially impacted the anodic electron transfer rate during the stable stage. As a result, higher power density meant faster electron transport and lower energy usage. However, more research is required on rhamnolipid concentration, anodic electron transfer, anode surfaces through electron microscopy, and the Bockris-Devanathan-Müller model. Furthermore, the anode electrode's performance could be improved even more by utilizing alternative carbonous materials, such as biochar.

Bioelectricity generation in MET systems is attractive as compared to other conventional systems. However, the electricity generated in this study is still modest and has to be improved. Optimizing MET architecture design and materials is necessary to boost electricity output. The use of bioelectrochemical systems to treat soil polluted with petroleum hydrocarbons was demonstrated in this study. Physico-chemical properties of soil can significantly influence MET performance (Ambaye et al., 2022). Further studies are required to facilitate the transfer of this technology to the field level. There is also a need to develop a more efficient process to lower the cost of soil restoration by adopting MET systems in various field applications independent of soil conditions.

Biochar and rhamnolipids can facilitate the phytoremediation process by speeding up the rate of petroleum hydrocarbon bioremediation by liberating oil from soil pores and boosting contact with soil microorganisms for breakdown during RL and biochar treatments (Wei et al., 2020). However, little research has been done on combined biosurfactant and biochar treatments, particularly with the bioelectrochemical system. Therefore, a more focused study is required to evaluate the remediation of petroleum hydrocarbon using biochar and rhamnolipids.

Biochar as an amendment in MET, the presence of redox-active quinone in biochar is responsible for extracellular electron transfer. These findings demonstrate that the dose of biochar plays a crucial role in determining the effectiveness of the biochar in the soil bioelectrochemical system remediation system in enhancing electricity generation and biodegradation of petroleum hydrocarbons from polluted soil. Moreover, biochar carries economic and various environmental benefits regarding energy demand and reduction in global warming potential (GWP). However, much more research is needed to improve the production process and evaluate the benefits of using biochar as a MET in soil remediation over its entire life cycle, despite its cost and life cycle advantages. Feedstock material with little economic value should be avoided at all costs, and production energy should be maximized. Biochar samples vary widely, so pyrolysis parameters must be adjusted to produce chars with high surface area and conductivity for MET and to have surface functional groups if biochar is to be used as an amendment material.

Biochar from rice straw showed high redox ability and soil conductivity, enhancing the extracellular electron transfer in the soil matrix in MET. More electrochemical analyses such as electron accepting capacity (EAC), electron-donating capacity (EDC), and electron exchange capacity (EEC) evidence are needed to illustrate the enhanced degradation, using biochar as solid electron mediators at the anode in MET.

8.2.3 Perspectives and scientific challenges on the environmental life cycle assessment

Life cycle assessment was carried out to evaluate the environmental impact under various conditions involved in the bioremediation of soil polluted with petroleum hydrocarbons through laboratory-scale experiments to select the most appropriate treatment strategy to help decision-makers and stakeholders through SimaPro 9.1 software. The findings are limited to the laboratory scale size of the assessed MET and bioslurry reactor and only assess the environmental impact. To check the system's feasibility, future studies, such as economic analysis on MET, bioslurry, and uncertainty and sensitivity analyses on the electricity mix variation on the remediation process on a pilot scale. Benefit-cost analysis is used to evaluate one technology/service and to compare the total economic benefits to the total economic costs related to its application. Combining both aspects - environmental and economic analyses - it would be possible to fully evaluate the MET and bioslurry reactor technology in case of promising outcomes to accelerate its industrial application. The results of the study should help stakeholders in the soil remediation process to compare different alternatives and to select the most appropriate treatment strategy.

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APPENDIX

Appendix I – Valorization of the PhD research work

Papers in peer-reviewed journals:

1. **Ambaye, T.G.,** Formicola, F., Sbaffoni, S., Franzetti, A. and Vaccari, M., 2023. Life cycle assessment of bioslurry and bioelectrochemical processes for sustainable remediation of soil polluted with petroleum hydrocarbons: An experimental study. Sustainable Production and Consumption, 36, p. 416-424. **(IF= 8.921).** https://doi.org/10.1016/j.spc.2023.01.021
2. **Ambaye, T.G**., Formicola, F., Sbaffoni, S., Prasad, S., Milanese, C., della Cuna, F.S.R., Franzetti, A. and Vaccari, M., 2023. Treatment of petroleum hydrocarbon contaminated soil by combination of electro-Fenton and biosurfactant-assisted bioslurry process. Chemosphere, p.138013. **(IF= 8.943).** https://doi.org/10.1016/j.chemosphere.2023.138013
3. **Ambaye, T.G**, Alif Chebbi, Francesca Formicola, Asia Rosatelli, Shiv Prasad, Franco Hernan Gomez, Silvia Sbaffoni, Andrea Franzetti, Mentore Vaccari. Ex-situ bioremediation of petroleum hydrocarbon contaminated soil using mixed stimulants: Response and dynamics of bacterial community and phytotoxicity, Journal of Environmental Chemical Engineering,2022,108814, **(IF=7.968).**https://doi.org/10.1016/j.jece.2022.108814.
4. **Ambaye, T.G**., Vaccari, M., Franzetti, A., Prasad, S., Formicola, F., Rosatelli, A., Hassani, A., Aminabhavi, T.M. and Rtimi, S., 2022. Microbial electrochemical bioremediation of petroleum hydrocarbons (PHCs) pollution: Recent advances and outlook. Chemical Engineering Journal, p.139372. **(IF= 16.744**). https://doi.org/10.1016/j.cej.2022.139372.
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7. **Ambaye, T.G**., Chebbi, A., Formicola, F., Prasad, S., Gomez, F.H., Franzetti, A. and Vaccari, M., 2022. Remediation of soil polluted with petroleum hydrocarbons, and their reuse for agriculture: Recent progress, challenges, and perspectives. Chemosphere, p.133572. **(IF= 8.943).** https://doi.org/10.1016/j.chemosphere.2022.133572
8. **Ambaye, T.G**., Vaccari, M., Prasad, S., van Hullebusch, E.D. and Rtimi, S., 2022. Preparation and applications of chitosan and cellulose composite materials. Journal of Environmental Management, 301, p.113850. **(IF= 8.91)** https://doi.org/10.1016/j.jenvman.2021.113850
9. **Ambaye, T.G**., Vaccari, M., Prasad, S. and Rtimi, S., 2021. Preparation, characterization and application of biosurfactant in various industries: A critical review on progress, challenges and perspectives. Environmental Technology & Innovation, 24, p.102090. **(IF= 7.758)** https://doi.org/10.1016/j.eti.2021.102090
10. **Ambaye, T.G**., Vaccari, M., van Hullebusch, E.D., Amrane, A. and Rtimi, S., 2021. Mechanisms and adsorption capacities of biochar for the removal of organic and inorganic pollutants from industrial wastewater. International Journal of Environmental Science and

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12. Ambaye, T.G., Rene, E.R., Nizami, A.S., Dupont, C., Vaccari, M. and van Hullebusch, E.D., 2021. Beneficial role of biochar addition on the anaerobic digestion of food waste: a systematic and critical review of the operational parameters and mechanisms. Journal of Environmental Management, 290, p.112537**. (IF= 8.91).** https://doi.org/10.1016/j.jenvman.2021.112537
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16. **Ambaye, T.G.,** Rene, E.R., Dupont, C., Wongrod, S. and Van Hullebusch, E.D., 2020. **Anaerobic digestion of fruit waste mixed with sewage sludge digestate biochar: influence on biomethane production**. Frontiers in Energy Research, 8, p.31. **(IF= 5.190).** https://doi.org/10.3389/fenrg.2020.00031
17. Chebbi, A., Franzetti, A., Formicola, F., **Ambaye, T.G.,** Gomez, F.H., Murena, B., De Marco, E., Beltrani, T., Sbaffoni, S. and Vaccari, M., 2022. **Insights into rhamnolipid-based soil remediation technologies by safe microorganisms:** A critical review. Journal of Cleaner Production, p.133088. **. (IF= 11.072).** https://doi.org/10.1016/j.jclepro.2022.133088
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One more article, based on Chapters 6 is under review

Three critical review articles are under the peer-review process.

Conferences

Oral presentations:

Ambaye, T.G., Formicola, F., Sbaffoni, S., Franzetti, A. and Vaccari, M., 2021. **Insights into rhamnolipid amendment towards enhancing microbial electrochemical treatment of petroleum hydrocarbon contaminated soil**, Oral presentation, SiCon Workshop su: Siti Contaminati. Esperienze negliinterventi di risanamento (SiCon 2022) Brescia, Italy, February 9-11, 2022.

Poster presentation:

Ambaye, T.G., Formicola, F., Sbaffoni, S., Franzetti, A. and Vaccari, M., 2021. **Insights into rhamnolipid amendment towards enhancing microbial electrochemical treatment of petroleum hydrocarbon contaminated soil**, poster presentation, SiCon Workshop su: Siti Contaminati. Esperienze negliinterventi di risanamento (SiCon 2022) Brescia, Italy, February 9-11, 2022.

Summer school presentations:

Ambaye, T.G., Formicola, F., Sbaffoni, S., Franzetti, A. and Vaccari, M., 2021. **Ex-situ bioremediation of petroleum hydrocarbon contaminated soil using mixed stimulants: Response and dynamics of bacterial community and phytotoxicity**, summer school on Circular economy in low- and middle-income countries: focus on water & waste at UNIBS, June 21 – June 25, 2021.

Ambaye, T.G., Formicola, F., Sbaffoni, S., Franzetti, A. and Vaccari, M., 2021. **Degradation of petroleum hydrocarbon contaminated soil by combination of electro-Fenton and biosurfactant-assisted biolsury process**, summer school on Circular economy in low- and middle-income countries: focus on water & waste at UNIBS, June 21 – June 25, 2021.

Ambaye, T.G., Formicola, F., Sbaffoni, S., Franzetti, A. and Vaccari, M., 2021. **Effect of rice-based biochar on petroleum hydrocarbons degradation and electricity generation in soil microbial electrochemical remediation systems**, summer school on Appropriate technologies for sustainable development: A focus on low- and middle-income countries, UNIBS, 20/06 – 24/06/2022.

Ambaye, T.G., Formicola, F., Sbaffoni, S., Franzetti, A. and Vaccari, M., 2021**. a feasibility study on the "Life cycle assessment of biolsury and bioelectrochemical process for sustainable remediation of soil polluted with petroleum hydrocarbon**, summer school on Appropriate technologies for sustainable development: A focus on low- and middle-income countries, UNIBS, 20/06 – 24/06/2022.

Appendix II –Supplementary data

Biochar's physical and chemical characteristics, as well as potential enhancement pathways for BES

The chemical and physical properties of the biochar mainly depend on the type of feedstock and the pyrolysis conditions such as heat transfer rate, temperature increase rate, pyrolysis pressure, pyrolysis final temperature, and residence time (Ambaye et al., 2020). According to Sathishkumar and Sanganyado (2020), the properties of biochar to be checked for the use of BC as an amendment in the BES process are the elemental composition, cation exchange capacity, and surface functional groups. These essential chemical properties contribute to the increased interactions between biochar and contaminants to enhance the electron transfer between the anode electrode and bacteria for generating electricity and degradation of organic pollutants in BES.

Rice straw from a village in the Pavia province, in the Lombardy region of Italy, was used to produce biochar. The rice straw was cleaned in deionized water before being dried for 24 hours in a 90°C oven. The material was then diced into 3–4 cm long pieces and pyrolyzed in a cylindrical quartz tube at 600 °C for 2 h with a 1.5 L min-1 N_2 flow, as illustrated in Fig. S1. Biochar was milled and sieved using a 100-mesh (0.15 mm) sifter after cooling to room temperature. According to the charring temperature, the resulting biochar was designated as BC600 °C. Table S1 summarizes the basic physical and chemical properties of BC600 °C.

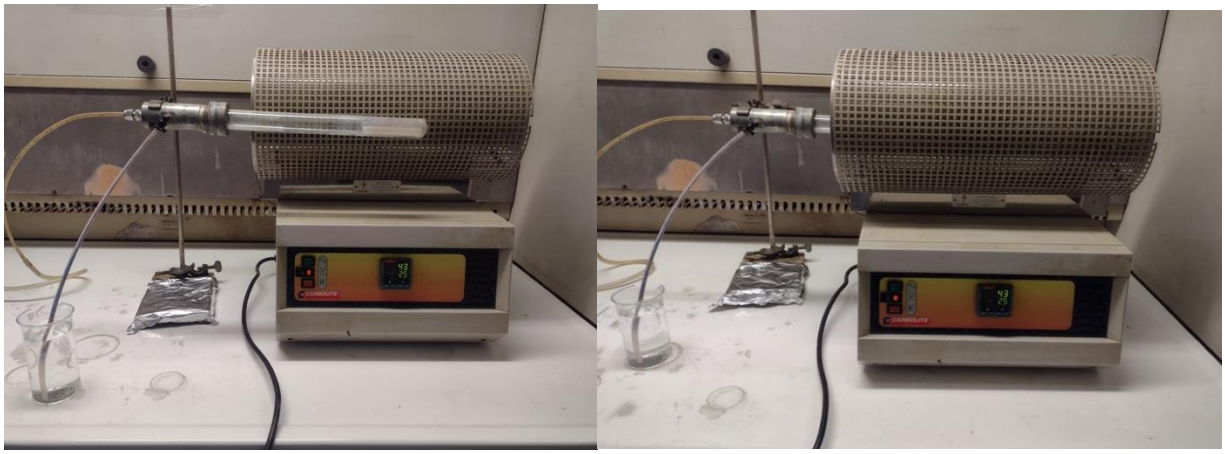

Fig. S1. The experimental apparatus used to produce biochar in this study (with the tube outside the oven to show the internal alumina boat and the tube inside).

Table. S1 Physicochemical properties of rice straw biochar (BC600)

Parameters	**Value**
C(%)	83.4
H(%)	2.41
N(%)	1.45
P (%)	2.10
O(%)	12.6
Ca(%)	0.013
Mg(%)	＜0.0001
Fe(%)	0.016
Cu(%)	＜0.0001
BET surface area (m^2/g)	97.05
BJH pore volume (cm^3/g)	0.034
BJH pore size (nm)	1.60
Electrical conductivity ($S \cdot cm^{-1}$)	5.2

Table S2 displays the physicochemical characteristics of biochar produced at 600 ^{o}C (BC600). According to the results of the SEM-EDS mapping, biochar is a porous carbon substance with equally distributed C, O, and N elements. FTIR spectroscopy evaluated the composition of different moieties on the surface of biochar. The FTIR spectra revealed the existence of OH groups (3462 cm^{-1}), aliphatic CH (2925 cm^{-1}), quinones with C=O stretching (1656 cm^{-1}), and COH (1445 cm^{-1}), as shown in Fig. S2.

The electron-donating and -accepting mechanism was probably aided by the phenolic -OH and quinone C=O groups. Peaks at 1618–1578 cm^{-1} in the pyrolyzed biochar at low temperatures were attributed to the C=O group on conjugated ketones and quinones (Sathishkumar and Sanganyado, 2020). Quinone and hydroquinone, redox-active components of the biochar, were more prevalent in BC 300. Some infrared peaks vanished as the pyrolysis temperature rose (Chen et al., 2018). A prior study hypothesized that the production of phenolic–OH redox-active chemicals in rice straw biochar required the presence of hemicelluloses and lignin components (Sathishkumar and Sanganyado, 2020). These quinones and phenols may be crucial in the electron transfer process leading to the breakdown of petroleum hydrocarbons.

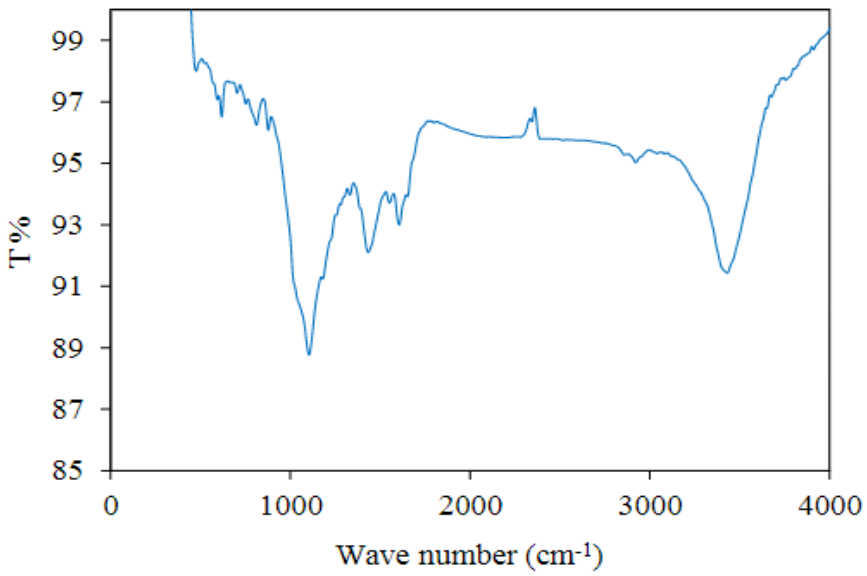

Fig. S2. Fourier transform infrared (FT-IR) analysis of biochar pyrolyzed at 600 °C in wave number the 4000–600 cm^{-1} range at 4 cm−1

Fig. S3 displays the outcome of characterizing the surface morphology of the biochar using a scanning electron microscope. The surface of BC 600 was smooth, with several clearly defined pores. High porosity is necessary for the capillary force to transfer outside ions into the interior pores of the biochar and for greater microbial mobility within the biochar. The biochar surface was roughened at high pyrolysis temperatures, most likely due to organic molecules volatilizing. According to earlier research, biochar frequently contains anoxic microsites that could encourage microbial development on the surface (Harter et al., 2014).

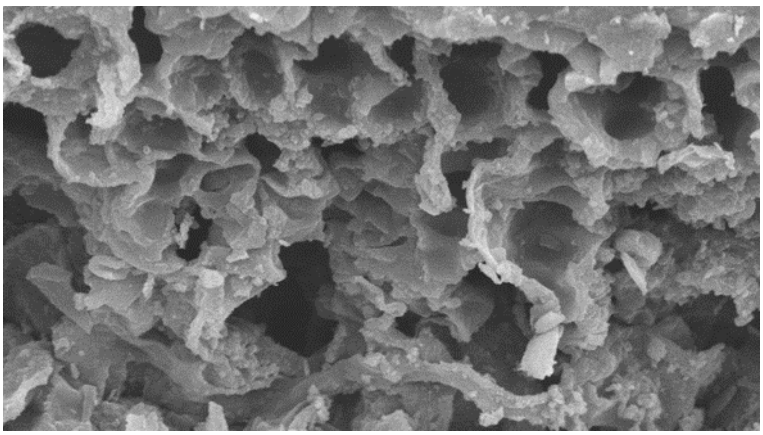

Fig. S3. Scanning electron microscope (SEM) image of biochar pyrolyzed at 600 °C.

The size of interactions between biochar and pollutants or nutrients is significantly influenced by the biochar's porosity and surface area (SA). Micro-, Meso-, and macro-pores are defined as pores with a size of 2 nm, 2–50 nm, and > 50 nm. As shown in Table S2, the generated biochar in this study has a porosity of 97 m^2/g and average pore size of 1.60 nm. The relative distribution of these pores affects interactions between biochar and ambient solutes, such as pollutants and nutrients. For instance, tiny molecules like gases and ordinary solvents can be absorbed by high efficiency micropores (2 nm) (Ambaye, 2021).

In general, oxidation and microbial degradation are less likely to affect biochar with rich aromatic structures. As a result, biochar's environmental resistance is correlated with its amount of aromatic condensation (Chen t et al., 2018). Compared to high-temperature biochar, low-temperature biochar is likely to contain less aromaticity (Chen et al., 2018).

Reference

Ambaye, T.G., Chebbi, A., Formicola, F., Prasad, S., Gomez, F.H., Franzetti, A. and Vaccari, M., 2022. Remediation of soil polluted with petroleum hydrocarbons, and their reuse for agriculture: Recent progress, challenges, and perspectives. Chemosphere, p.133572.

Ambaye, T.G., Vaccari, M., van Hullebusch, E.D., Amrane, A. and Rtimi, S., 2021. Mechanisms and adsorption capacities of biochar for the removal of organic and inorganic pollutants from industrial wastewater. International Journal of Environmental Science and Technology, 18(10), pp.3273-3294.

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