



UNIVERSITY OF MOLISE
Department of Biosciences and Territory

DOCTORAL THESIS

BIOLOGY AND APPLIED SCIENCE
Curriculum BIOLOGY
XXXVII Cycle

***ISOLATION AND CHARACTERIZATION OF HYDROCARBONOCLASTIC
BACTERIAL STRAINS AND THEIR POTENTIAL APPLICATION IN
BIOREMEDIATION***

S.S.D. BIOS-15/A - Microbiologia

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Academic year 2023/2024



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La natura non ha fretta, eppure tutto si realizza

(Lao Tzu)

I hereby declare that this thesis, submitted as partial fulfilment to obtain the academic degree of Doctor of Philosophy (Ph.D.) in Biology and Applied Science, is my own unaided work.

I have not used sources other than those indicated, and all direct and indirect sources are acknowledged as references. Parts of this dissertation have been published in international journals and/or conference articles.

January 13, 2026

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Abstract

The ongoing increase in energy demand and industrial development has resulted in a substantial discharge of hydrocarbons and heavy metals into the environment. These persistent contaminants have a profound impact on biogeochemical cycles, engendering significant ecological and health concerns. Conventional remediation technologies (physicochemical treatments) are often expensive, invasive, and sometimes incapable of restoring ecological functionality. In this scenario, bioremediation represents a *green* approach that leverages the metabolic capabilities of microorganisms to degrade or immobilize contaminants. This approach is in line with circular economy strategies and Nature-Based Solutions promoted by European policies.

This study explored two complementary lines of research to address the issue under discussion. The first focused on the isolation and characterisation of hydrocarbon-oxidising bacterial strains from sources naturally contaminated by petroleum hydrocarbons. The second involved the analysis of their genetic makeup and the assessment of their potential application as bioremediation tools, both as a microbial consortium alone or in synergy with plants, to enhance current bioremediation practices.

This approach is predicated on the observation that chronically contaminated environments can serve as reservoirs of microbial biodiversity with unique metabolic capabilities, and that the interaction between microorganisms and plants can enhance the efficiency of decontamination processes. The naturally hydrocarbon-contaminated springs of Tramutola, in the Agri Valley (Basilicata region, Italy), could function as a natural laboratory, wherein the presence of hydrocarbons and gases (H₂S) has led to the selection of hydrocarbon-oxidising microorganisms. Water and soil samples collected at this site were enriched in Bushnell–Haas medium with diesel oil as the sole carbon source. Four strains - *Gordonia amicalis* strain S2S5, *Rhodococcus erythropolis* strain S2W2, *Acinetobacter puyangensis* strain S1W1, and *A. tibetensis* strain S2S8 - were selected for the purpose of investigating their capacity for growth on diesel oil and their emulsification properties. Complete genomic sequencing revealed the presence of genes associated with the degradation of aliphatic and aromatic hydrocarbons and with the detoxification of heavy metals, thus confirming the biotechnological potential of these microorganisms.

The four strains were assembled into a microbial consortium and used, in an initial trial carried out in lake water mesocosms artificially contaminated with diesel oil, to evaluate their bioaugmentation capacity. Following a 28-day period, net of losses due to abiotic factors (11.84%), the bioaugmented mesocosm removed 19.11% of hydrocarbons, compared to 6.00% obtained through natural attenuation, thereby demonstrating superior efficacy. Metagenomic analyses revealed a remodelling of the microbial community, with an increase in the relative abundances of *Rhodococcus*, *Gordonia*, and other degrading taxa and a reduction in alpha-diversity.

The subsequent research then addressed the application to complex solid matrices. An historical industrial soil in Taranto (Apulia region, Italy), which had been historically contaminated with polychlorinated biphenyls (PCBs) and heavy metals, was selected for the experimental setup of mesocosms. The microcosms comprised four distinct conditions: untreated soil, bioaugmentation with the microbial consortium, planting of *Lavandula angustifolia*, and a combined treatment (consortium and plant). Following a 90-day period of observation, the combination of treatments resulted in the most significant reductions in lead (44.7%) and tin (66.9%), while concurrently leading to an increase in soil pH. Additionally, the combination treatment yielded the highest soil quality index (SQI = 0.73 versus 0.32 for the control). The integration of plant and microbial strains resulted in the stimulation of dehydrogenase activity, an increase in microbial abundance, and the selection of adapted communities. In contrast, bioaugmentation alone exhibited a reduced degree of persistence. The application of 16S rRNA gene sequencing analysis and functional prediction has revealed an enrichment of functions related to metal efflux, siderophore, and biosurfactants production in the combined treatment. This finding demonstrated the synergy between *L. angustifolia* and the bacterial consortium.

The present study has demonstrated that natural hydrocarbon contaminated environments can constitute reservoirs of microbial biodiversity useful for environmental biotechnology and that the isolated strains can offer high potential for bioremediation applications. The application of bioaugmentation, through the introduction of a selected microbial consortium, has been demonstrated to enhance hydrocarbon removal in contaminated waters. In the context of multi-contaminated soils, the integration of bioaugmentation with aromatic plants has been shown to

address the complexity of the soil matrix, thereby promoting the stabilisation of heavy metals and the recovery of soil quality. The thesis is divided into chapters that address the following topics: a review of the existing literature, the isolation and characterisation of hydrocarbonoclastic bacterial strains, bioaugmentation experiments carried out by using artificially hydrocarbon-contaminated lake waters, and the experimentation conducted on multi-contaminated soils with the combined use of the bacterial consortium and *L. angustifolia*. The thesis concludes with a summary of the scientific and applicative implications deriving from this approach.

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1 INTRODUCTION

1.1. ENVIRONMENTAL POLLUTION: THE CHALLENGE OF MULTI-CONTAMINATION

Environmental pollution has become one of the defining issues of the modern era, exerting profound impacts on natural ecosystems, public health, and global socio-economic development (Atakpa *et al.*, 2022), as conceptually illustrated in Figure 1.

Pollutants can be classified into categories - chemical, physical, physiological and biological (Englande *et al.*, 2015) - and their release into the environment by human activity has been observed to exceed the natural capacity of ecosystems to buffer and detoxify harmful compounds (Siddiqua *et al.*, 2022). The Italian legislative document that establishes environmental protection regulations is Legislative Decree No. 152 of April 3, 2006, which came into force on April 29, 2006. Title V of Part Four delineates the regulations pertaining to the remediation of contaminated sites. The decree defines a contaminated site as “a site in which the threshold concentrations of risk (CSR) are exceeded”, and the threshold concentrations of risk (CSR) as “the levels of contamination of environmental matrices, to be determined on a case-by-case basis with the application of the risk analysis procedure [...], the exceeding of which requires safety measures and remediation. The concentration levels thus defined are considered to be the acceptability levels for the site.”.

The consequences of such contamination are not limited to environmental degradation, but extend to human well-being (Shetty *et al.*, 2023), as when present in aerosols, contaminants can be inhaled, causing respiratory diseases (Damiati *et al.*, 2025), or they can be transferred from the soil to crops and subsequently enter the food chain, increasing risks to food safety and human health (Shetty *et al.*, 2025). Pollutants are typically categorised into two distinct classifications: namely, biodegradable and non-biodegradable pollutants. The former consists of sewage effluents and organic matter, both of which are known to decompose easily under normal circumstances. Non-biodegradable substances are defined as those that are not subject to degradation by microorganisms. During the last century, most organic pollutants derived

from the rapid growth of industrialisation, urbanisation, and intensive agriculture (Wasi *et al.*, 2013).

The European Environment Agency (EEA), the most recent update of which was in December 2024, estimates 2.8 million potentially contaminated sites across Europe, of which 1.4 million have actually been registered but are showing slow remediation progress. Of these, a mere 8.3% of the registered sites have been remediated, and 0.7% of the total registered sites are currently undergoing remediation.

Italy has approximately 42 Sites of National Interest (SIN), covering 148,594 hectares of land and 77,136 hectares of marine land. Of these, 11.4% of the registered contaminated sites have had their Risk Assessments approved, 53.4% have had their Remediation Proposals approved, and only 14.5% have had their remediation completed, and certification is pending.

A critical aspect of pollution is the simultaneous presence of different contaminants (organic and inorganic) within the same environmental matrix. This scenario is increasingly common in industrial areas, mining regions, and oilfields, where hydrocarbons derived from fossil fuel exploitation frequently coexist with heavy metals released from metallurgical, chemical, and agricultural activities (Lee *et al.*, 2025).

The co-existence of different classes of pollutants creates complex and often synergistic effects. For instance, hydrocarbons have been observed to form hydrophobic barriers that limit the diffusion of oxygen and nutrients (Kamranifar *et al.*, 2025), while the presence of heavy metals has been shown to inhibit key enzymes and destabilise cellular homeostasis, reducing the degradative capacity of microbial communities (Angon *et al.*, 2024; Rashid *et al.*, 2023). The combined effect of stress on microbial communities has been demonstrated to be deleterious, resulting in a reduction in biodiversity and an impediment to the intrinsic capacity of soils to self-remediate (Matei *et al.*, 2025; Sánchez-Corona *et al.*, 2025). The ecological consequences of multiple contamination are significant, with soils subjected to such stress often showing a reduction in microbial biomass (Szili-Kovács *et al.*, 2024) and diversity (Dixit *et al.*, 2024), reduced enzymatic activity (Flores *et al.*, 2025), alterations in biogeochemical cycles (Zaman *et al.*, 2025), and a loss of soil fertility (Ancona *et al.*, 2022; Lee *et al.*, 2020). Consequently, a decline in soil fertility has been demonstrated to impede the capacity of plants to support and grow (Ilinskiy *et al.*, 2023). In aquatic systems, the release of hydrocarbons and

metals has been demonstrated to disrupt planktonic communities (Mekonnen *et al.*, 2024), reduce primary productivity, and threaten biodiversity at multiple trophic levels.

From a socioeconomic perspective, multi-contamination events can lead to a loss of ecosystem services, decreased land values, and the necessity for costly remediation (Zou *et al.*, 2024). This is particularly problematic in low- and middle-income countries, where a lack of technical capacity and adequate regulatory frameworks hinders the effective and sustainable management of contaminated sites (O'Brien *et al.*, 2020). Multi-contamination represents a significant challenge for the modern environmental science community. As stated in the proposed EU Directive on Soil Monitoring and Resilience (Soil Monitoring Law, 2024) and the Water Framework Directive (WFD 2000/60/EC), in order to achieve the soil health objective by 2050 and to meet international commitments regarding zero land take and land degradation neutrality, it is necessary to reduce pollutant concentrations, restore ecological functions, and ensure the safe reuse of soil and water, promoting good environmental quality.

The present PhD research is positioned within this context, with a particular focus on two classes of contaminants: petroleum hydrocarbons (organic contaminants) and heavy metals (inorganic). These contaminants are distinguished by their persistence, toxicity, and ubiquity, and they frequently coexist, giving rise to complex contamination scenarios.

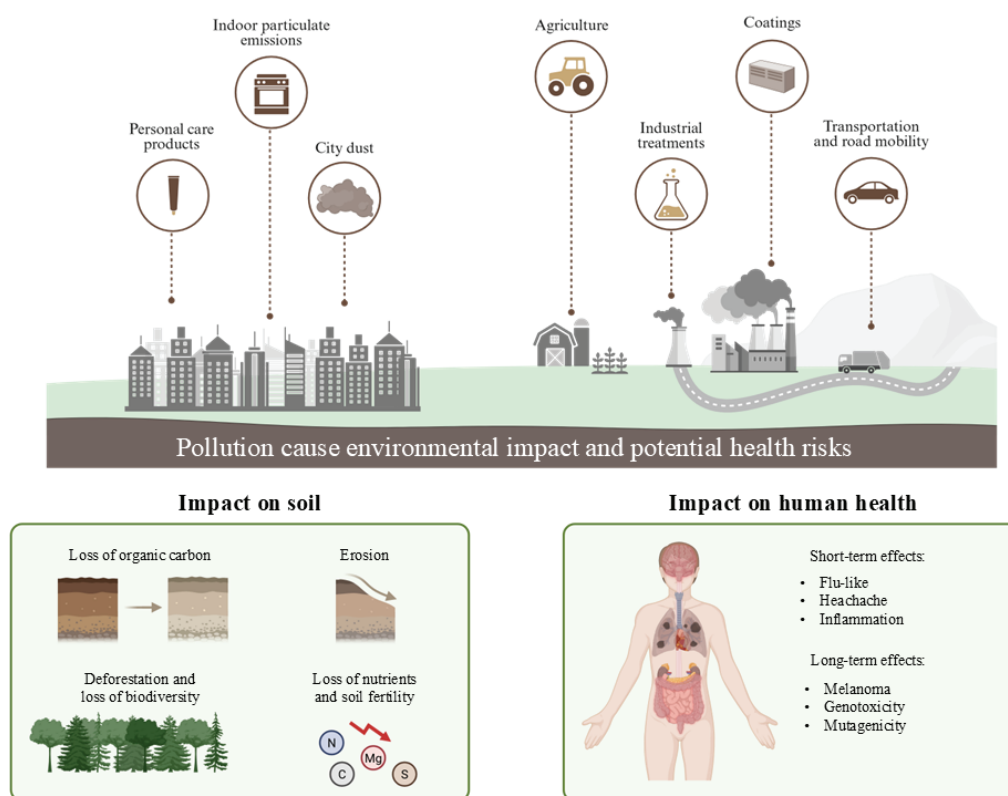


Figure 1 - The conceptual framework illustrates the origin of environmental pollution from multiple anthropogenic activities, the resulting degradation of soil properties and ecosystem services, and the potential short- and long-term impacts on human health. It emphasises the interconnected nature of environmental and public health risks.

1.2. PETROLEUM HYDROCARBONS: ENVIRONMENTAL IMPACT AND POTENTIAL HEALTH RISKS

Petroleum has been considered one of the world's most important raw materials since the beginning of the 20th century due to its transportable and easily usable nature. As a direct consequence of fossil fuel exploitation, refining, storage, and distribution, it was the most widespread and persistent organic contaminant released into the environment (García-Rincón *et al.*, 2024). Petroleum is a natural and heterogeneous mixture of hydrocarbons consisting mainly of alkanes with different chain lengths and branching points, cycloalkanes, monoaromatic or polycyclic aromatic hydrocarbons (PAHs). In addition to hydrocarbons, petroleum may contain resins and asphaltenes in smaller percentages. These components contain nitrogen, sulphur and oxygen, while traces of phosphorus and heavy metals, such as nickel and vanadium, may also be present (McGenety, 2014). Polycyclic aromatic hydrocarbons (PAHs) are a group of over one hundred organic compounds with two or more fused aromatic rings arranged in various configurations. These compounds are widely distributed in the environment as complex chemical mixtures (Teixeira *et al.* 2024). PAHs are classified as persistent organic pollutants (POPs) (United Nations Economic Commission for Europe, 1998) and their release into the environment has been shown to have several ecological impacts.

The environmental fate of these compounds is governed by several processes, including physical process such as volatilisation, chemical process such as oxidation, and biological process such as biodegradation. However, the high hydrophobicity of high molecular weight compounds limits their solubility and bioavailability, thereby slowing down their degradation. Their toxicity depends precisely on their bioavailability, as well as the concentration and duration of exposure. Once absorbed by living organisms, hydrocarbons interact with cellular receptors, causing lethal effects, especially in acute exposures (e.g., oil spills), or sub-lethal effects in prolonged exposures. Effects include nervous system damage, physiological and behavioural changes, lesions, developmental defects, and respiratory problems (Pandolfo *et al.*, 2023; Patel *et al.*, 2020). Hydrocarbon toxicity affects humans, plants, animals, and microorganisms, impacting biodiversity and ecosystem functioning.

In soils, the presence of hydrocarbons can form layers that are hydrophobic, thereby reducing both gas exchange and water infiltration. Hydrocarbon contamination also interferes with nutrient availability, particularly nitrogen and phosphorus, further limiting microbial growth and soil fertility (Mekonnen *et al.*, 2024). In aquatic ecosystems, the presence of oily films has been shown to reduce oxygen transfer at the air-water interface and to block sunlight penetration. This, in turn, can lead to a decline in primary productivity and changes in community composition across trophic levels (García-Rincón *et al.*, 2024).

1.3. THE ROLE OF HYDROCARBON-OXIDISING BACTERIA

Microorganisms such as bacteria, fungi, and algae have the ability to degrade hydrocarbons, but bacteria are reported as primary degraders and most active agents in the degradation of petroleum pollutants.

Bacteria capable of oxidising and degrading hydrocarbons represent a very important ecological and biotechnological resource for removing petroleum pollutants from contaminated ecosystems. These microorganisms, which are ubiquitous in nature, can use hydrocarbon compounds as their sole source of carbon and energy (Hossain *et al.*, 2021), playing a fundamental role in biogeochemical cycles and the natural attenuation of environmental contamination. The significance of these organisms has increased in response to the intensification of anthropogenic activities associated with the petroleum industry, which has resulted in a substantial escalation in soil and water contamination on a global scale.

The capacity to degrade hydrocarbons has been documented in a variety of bacterial genera, encompassing both Gram-negative and Gram-positive bacteria from diverse phyla (Das *et al.*, 2025). Among the *Proteobacteria*, the most extensively studied genera include *Pseudomonas*, *Acinetobacter*, *Alcanivorax*, *Marinobacter*, and *Burkholderia*. In the phylum *Actinobacteria*, the genera *Rhodococcus*, *Gordonia*, *Mycobacterium*, *Nocardia*, and *Dietzia* are of relevance for bioremediation applications (Xu *et al.*, 2018).

The genus *Rhodococcus* has been demonstrated to possess a high degree of metabolic plasticity, which enables it to degrade a variety of alkanes, including linear, branched, and cyclic structures, as well as polycyclic aromatic hydrocarbons (PAHs). Notably, *Rhodococcus*

maintains its activity under adverse environmental conditions, such as low temperatures (Thi Mo *et al.*, 2022; Ivshina *et al.*, 2022). The genus *Gordonia* has demonstrated a remarkable ability to degrade long-chain alkanes (Liu *et al.*, 2021) and produces ester-peptide biosurfactants that facilitate the absorption of hydrophobic substrates.

Regarding the degradation of PAHs, particular attention should be paid to the genus *Sphingomonas*, frequently isolated from oil-contaminated soils, which possesses a wide repertoire of catabolic pathways for the metabolism of aromatic compounds via dioxygenases and dehydrogenase enzymes (Zhou *et al.*, 2022).

A functional characteristic of significant biotechnological relevance of hydrocarbon-oxidising bacteria is their ability to produce biosurfactants (Rather *et al.*, 2025), amphipathic molecules that reduce surface and interfacial tension, thereby increasing the bioavailability of hydrophobic substrates. Surfactants improve the dispersion of contaminants in the aqueous phase and increase their bioavailability. Furthermore, as reported in the literature (Lee *et al.*, 2018), biosurfactants can replace synthetic surfactants and also offer the advantages of being less toxic, more biodegradable, and inexpensive to produce.

The classification of biosurfactants is based on their chemical structure and can be divided into the following categories: glycolipids (e.g. rhamnolipids, trehalose lipids, sophorolipids), lipopeptides, phospholipids, and polymers (Kaur *et al.*, 2024).

Rhamnolipids represent the most extensively studied bacterial biosurfactants. These glycolipids consist of one or two rhamnose units linked to β -hydroxy fatty acid chains and exhibit excellent emulsifying properties, low toxicity, and high biodegradability (Cerqueira dos Santos *et al.*, 2024). Rhamnolipids have been demonstrated to reduce oil-water surface tension, thereby facilitating the emulsification of hydrocarbons and their access to bacterial enzyme systems (Randhawa and Rahman, 2014).

Trehalose lipids, produced by *Actinobacteria* of the genus *Rhodococcus* (Andreolli *et al.*, 2023), are biosurfactants with versatile biochemical properties. Furthermore, these biosurfactants have been demonstrated to promote the formation of biofilms, thereby enhancing the efficiency of hydrocarbon degradation (Elayaperumal *et al.*, 2025).

The synthesis of biosurfactants is intimately linked to the development of biofilms at the oil-water interface. Biofilms constitute a microenvironment conducive to degradation activity,

increasing the contact area between bacterial cells and the hydrophobic substrate and protecting microorganisms from unfavourable environmental conditions.

It is evident that certain bacterial strains possess the capacity for broad-spectrum degradation (Rafeeq *et al.*, 2023). However, the complete mineralisation of petroleum hydrocarbons, which are intricate mixtures, typically necessitates the synergistic action of microbial consortia comprising populations with complementary enzymatic activities (Ibo *et al.*, 2024).

The metabolic synergies that occur within consortia can manifest themselves through different mechanisms. Some bacteria can produce biosurfactants, which increase the bioavailability of hydrocarbons (Sah *et al.*, 2022; Zahed *et al.*, 2022). Others initiate the oxidative attack, while still others are responsible for the metabolism of the intermediates that are produced during the process of degradation. This division of metabolic labour reflects the ecological principle that efficient hydrocarbon removal in nature is the result of the coordinated activity of diverse microbial communities rather than individual species (Qattan *et al.*, 2025).

The degradation of petroleum hydrocarbon pollutants by bacterial species has been extensively documented, and the metabolic pathways involved have been elucidated (Abbasian *et al.* 2015; Meckenstock *et al.* 2016). Initially, it was long considered to be an exclusively aerobic process, but subsequent research identified anaerobic degradation mechanisms (Abbasian *et al.*, 2015) that utilise different enzymes and hydrocarbon activation mechanisms.

1.4. METABOLIC PATHWAYS OF HYDROCARBON DEGRADATION

The process of microbial degradation of hydrocarbons occurs through specific metabolic pathways (Espinosa-López *et al.*, 2025). These pathways differ substantially depending on the availability of molecular oxygen. While aerobic pathways use oxygen as a co-substrate in initial oxidative reactions (Wang *et al.*, 2013), anaerobic pathways employ alternative activation mechanisms that do not require oxygen (Bian *et al.*, 2015).

Aerobic degradation of aromatic and aliphatic hydrocarbons (Figure 2.A) has proven to be the most effective method, however, it requires large amounts of oxygen and requires the intervention of enzymes, oxygenases (Melo *et al.*, 2025; Rojo *et al.*, 2020), which catalyse the incorporation of one or two molecular oxygen atoms into the substrate as OH groups (Niet *et al.*, 2014).

Subsequently, in the degradative metabolism of aliphatic hydrocarbons, two dehydrogenase enzymes (alcohol dehydrogenase and aldehyde dehydrogenase) sequentially reduce the primary alcohol generated by monooxygenase and the resulting aldehyde. Aldehyde dehydrogenase inserts (using H₂O as a donor) an additional oxygen atom, generating a simple fatty acid that will follow the fatty acid degradation pathway, namely β -oxidation, which leads to the formation of Acetyl-CoA molecules, channelled into the tricarboxylic acid cycle (or Krebs cycle). The final products are CO₂ and H₂O, and in this way, the organic contaminant molecule is mineralized by bacterial metabolism. Starting from fatty acids with an even number of carbon atoms, acetyl-CoA molecules are obtained; if the number is odd, a propionyl-CoA molecule remains, which is then converted into succinyl-CoA, an intermediate in the tricarboxylic acid cycle (Brzeszcz *et al.*, 2018).

The aerobic degradation of aromatic hydrocarbons involves the intervention of mono- and dioxygenase enzymes. The first reaction is hydroxylation by the dioxygenase; through the formation of a cis-dihydrodiol, a catechol molecule is obtained (Hu *et al.*, 2024; Tesfaye *et al.*, 2025). The catechol ring is then opened by a second dioxygenase that performs the opening at the ortho- (intradiol) or meta- (extradiol) position, generating intermediates that are subsequently converted into molecules of central metabolism.

Despite these mechanisms, the degradation of high-molecular-weight PAHs remains slow and often incomplete, explaining their persistence in contaminated environments (Pandolfo *et al.*, 2023).

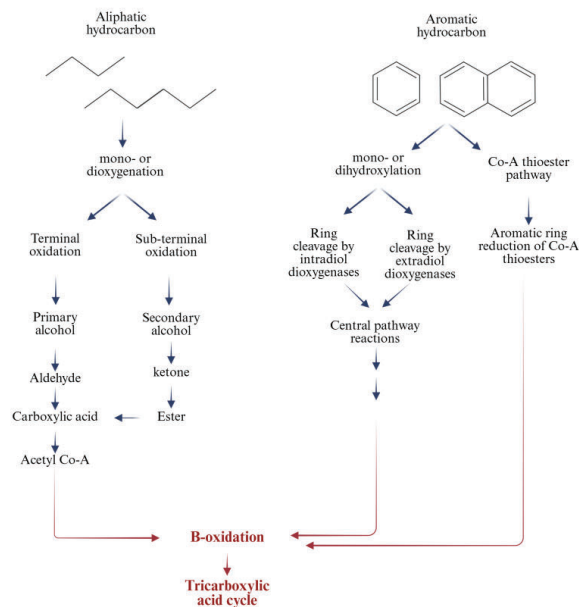
In environments where oxygen is scarce, bacteria employ alternative activation strategies to perform anaerobic hydrocarbon degradation (Ji *et al.*, 2019) (Figure 2.B).

Anaerobic pathways, though slower, are crucial for sustaining biodegradation in oxygen-limited environments, ensuring that hydrocarbons do not accumulate indefinitely in sediments or aquifers. Furthermore, many hydrocarbonoclastic bacteria can switch between aerobic and anaerobic metabolism, depending on environmental conditions, thereby increasing their ecological resilience (Qattan, 2025).

Recent advances in omics technologies - including metagenomics, transcriptomics, and metabolomics - have expanded our understanding of hydrocarbon degradation pathways. These approaches have revealed not only novel enzymes and metabolic intermediates but also the complex regulatory networks that control hydrocarbon catabolism under fluctuating conditions (Karnwal *et al.*, 2024). For instance, studies have shown that microbial consortia often employ division of labour, where one species initiates degradation while others metabolise the intermediates, increasing the overall efficiency of the process (Hu *et al.*, 2024).

In summary, the microbial degradative metabolism of petroleum hydrocarbons is a complex but highly adaptable system, encompassing both aerobic and anaerobic pathways. The efficiency and versatility of these processes explain the central role of microorganisms in natural attenuation and provide the mechanistic basis for their use in bioremediation. However, the persistence of certain hydrocarbon fractions, especially high-molecular-weight PAHs, underlines the need for continue research into optimising microbial degradation and integrating it with complementary remediation strategies.

A AEROBIC DEGRADATIVE METABOLISM



B ANAEROBIC DEGRADATIVE METABOLISM

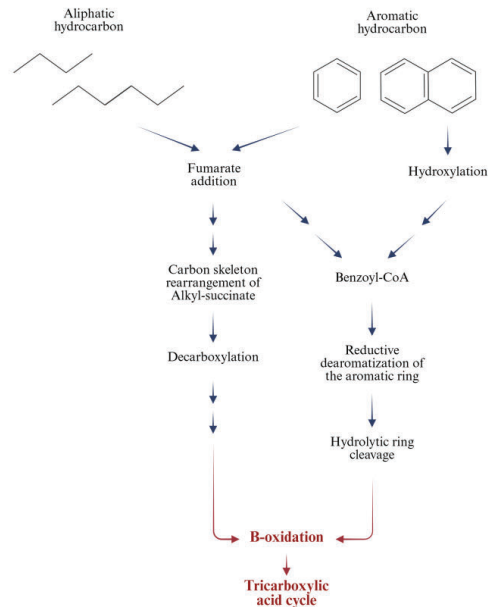


Figure 2 - The following schematic illustrates the aerobic and anaerobic hydrocarbon degradation pathways for aliphatic and aromatic compounds. A shows the aerobic pathways, while B shows the anaerobic pathways.

1.5. HEAVY METALS: ENVIRONMENTAL IMPACT, PERSISTENCE AND TOXICITY

Heavy metals (HMs) are a group of metallic elements with high density and high atomic mass. They represent a major class of environmental contaminants due to their persistence, toxicity, and ability to accumulate in living organisms (Nnaji *et al.*, 2024). The chemical form of the metal, as well as the environmental conditions (pH, salinity, redox state, and organic matter content), have been demonstrated to regulate its bioavailability and, consequently, toxicity (Sanchez-Corona *et al.*, 2025). Unlike organic pollutants such as hydrocarbons, which can be metabolised by microorganisms, heavy metals cannot be completely degraded (Cheng *et al.*, 2025; Jomova *et al.*, 2025). Instead, they persist indefinitely in soils and sediments, where they can undergo speciation, complexation, or redox transformations that influence their mobility and bioavailability (Adebayo *et al.*, 2025), leading to biodiversity loss and land degradation (Bender *et al.*, 2025). Their recalcitrant nature means that even low concentrations can pose long-term ecological and health risks, making them particularly problematic in multi-contaminated sites where they often co-occur with organic pollutants such as petroleum hydrocarbons or PCBs (Ancona *et al.*, 2021; Ancona *et al.*, 2017).

The most concerning heavy metals include cadmium (Cd), chromium (Cr), mercury (Hg), arsenic (As), lead (Pb), nickel (Ni), copper (Cu), and zinc (Zn), which are widely used in industrial production, mining, and agriculture. Many of these elements are not only toxic at elevated concentrations but also interfere with essential metabolic processes at trace levels (Ancona *et al.*, 2022). For example, HMs disrupt enzymatic activity and induces oxidative stress, while mercury binds to thiol groups in proteins, causing irreversible damage to cellular structures (Ancona *et al.*, 2021; Barra Caracciolo *et al.*, 2020). Because of their high affinity for organic ligands and their ability to bioaccumulate in food webs, heavy metals pose a significant risk to human health, with chronic exposure linked to carcinogenesis, neurological impairment, kidney dysfunction, and developmental abnormalities (Bora and Deka, 2025). The major risks to human health are reported in Table 1.

Table 1 - *Toxic effects of some heavy metals (HMs) on human health.*

Heavy Metals (HMs)	EPA Regulatory Limit (ppm)	Toxic Effect
Ag	0.10	Breathing problems, long and throat irritation and stomach pain
As	0.01	Alteration of oxidative phosphorylation and ATP synthesis
Ba	2.00	Cardiac arrhythmias, respiratory failure, gastrointestinal dysfunction, muscle twitching and elevated blood pressure
Cd	5.00	Chronic obstructive pulmonary disease (COPD), carcinogenic and mutagenic, interferes with the endocrine system
Cr	0.10	Inflammation of the respiratory tract, hair loss, damage sperm and the male reproductive system, decreased pulmonary function
Cu	1.30	Brain and kidney damage
Hg	2.00	Autoimmune diseases, depression, drowsiness, fatigue, hair loss, insomnia, memory loss, restlessness, visual disturbances, tremors, behavioral changes, brain, lung, and kidney damage
Ni	0.20	Allergic skin diseases, carcinogenic to the lungs, nose, sinuses, and throat if chronically inhaled, immunotoxic, neurotoxic, genotoxic, reduces fertility, hair loss
Pb	15.00	In children, it causes developmental delays, reduced intelligence, loss of short-term memory, learning difficulties and coordination; it increases the risk of cardiovascular disease.
Sn	Not determined	Nausea, vomiting, and diarrhea, and interfere with iron and copper metabolism (heme and cytochrome P450).
Zn	0.50	Gastrointestinal distress, neutropenia, neurological issues, reduced immune function

From an ecological perspective, heavy metal contamination can have a significant impact on soil function. Elevated concentrations have been shown to reduce microbial diversity and compromise the enzymatic processes involved in nutrient cycling, leading to a decline in soil fertility and biological activity (Barra Caracciolo *et al.*, 2020). In the agricultural sector, this can result in reduced crop productivity and the potential transfer of toxic metals into edible plant tissues, thereby compromising food security (Ancona *et al.*, 2021).

Heavy metals play complex roles in bacterial communities. Some are essential micronutrients, enabling crucial metabolic processes; others have harmful effects and

profoundly alter microbial communities. Heavy metals have the capacity to displace essential metals in proteins, impede protein synthesis and enzymes, and promote the formation of reactive oxygen species. This process has been shown to result in lipid peroxidation and membrane and DNA damage (Sanchez-Corona *et al.*, 2025; Barra Caracciolo and Terenzi, 2021). It has also been observed that metal ions exhibit a high affinity for chemical groups, such as sulfhydryl, phosphate, and hydroxyl groups. This interaction leads to competition for essential nutrients, thus altering ion homeostasis and inducing osmotic stress (Barra Caracciolo and Terenzi, 2021).

Heavy metal stress exerts strong selective pressure on microbial communities, favouring those microorganisms that have evolved adaptive strategies capable of not only ensuring survival but also enabling growth in environments characterised by highly toxic metal concentrations. These microorganisms possess specific metal resistance genes, frequently located on plasmids, transposons, and other mobile genetic elements. These genes can be transferred horizontally between different species, contributing to the spread of adaptive traits (Sanchez-Corona *et al.*, 2025). Bacterial tolerance to heavy metals is the result of a number of molecular and physiological mechanisms. These include the limitation of metal ion uptake, active efflux through highly specific transport systems, metal sequestration through binding proteins and chelating agents, and modification of metal speciation through redox transformations and precipitation processes into less bioavailable and less toxic forms (Figure 3) (Roy *et al.*, 2023). These responses are finely controlled by complex metal-sensitive gene regulatory systems, which modulate the expression of genes involved in detoxification and maintaining cellular homeostasis as a function of environmental conditions. In addition to this, these adaptive strategies represent a resource of great interest for the field of bioremediation, thanks to the ability of microorganisms to immobilise or transform metals and to interact synergistically with plants in the rhizosphere, improving the efficiency of phyto- and bioremediation processes (El-Baz *et al.*, 2025).



1.6. BIOREMEDIATION TECHNIQUES: A SUSTAINABLE ALTERNATIVE

There is an ever-increasing interest and need to remediate contaminated sites. Over the decades, a wide range of strategies has been developed to decontaminate polluted environmental matrices, resulting in the availability of remediation technologies. An overview of the main remediation technologies, ranging from conventional physico-chemical approaches to biological and nature-based solutions, is schematically presented in Figure 4.

Conventional approaches, based on physical and chemical interventions (e.g., excavation, incineration, washing, or stabilization), have been shown to entail substantial energy consumption, frequently incur exorbitant expenses, and yield residual materials that necessitate additional processing (Singh *et al.*, 2021; Tang *et al.*, 2024). Moreover, there is an incapacity to restore the ecological functionality of soil and biological integrity (Aparicio *et al.*, 2022).

Physico-chemical remediation technologies include chemical treatments, which typically rely on redox reactions to transform contaminants into less toxic or less mobile compounds; physical treatments, which aim to separate contaminants from the solid or liquid matrices and concentrate them for subsequent disposal or treatment; and thermal treatments, which induce contaminant desorption or volatilization, promote destruction via pyrolysis, or immobilize pollutants through vitrification of the solid matrix.

Despite their effectiveness in contaminant removal, these approaches often result in substantial environmental disturbance and limited long-term sustainability.

As a result, increasing research efforts have focused on biological and nature-based solutions that integrate efficiency with sustainability and cost-effectiveness.

The bioremediation process involves the utilisation of the metabolic capabilities of microorganisms and plants to facilitate the degradation or transformation of organic contaminants, as well as the immobilisation of inorganic pollutants (Qattan *et al.*, 2025). Unlike physicochemical methodologies, biological processes are predominantly applied *in situ* and are designed either to mineralize organic compounds or stabilise metals, thereby limiting the transfer of contaminants to other environmental compartments (Chicca *et al.*, 2022; Sharma *et al.*, 2023). This distinction is relevant because biological processes interact differently with organic and inorganic contaminants. Organic pollutants, such as hydrocarbons, can be

completely mineralized to CO₂ and H₂O through complex enzymatic pathways. In contrast, heavy metals are non-degradable. Bacteria can modify their chemical speciation, mobility, and bioavailability (Khatoon *et al.*, 2024), and have the capacity to reduce metal toxicity through processes such as redox reactions, precipitation, complexation or intracellular sequestration (Monroy-Licht *et al.*, 2025; Zhou *et al.*, 2023).

In addition, hyperaccumulating plants have been observed to extract and concentrate metals in biomass for subsequent harvesting (Abo-Alkasem *et al.*, 2023; Curiel-Alegre *et al.*, 2022).

The combined use of both microorganisms and plants has proven particularly effective in managing sites multiply contaminated by hydrocarbons and metals. Plants stimulate the growth and activity of degrading microbial communities through the release of root exudates (Ancona *et al.*, 2017; Ancona *et al.*, 2021; Barra Caracciolo *et al.*, 2020), which serve as carbon source and signalling molecules. These interactions accelerate hydrocarbon degradation and metal transformation, contributing to restore soil fertility and functionality. Experiments with clonal poplars in southern Italy have demonstrated that such systems have the capacity to immobilise metals and to stimulate the microbial community responsible for the degradation of polychlorinated biphenyls (PCBs) and hydrocarbons, thereby enhancing soil quality (Ancona *et al.*, 2017; Ancona *et al.*, 2021). An innovative approach is the use of aromatic plant species (Pandey *et al.*, 2024) such as lavender, rosemary, and sage, proposed as suitable candidates due to their stress tolerance, deep root systems, and ability to release secondary metabolites that promote beneficial microbial consortia.

In summary, the combination of microbial biotechnology and phytotechnology is a promising approach for the remediation of contaminated matrices. The inherent complexity of contaminants, namely hydrocarbons and heavy metals, necessitates an integrated and adaptable approach. This approach must take into consideration local microbial biodiversity, the selection of plant species, and the potential utilisation of organic amendments, such as biochar and compost, or chelating agents. The ensuing sections will analyse two key strategies for enhancing these processes: bioaugmentation/biostimulation and plant-assisted bioremediation.

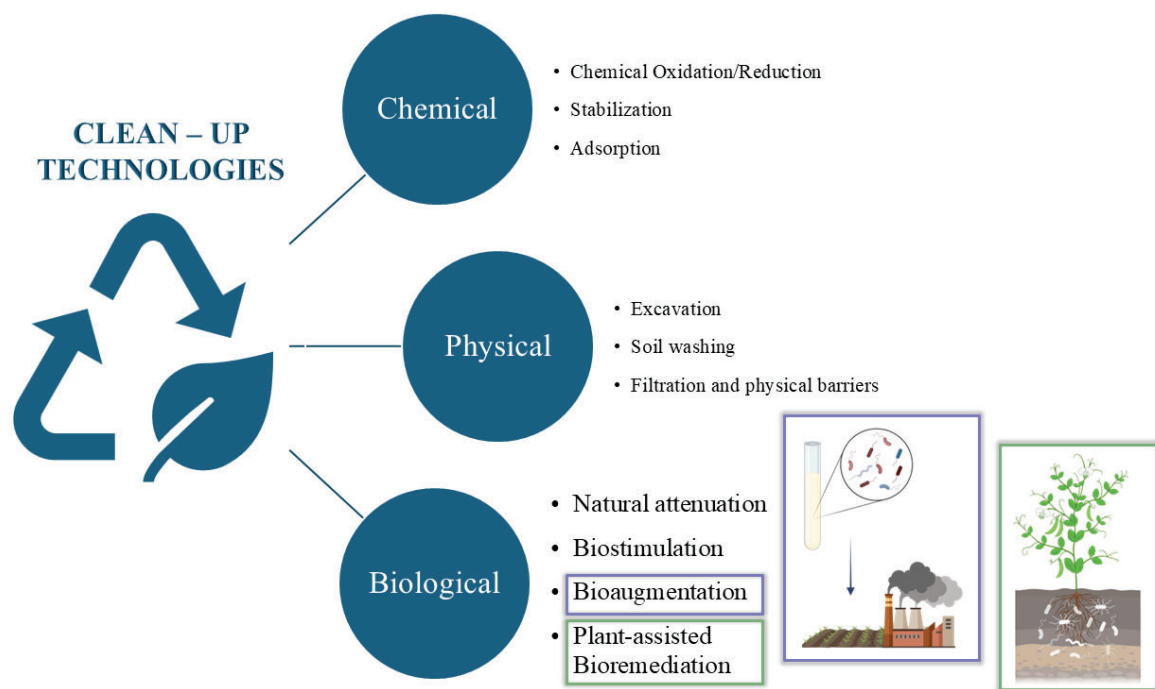


Figure 4 - Conceptual classification of conventional and biological remediation technologies, highlighting the role of bioaugmentation, biostimulation, and plant-assisted bioremediation as sustainable approaches for the management of contaminated soils.

1.7. BIOAUGMENTATION AND BIOSTIMULATION

Bioaugmentation and biostimulation are two complementary approaches that are widely used to enhance bioremediation using microorganisms (Jiang *et al.*, 2016) (Figure 5). Bioaugmentation is defined as the process of inoculating contaminated environment with selected microbial strains or consortia that possess high degradative capacity (Villaverde *et al.*, 2019), which can also be isolated from other polluted sites. The purpose of bioaugmentation is to increase the population of specific degraders that can attack the target pollutant (Chicca *et al.*, 2022; Das *et al.*, 2023).

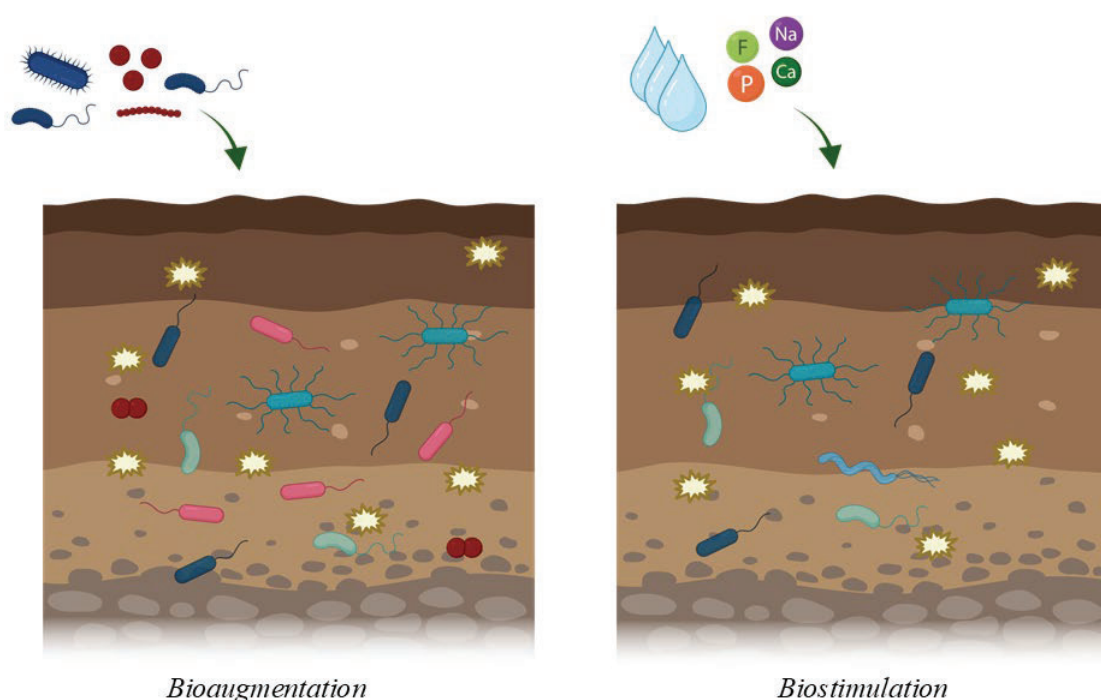


Figure 5 - Diagram of two microbiological approaches for the bioremediation of contaminated soils. On the left is bioaugmentation and on the right biostimulation.

Conversely, biostimulation aims to stimulate the metabolic activity of the indigenous microbial community already present in the environment by optimising environmental conditions. This typically involves limiting nutrients (sources of nitrogen, phosphorus, trace elements) and regulating the oxygen supply (e.g. through aeration or electron-accepting oxidising compounds) (Udume *et al.*, 2023). In certain cases, biosurfactants or co-substrates

are used to increase the bioavailability of hydrophobic contaminants (Chen *et al.*, 2020). The objective of both approaches is to enhance the rate and extent of biodegradation in comparison to the process that would occur through natural attenuation alone.

The utilisation of microbial consortia in bioaugmentation, as opposed to individual strains, is favoured due to their enhanced metabolic plasticity, which surpasses that of a single strain. However, both selected strains and constructed microbial consortia are likely to be successful in remediation because they are already adapted to the potentially hostile environmental growth conditions, which they will encounter during the process (Chicca *et al.*, 2022; Curiel-Alegre *et al.*, 2022). In the context of field interventions, the utilisation of native microorganisms, which have already adapted to the contaminant and the prevailing ecological niche, is considered optimal (Sharma *et al.*, 2024), especially when the concentration of pollutants to be removed is excessively high. The most effective method of contaminant degradation appears to be the isolation of microorganisms or consortia of microorganisms from sites that have been subject to long-standing historical contamination (Shokranian *et al.*, 2025; Goma-Tchimbakala *et al.*, 2023).

In order for the process to be successful, it is essential that the microorganisms are provided with optimal conditions for growth and development, including the injection of macronutrients, such as nitrogen and phosphorus, and other supplemental components, into the remediation sites to induce accelerated growth (Tyagi *et al.*, 2011).

The utilisation of biostimulation as a remediation technology constitutes an appealing approach for the bioremediation of hydrocarbon-contaminated sediments, primarily due to its capacity to promote well-adapted native microbial communities (especially in cases where the contamination is of a historical nature), which, however, may be deficient in crucial nutrients.

The degradation of petroleum hydrocarbons by a specific native microbial population or a consortium of indigenous microbes has been shown to be enhanced by the addition of specific nutrients to the soil or sediment (Li *et al.*, 2023). In addition to sources of phosphorus and nitrogen, it is often necessary to add surfactant molecules to the contaminated site to make the contaminant more bioavailable, although there is no consensus on whether the results are always positive (Szulc *et al.*, 2014).

It is conceivable that bacterial species which can degrade hydrocarbons may also possess the capacity to produce biosurfactants, thus rendering the bacterial strain more appealing for the purpose of bioremediation through bioaugmentation. Furthermore, biostimulation also represents a simple and cost-effective method for enhancing the natural biodegradation of pollutants present at sites requiring remediation.

1.8. PLANT-ASSISTED BIOREMEDIATION (PABR)

Plant-Assisted Bioremediation (PABR) can be regarded as the subsequent stage in the development of phytoremediation, whereby the cooperation between plants and rhizosphere microorganisms is employed for the purpose of decontaminating soil and water. In the rhizosphere, the soil layer immediately adjacent to the roots, plants release sugars, amino acids, and organic acids that serve as a substrate for microbial communities (Barra Caracciolo *et al.*, 2021) (Figure 6).

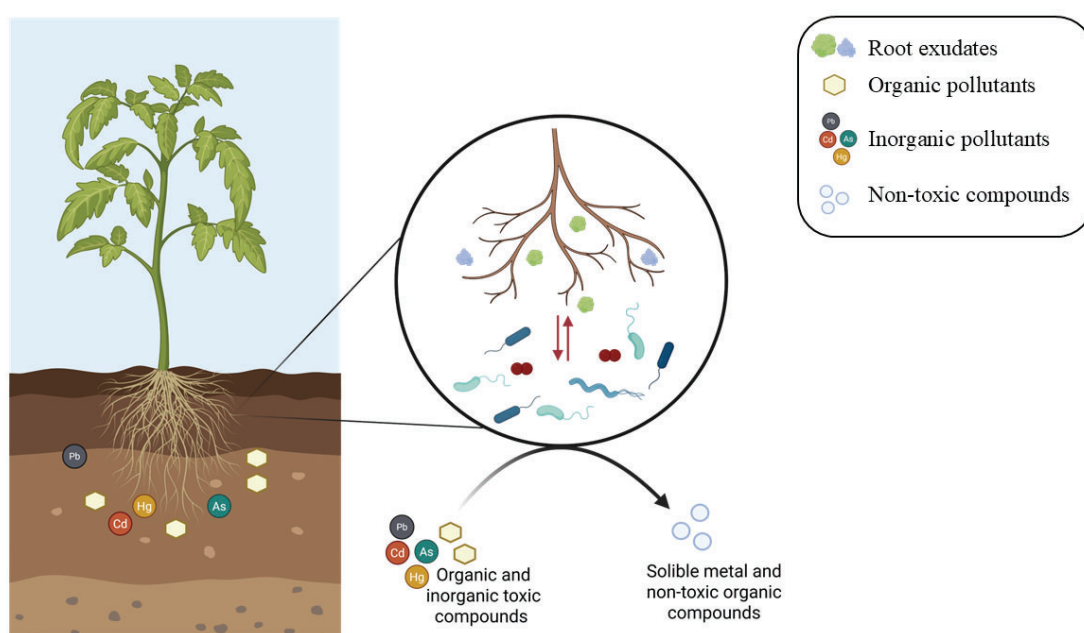


Figure 6 - A schematic description of cooperation between plants and rhizosphere microorganisms in Plant-Assisted Bioremediation.

These communities provide nutrients, protection from chemical stress, and degrade contaminants near the root system (Tang *et al.*, 2024; Curiel-Alegre *et al.*, 2022). In the context of organic pollutants, PABR is characterised by the process of rhizodegradation, whereby

microbial communities are stimulated by root exudates, accelerating their degradation (Conlon *et al.*, 2025). The root system provides physical support for the formation of biofilms (Liu *et al.*, 2025), increases soil porosity, facilitating the diffusion of contaminants and nutrients (Barra Caracciolo and Terenzi, 2021).

In the context of inorganic pollutants, such as heavy metals, PABR functions through multiple mechanisms, including phytoextraction (i.e. the absorption and accumulation of metals in leaves or stems), phytostabilization (i.e. immobilization in the soil), and rhizofiltration (i.e. the absorption from contaminated water). It has been established that microorganisms within the rhizosphere can produce siderophores and organic acids (Thepbandit and Athinuwat, 2024). The function of these substances is to solubilise metals, thereby increasing their availability for uptake. In addition, they can precipitate metals into less soluble forms (e.g. sulphides), thus reducing their mobility and toxicity. The interaction between hyperaccumulating plants and metal-tolerant microbial microorganisms facilitates the effective sequestration, degradation, or stabilization of contaminants such as As, Pb, Cd, Hg, and Cr. Plant-microbe synergistic association promotes the regeneration of ecosystem functions and represents a sustainable solution for agriculture and environmental protection (Dubey *et al.*, 2025). It has been demonstrated that rhizospheric microorganisms, including *Pseudomonas*, *Burkholderia*, *Bacillus*, and mycorrhizal fungi, work together to enhance metal uptake and detoxification (Joshi *et al.*, 2023) and release auxins, siderophores, and ACC deaminases, which promote root growth and reduce plant stress (Sharma *et al.*, 2025).

The formation of biofilms in the rhizosphere has been demonstrated to facilitate metabolic exchanges and cross-feeding, thereby supporting community resilience and organic and inorganic pollutants complete degradation (Dadhich *et al.*, 2025; Hnini *et al.*, 2025).

From an application perspective, plant-assisted bioremediation (PABR) provides both ecological and economic advantages compared with conventional remediation technologies. PABR minimizes soil disturbance and preserves soil structure, while simultaneously promoting stabilization of contaminants and the gradual restoration of soil fertility. Furthermore, it reduces erosion, improves soil physical properties, and promotes nutrient cycling, thus contributing to the restoration of ecosystem functions and supporting biodiversity.

In addition to environmental benefits, PABR offers significant economic opportunities through the valorisation of plant biomass. The use of non-food and non-forage crops, such as aromatic plants, allows contaminated sites to be managed without entering the food chain, while also enabling the production of valuable secondary products, including essential oils and other bioactive compounds.

Nevertheless, there are still critical issues to be addressed, including the selection of the most suitable plant species, the time required for plant growth, the management of contaminated biomass, and the adaptation to multiple contaminants. Addressing these issues will require multidisciplinary studies combining microbiology, agronomy, and environmental engineering, as well as the development of omics-based monitoring systems to follow the evolution of microbial communities and the effectiveness of processes over time.

1.9. *LAVANDULA ANGUSTIFOLIA*: AN IDEAL CANDIDATE FOR PLANT-ASSISTED BIOREMEDIATION

Lavandula angustifolia Mill. is a perennial aromatic shrub that belongs to the *Lamiaceae* family (Caprari *et al.*, 2021). It is cultivated in several regions for ornamental, medicinal, and industrial purposes, including the Mediterranean basin and other temperate regions (Simsek *et al.*, 2024). Beyond its traditional applications in perfumery and phytotherapy (Hlihor *et al.*, 2022), lavender has recently attracted increasing attention as a promising species for phytoremediation (Pandey *et al.*, 2024; Pirsandib *et al.*, 2022) due to its ecological resilience, capacity to interact with soil microbial communities, and economic value derived from essential oils. It is evident that a combination of characteristics inherent in this plant render it particularly well-suited for the purpose of contaminant remediation in soils exhibiting multiple contaminants.

From an agronomic perspective, lavender demonstrates a high level of adaptability to marginal and degraded environments, exhibiting tolerance to abiotic stresses such as drought, salinity, and nutrient-poor conditions, which are frequently observed in polluted sites (Pandey *et al.*, 2024). Its perennial growth habit, robust root system, and ability to persist for several years without replanting ensure soil cover and long-term stabilization, reducing erosion and

promoting rhizosphere activity essential for pollutant transformation (Simsek *et al.*, 2024; Hlihor *et al.*, 2022). It has been demonstrated through experimental studies that *L. angustifolia* possesses the capacity to tolerate and, to a certain extent, accumulate heavy metals such as cadmium, lead, copper, and zinc, without substantial reductions in biomass or essential oil yield (Stefan *et al.*, 2025). It is important to note that analyses consistently report that metals do not transfer into the essential oils, which remain uncontaminated and thus safe for industrial applications (Hlihor *et al.*, 2022; Kaveh, 2025). This characteristic confers a substantial advantage over food crops, as it eliminates the risk of contaminant entry into the food chain, while still enabling the economic valorisation of biomass from polluted soils.

It is important to note the role of lavender in shaping the microbial community of the rhizosphere. As with other aromatic and medicinal plants, *L. angustifolia* releases a complex array of root exudates rich in sugars, amino acids, phenolics, and terpenoids, which modulate the composition and activity of soil microorganisms (Rasouli *et al.*, 2022; Barra Caracciolo *et al.*, 2020). These compounds have been demonstrated to enhance the proliferation of plant growth-promoting rhizobacteria and microbial consortia, thereby facilitating the detoxification/degradation of inorganic and organic contaminants in contaminated sites (El-Baz *et al.*, 2025). Furthermore, the secondary metabolites produced by lavender possess strong antioxidant and antimicrobial properties that contribute to stress tolerance under adverse conditions, mitigate oxidative damage induced by pollutants, and influence the ecological balance of the rhizosphere by suppressing pathogens and favouring beneficial microbes (Crisan *et al.*, 2023; Angelova *et al.*, 2015). This interplay between physiological adaptations and rhizospheric interactions exemplifies the multifunctional contribution of lavender to soil recovery processes.

The cultivation of lavender on polluted or marginal lands also offers unique socio-economic benefits. In contrast to many hyperaccumulator plants that are devoid of commercial value, lavender is a highly lucrative crop due to the surging demand for its essential oil in the pharmaceutical, cosmetic, and food industries (Simsek *et al.*, 2024). Furthermore, lavender provides ecosystem services that extend beyond the scope of phytoremediation, encompassing the conservation of biodiversity through pollinator support, carbon sequestration, and the protection of soil against erosion (Pandey *et al.*, 2024; Zaman *et al.*, 2024).

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2 OBJECTIVES

In light of the considerations discussed above, the present doctoral research has been conceived with the overarching aim of investigating sustainable strategies for the remediation of environments affected by complex contamination, focusing on the integration of microbial and plant-based approaches. The study was grounded in the recognition that the simultaneous presence of contaminants represents one of the most challenging scenarios in environmental biotechnology, requiring interventions that are both effective and ecologically sustainable.

The first objective of this work was the isolation and characterisation of hydrocarbonoclastic bacteria from naturally hydrocarbon-contaminated environments. These microorganisms have been key agents in the biodegradation of hydrocarbons, and their study has provided insights into catabolic versatility and adaptive traits that may be exploited for remediation purposes.

Following the isolation step, selected strains were subjected to a multilevel characterisation. This included physiological and biochemical profiling, assessment of salinity tolerance, and whole genome sequencing to clarify their genetic potential for hydrocarbon degradation and environmental adaptation. Additionally, mesocosm-scale experiments were conducted to evaluate the biodegradation performance of selected strains under controlled contamination conditions. This objective formed the foundation of the research, enabling the construction of a robust microbial toolkit suitable for subsequent bioaugmentation strategies.

The second objective was the evaluation of plant–microbe interactions, with particular emphasis on the synergistic potential of *Lavandula angustifolia* combined with a selected bacterial consortium. The choice of lavender was motivated by its ecological resilience, low metal translocation to economically valuable products, and capacity to stimulate rhizosphere microbial communities. Microcosm experiments were designed to test this integrated approach on heavy metal-contaminated soils, assessing a broad set of parameters that include soil physico-chemical properties, enzymatic activities, microbial abundance, and indices of soil quality. This part of the research explored how microbial inoculants and aromatic plants can act together to enhance soil functionality while opening opportunities for economic valorisation.

The third objective was to clarify the ecological and molecular responses of soil microbial communities to different remediation strategies through metabarcoding-based analyses. High-throughput sequencing of soil microbial DNA enabled detailed characterisation of community diversity, structure, and changes in response to different bioremediation treatments. By integrating molecular data with soil quality indicators and functional parameters, this objective provided insights into the ecological sustainability of remediation practices. Specifically, it allowed us to assess how plant-microbe consortia reshape microbial communities, promote the restoration of ecosystem functions, and contribute to long-term soil resilience.

These objectives form a coherent and progressive framework. It begins with the identification and genomic characterisation of microbial resources. It continues with their functional validation and experimental application in synergy with plants. Finally, it ends with the molecular-scale assessment of ecosystem responses. This multidisciplinary approach could generate new insights into the feasibility, efficacy, and scalability of integrated bio- and phytoremediation strategies for multi-contaminated soils. This would highlight their ecological, technological, and socio-economic relevance.

The overall PhD research work has been published as scientific articles and it is summarised as it follows:

1. Natural Hydrocarbon-Contaminated Springs as a Reservoir of Microorganisms Useful for Bioremediation: Isolation and Multilevel Analysis of Hydrocarbonoclastic Bacteria from the Agri Valley (Southern Italy).

Sustainability 2025, 17, 3083. <https://doi.org/10.3390/su17073083>

2. Synergistic Effects of *Lavandula angustifolia* and a Bacterial Consortium on Bioremediation of a Heavy Metal-Contaminated Soil.

Plants 2025, 14, 2734. <https://doi.org/10.3390/plants14172734>

3. Metagenomic Insights into Plant–Microbe Synergies for heavy metals contaminated soil remediation.

The paper is submitted - Manuscript Number NBIOT-D-26-00013 - to *New Biotechnology Journal* (Elsevier).

3 CHAPTER I

ISOLATION AND CHARACTERISATION OF HYDROCARBON- OXIDISING BACTERIAL STRAINS

PREFACE

The present study constitutes the primary published contribution of my doctoral research. Its central focus is the isolation and multilevel characterisation of hydrocarbon-oxidising bacterial strains from naturally contaminated environments in the Agri Valley (Basilicata region, Southern Italy). The rationale behind focusing on this specific geographical area pertains to its unique hydrogeological and environmental characteristics. The Agri Valley boasts a number of natural hydrocarbon springs, the existence of which was first documented at the close of the 19th century. The Agri Valley is an intramountain valley, 30 km long and 12 km wide, with a northwest/southeast orientation. The study concentrated on a specific area of the valley, encompassing two springs (S1 and S2) with natural hydrocarbon outcrops. Such contexts represent a privileged niche for microbial communities that have evolved under persistent hydrocarbon exposure, developing metabolic traits of considerable biotechnological interest.

Hydrocarbon contamination poses a critical threat to environmental health and ecosystem services, necessitating the development of efficient and sustainable remediation strategies. Despite their widespread application, conventional physicochemical methods are frequently associated with substantial financial costs, invasiveness, and deleterious environmental impact.

Conversely, bioremediation proffers a more ecologically compatible alternative, capitalising on the intrinsic capacities of microorganisms to completely degrade or transform hydrocarbons into less harmful compounds.

However, the success of biological approaches is contingent upon a comprehensive understanding of the physiology, genetics, and ecological interactions of the microbial taxa involved. In particular, the identification and characterisation of native hydrocarbon-oxidising bacteria have represented a crucial step towards the design of microbial consortia with enhanced bioremediation potential.

The research article integrated classical microbiological methods for the isolation of hydrocarbon-oxidising bacterial strains with genomic analyses to explore the microbial diversity associated with hydrocarbon-contaminated springs and their surrounding soils. The study also evaluates the effectiveness of bioaugmentation using a four-strain microbial consortium to remove hydrocarbons from lake waters artificially contaminated with diesel fuel in mesocosm experiments. This provides a comprehensive perspective on the capabilities of selected bacterial strains (Figure 7). This study combines phenotypic characterisation, genome sequencing and functional prediction to achieve two objectives. Firstly, it advances the taxonomic and ecological understanding of hydrocarbonoclastic bacteria. Secondly, it addresses their practical role in bioaugmentation strategies.

The objective of this research was to underscore the promise of naturally contaminated sites as reservoirs of novel microbial species with potential applications in environmental biotechnology. The research also sought to characterise hydrocarbon-containing bacteria isolated from naturally hydrocarbon-contaminated springs and surrounding soils. Furthermore, the study evaluated the effectiveness of bioaugmentation using a four-strain microbial consortium or in association with plants, in different environmental contexts, such as lake waters.

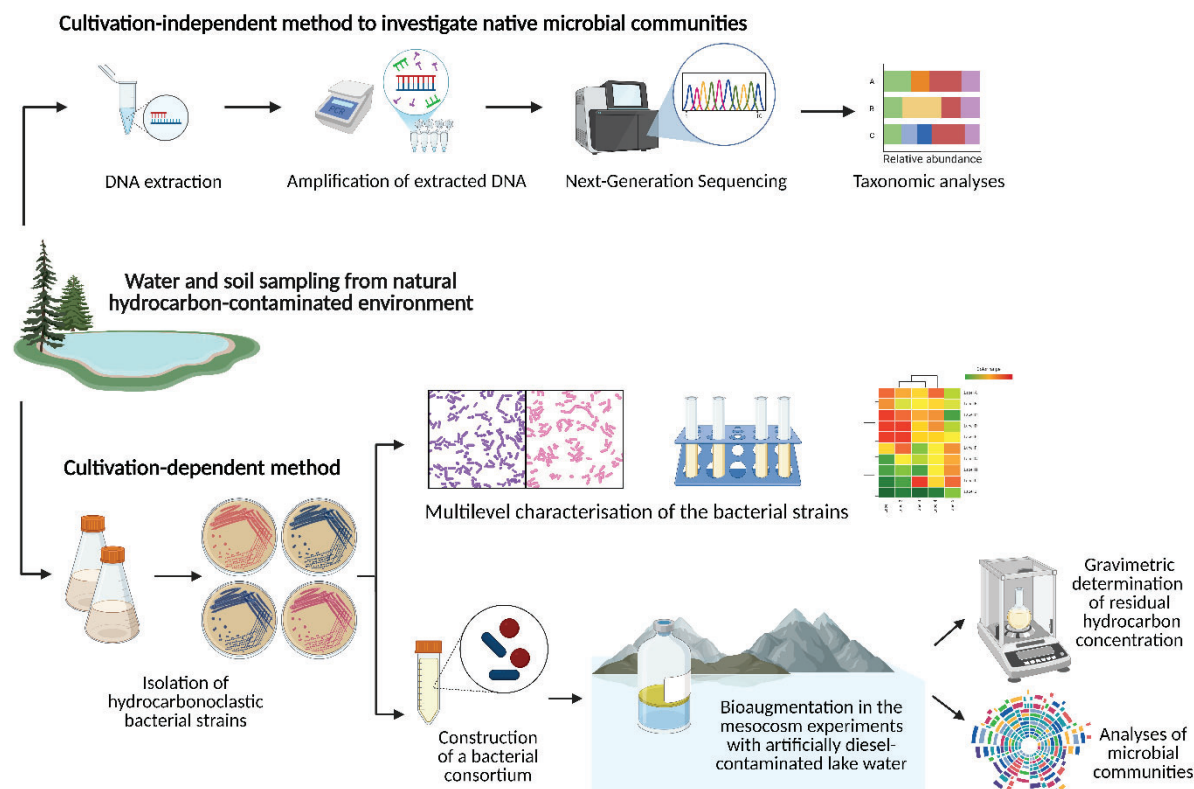


Figure 7- The graphical abstract of the work carried out comprises an integration of classical microbiological methods (culture-dependent) with metagenomic analyses (culture-independent), beginning with water and soil sampling from the naturally hydrocarbon-contaminated site. It then progresses to mesocosm experiments, providing a comprehensive perspective on the capabilities of selected bacterial strains.

3.1. Natural Hydrocarbon-Contaminated Springs as a Reservoir of Microorganisms Useful for Bioremediation: Isolation and Multilevel Analysis of Hydrocarbonoclastic Bacteria from the Agri Valley (Southern Italy)

Article

Natural Hydrocarbon-Contaminated Springs as a Reservoir of Microorganisms Useful for Bioremediation: Isolation and Multilevel Analysis of Hydrocarbonoclastic Bacteria from the Agri Valley (Southern Italy)

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Abstract: This research aimed to characterise hydrocarbonoclastic bacteria isolated from naturally hydrocarbon-contaminated springs and the surrounding soils in the Agri Valley (Southern Italy) and to assess the effectiveness of bioaugmentation using a four-strain microbial consortium for removing hydrocarbons from artificially diesel-contaminated lake waters in mesocosm experiments. Four novel bacterial strains were selected for the experimentation: *Gordonia amicalis* S2S5, *Rhodococcus erythropolis* S2W2, *Acinetobacter tibetensis* S2S8, and *Acinetobacter puyangensis* S1W1. The four isolates can use diesel oil as their sole carbon source, and some exhibited a relatively high emulsifying capacity and ability to adhere to hydrocarbons. Furthermore, genome analyses revealed the presence of genes associated with the degradation, detoxification, and transport of various contaminants. Mesocosm experiments demonstrated that the bioaugmentation enhanced the capacities of the native lake microbial communities to remove hydrocarbons, although drastic changes in their composition (analysed through Next-Generation Sequencing—NGS) were observed. Taken together, these results suggest that naturally contaminated environments can serve as a valuable reservoir of microorganisms with significant biotechnological potential, particularly in the field of bioremediation. However, a complete understanding of the ability of the isolated bacterial strains to efficiently degrade contaminants requires further research to fully assess their capabilities and limitations across different settings.

Keywords: oil contamination; hydrocarbonoclastic bacteria; bioremediation; bioaugmentation; microbial consortium



Academic Editor: Agostina Chiavola

Received: 14 February 2025

Revised: 24 March 2025

Accepted: 25 March 2025

Published: 31 March 2025

Citation: Cavone, C.; Monaco, P.; Fantasma, F.; Rizzo, P.; Tarracchini, C.; Petraro, S.; Ventura, M.; Milani, C.; Celico, F.; Naclerio, G.; et al. Natural Hydrocarbon-Contaminated Springs as a Reservoir of Microorganisms Useful for Bioremediation: Isolation and Multilevel Analysis of Hydrocarbonoclastic Bacteria from the Agri Valley (Southern Italy). *Sustainability* **2025**, *17*, 3083. <https://doi.org/10.3390/su17073083>

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1. Introduction

Environmental hydrocarbon contamination is a critical global challenge, with far-reaching implications for ecosystem sustainability [1]. Hydrocarbons, which are ubiquitous

and can have long-term toxic effects on living organisms, pose a significant concern for environmental health and the future of Earth's ecosystems [2–4]. These chemical compounds, primarily derived from crude oil and its derivatives, serve dual roles as both essential resources and environmental hazards [5].

Crude oil and its by-products are strategic commodities that drive modern society's socio-economic and industrial advancements, representing key energy sources for transportation, agriculture, and manufacturing [6,7]. As a result, due to the crucial role of crude oil in shaping various aspects of modern life, global demand for this resource continues to grow steadily [8].

The International Energy Agency (IEA) highlighted this persistent world oil demand in its November 2024 Oil Market Report (OMR), projecting an increase by 920 thousand barrels per day (kb/d) in 2024, reaching a total consumption of 102.8 million barrels per day (mb/d), and nearly 1 million barrels per day (mb/d) in 2025, leading to a total consumption of 103.8 mb/d. These figures demonstrate the enduring reliance on hydrocarbons as a fundamental component of global energy systems [9].

Despite their utility, the extensive exploitation of hydrocarbons has led to significant environmental consequences [10–12].

One of the most pressing concerns is oil spills, which can occur due to both natural processes and human activities [13,14]. The environmental impacts of such contamination are diverse, affecting soil, water, air quality, and biodiversity [15]. Consequently, hydrocarbon pollution represents a significant ecological burden, requiring comprehensive, interdisciplinary approaches to mitigate its impact [16].

Addressing these challenges necessitates the development of advanced strategies focused on balancing hydrocarbon utilisation and environmental protection [17,18].

Among the various remediation strategies, physico-chemical approaches such as chemical oxidation and soil washing are costly, invasive, and often associated with undesirable side effects, including the generation of toxic by-products. Biological approaches, in contrast, offer a more environmentally friendly and cost-effective alternative. These strategies leverage the unique capabilities of microorganisms and plant species to remove or degrade persistent and hazardous pollutants [19], presenting a sustainable solution [15,20,21].

Microorganisms play a significant role in maintaining ecosystem stability and can be employed to mitigate oil pollution. For instance, bacteria can adapt to oil-contaminated environments, utilising hydrocarbons as a source of carbon and energy for growth [22].

Specific enzymatic biodegradation mechanisms, in the framework of aerobic or anaerobic metabolism, facilitate their transformation into less harmful by-products or mineralisation and conversion into CO₂, CH₄, and H₂O [23].

Bioremediation strategies may be classified into two principal categories: biostimulation, which enhances environmental conditions (e.g., pH and nutrient availability) to optimise microbial activity [18,24,25], and bioaugmentation, which involves the introduction of specific microbial strains or consortia to improve remediation effectiveness [21,26,27]. Nevertheless, the large-scale application of biological approaches faces several challenges. These include the following: (1) the environmental variability, which may limit the effectiveness of introduced microorganisms [15,21]; (2) the toxicity of contaminants, which may inhibit microbial growth [28]; and (3) the complexity of contaminants, which often requires cooperative interactions between different microbial species, highlighting the need for a deeper understanding of microbial community composition and dynamics [21,28].

Moreover, integrating advanced technologies such as genetic engineering and metagenomic analysis could significantly enhance the efficacy and sustainability of bioremediation strategies, despite the challenges involved.

The primary aims of this study were to isolate and characterise hydrocarbonoclastic bacteria from naturally hydrocarbon-contaminated springs located in the municipality of Tramutola (Agri Valley, Southern Italy), and to preliminarily assess their potential for remediating polluted environments. This was accomplished through a combination of traditional microbiological techniques, genome analyses, and mesocosm experiments. Despite the challenges posed by oil pollution, indigenous bacteria that have evolved in petroleum-enriched ecosystems—capable of metabolising or degrading hydrocarbons for growth and reproduction—may, in fact, represent promising candidates for the remediation of oil-contaminated sites and the enhancement of purification efficiency [29].

2. Materials and Methods

2.1. Study Area and Sample Collection

A sampling campaign was conducted in June 2022 at two natural hydrocarbon springs, designated as S1 and S2 (S1: 40.3228 N, 15.7598 E; S2: 40.3227 N, 15.7585 E), located within the municipality of Tramutola (Basilicata region, Southern Italy) (Figure 1), to isolate hydrocarbonoclastic bacteria and characterise the native microbial communities. The springs are situated in the Agri Valley, a prominent northwest–southeast oriented intramountain basin that extends approximately 30 km in length and has an average width of 12 km [29]. This valley is geologically significant, as it hosts Europe’s largest onshore oil and gas field, with complex geological structures [30].

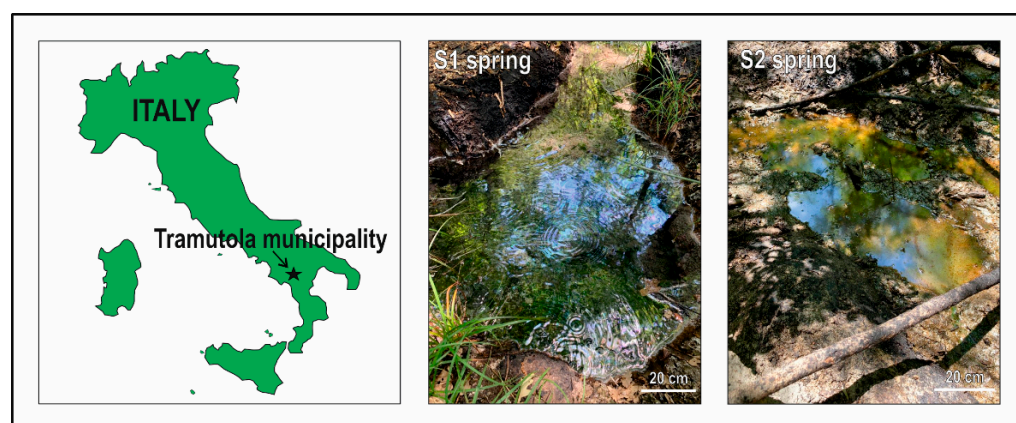


Figure 1. Localisation of the study area (on the left) and hydrocarbon springs S1 and S2 (on the right).

Water and soil samples were collected from S1 and S2 springs and surrounding areas for microbial community analysis and isolation of hydrocarbon-degrading bacterial strains. Water samples (2 L) were collected from each spring in sterile 1 L bottles, while 20 g of soil was sampled using sterile spoons and stored in 50 mL tubes. To maintain sample integrity, they were immediately refrigerated and transported to the laboratory.

2.2. Isolation and Genome Sequencing of Hydrocarbon-Oxidising Bacterial Strains

To isolate hydrocarbon-oxidising bacterial strains, a slightly modified version of the method described previously by Rizzo et al. [29] was followed. Aliquots of water samples (10 mL) and soil samples (1 g) were inoculated in 40 mL Bushnell–Haas medium (MgSO_4 0.2 g/L; CaCl_2 0.02 g/L; KH_2PO_4 1.0 g/L; K_2HPO_4 1.0 g/L; NH_4NO_3 1.0 g/L; FeCl_3 0.05 g/L), supplemented with diesel oil at a concentration of 2% v/v as the sole carbon source. The diesel oil was purchased from an Eni Spa petrol station, an Italian multinational corporation operating in the oil and gas sector. Cultures were incubated at 28 °C for 7 days with agitation (100 rpm). The enrichment step was repeated for four cycles. From the first enrichment cycle, 100 μL aliquots of the cultures were spread on Bushnell–Haas (BH) agar

medium supplemented with diesel oil (2% *v/v*) as a carbon source. The colonies grown on the plates were repeatedly streaked over the entire surface of fresh BH agar medium supplemented with diesel oil and finally onto LB agar plates to ensure that pure cultures were obtained.

The isolated hydrocarbonoclastic bacterial strains were initially characterised phenotypically through the analysis of colony morphology, microscopic observations, Gram staining, and catalase production tests. Four strains (named S1W1, S2W2, S2S5, and S2S8), which exhibited distinct phenotypic traits and faster growth in BH medium supplemented with diesel oil (optical density measurements at a wavelength of 600 nm), were selected for further investigation and subjected to 16S rDNA partial sequencing.

The obtained 16S rDNA sequences were then compared with those stored in the GenBank database at the NCBI (National Center for Biotechnology Information) by using the BLAST v.2.13.0 (Basic Local Alignment Search Tool) program.

To obtain a comprehensive genetic characterisation, the genome of the four selected isolated bacterial strains was sequenced using the MiSeq Software v4.0 (Illumina, San Diego, CA, USA) at GenProbio srl, Parma, Italy. Individual genomic libraries were obtained using the Nextera XT preparation kit and loaded into a MiSeq flow cell (MS-102-3003, Illumina) with 500-cycle sequencing and paired-end reads of 250 bp. The MiSeq sequencer was used for sequencing according to the manufacturer's instructions. The raw reads in FASTQ format obtained from the genome sequencing phase were filtered and assembled using the MEGAnnotator2 pipeline [31]. After quality control, the assembled reads were assigned to taxonomic categories using the taxonomic assignment module integrated in MEGAnnotator2 [31]. Subsequently, the obtained contigs were grouped according to the assigned taxonomy. Accounts smaller than 5000 bp were discarded. The whole-genome sequences decoded in this study have also been deposited in the NCBI Sequence Read Archive under the accession number PRJNA1223154.

2.3. Comparative Genome Analysis of the Selected Hydrocarbon-Oxidising Bacterial Strains

The assembled genomes were then annotated by the MEGAnnotator2 pipeline [31] using the Prokka software v1.14.6 [32] to identify and annotate the gene content of bacterial strains obtained from NCBI, as well as microorganisms isolated from the natural hydrocarbon springs and the surrounding soils. This process also enables the prediction of protein-coding sequences from the genomic data.

Protein sequences shared among all genomes of the same species were selected to reconstruct the core genome of each species under analysis. Protein domains with known functions for each of these sequences were evaluated using the InterPro database. In addition, shared protein families among phylogenetically close strains were selected to reconstruct a common central genome. Specifically, the PanX pipeline (pan-genomic analysis and exploration) [33] was employed to analyse the core genes of species closely related to those isolates in this study. The resulting phylogenetic relationships were visualised using iTOL v6.8.1 (interactive Tree Of Life) [34], an online tool for annotating, managing, and graphically representing phylogenetic trees.

2.4. Prediction of Genes Involved in Toxic Compound Degradation

To explore the arsenal of genes coding for enzymes involved in processes leading to the degradation of toxic compounds, heavy metals, PCBs, and possible transporters of these substances, these structures were searched through a literature search. Gene sequences encoding these enzymes were identified from the MetaCyc database [35] and downloaded from NCBI. Their presence in the genomes selected for this study was evaluated through DIAMOND [36]. The BLASTp function is based on an optimised local database focused

on the analysis of coding sequences for enzymes involved in the degradation of toxic compounds. For DIAMOND hits, these options were used: `--evaluate  $1 \times 10^{-25}$` , `--subject-cover 80`, `--query-cover 80`, `--sensitive`, and `--max-target-seqs 1`. The Artemis program was used to evaluate the gene surrounds of the sequences identified through the degradation database, while the genetic similarities of the sequences were checked using the Clustalx 2.1 software [37].

2.5. Emulsification Properties and Microbial Adhesion to Hydrocarbons Assays

The experimental protocol described by Rani et al. [38] was followed for the determination of the emulsifying capacity (CE%) and emulsion index (EI%). The isolated bacterial strains were grown in both BH and LB medium containing 2% *v/v* diesel oil and incubated at 28 °C with agitation (100 rpm). After three incubation days, the cultures were centrifuged at $7500 \times g$ for 5 min at 15 °C. The cell-free supernatants (2 mL) were injected into glass test tubes, 200 µL of diesel fuel was added, and tubes were vigorously vortexed for 30 s and left for 10 min. Samples showing an emulsion were selected, and once the emulsion had stabilised, more diesel oil (200 µL) was added; the procedure was repeated until a distinct and clear fraction of non-emulsified diesel oil was observed on the emulsion surface.

The emulsifying capacity (EC%) was calculated using the following formula (Equation (1)):

$$EC = \frac{V_{total\ oil\ used}}{V_{initial\ aqueous\ phase}} \times 100 \quad (1)$$

The emulsion index (EI%) was calculated after the emulsifying capacity test; the change in the emulsion layer was measured after 24 and 48 h (EI_{24} and EI_{48} , respectively) using the following formula (Equation (2)):

$$EI = \frac{Height\ of\ the\ emulsion\ layer}{Height\ of\ the\ total\ mixture} \times 100 \quad (2)$$

Hydrophobicity was measured following the method of Choundhary et al. [39] with some modifications.

An overnight culture of each isolate (50 mL) (incubated at 28 °C with agitation at 100 rpm) was harvested by centrifugation at 3500 rpm at 25 °C for 10 min. The supernatants were discarded, and the cell pellets were washed twice in PUM buffer and suspended in the same buffer (K_2HPO_4 22.2 g/L; KH_2PO_4 7.26 g/L; urea 1.8 g/L; $MgSO_4 \times 7H_2O$ 0.2 g/L).

The initial absorbance (A_0) of the bacterial suspensions was adjusted to 0.5 at 600 nm. The bacterial suspensions (5 mL) were transferred into tubes containing diesel oil (200 µL) and incubated at 28 °C for 10 min; they were then vortexed for 2 min. The suspensions were kept undisturbed for 1 h for phase separation.

Finally, the aqueous phase was carefully removed using a Pasteur pipette, and the final absorbance (A_1) was recorded at 600 nm using a spectrophotometer. The percentage of hydrophobicity was calculated using the following formula (Equation (3)):

$$H = 1 - \left(\frac{A_0 - A_1}{A_0} \right) \times 100 \quad (3)$$

2.6. Water and Soil DNA Extraction and 16S rDNA Gene Sequencing

Spring water and soil samples collected at springs S1 and S2 were used for microbial community analyses. Water samples (1 L) were filtered through sterile mixed cellulose ester membranes (S-Pak™ Membrane Filters, 47 mm in diameter, 0.22 µm pore size, Millipore Corporation, Billerica, MA, USA) within 24 h of collection.

DNA extractions from filters and soils were performed using the DNeasy PowerSoil Pro Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions.

To evaluate DNA integrity and quantity, 2 μ L of extracted DNA was visualised on a 1% agarose gel (*w/v*). Gel electrophoresis was conducted in $0.5\times$ TAE buffer at 100 V for 40 min, using a 1 kb DNA ladder as a molecular weight marker (Thermo Fisher Scientific, Waltham, MA, USA).

The microbial community profiles in the samples were generated using Next-Generation Sequencing (NGS) technologies at the BMR Genomics Srl Laboratory, according to Caprari et al. [40,41]. Partial 16S rDNA gene sequences were obtained from the extracted DNA through polymerase chain reaction (PCR) using the primer pair Pro341F and Pro805R, targeting both the bacterial and archaeal V3–V4 hypervariable regions [42] and evaluated in 1.5% agarose gel. The PCR reaction was conducted as follows: 1 min of denaturation at 94 °C, 25 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C and extension for 45 s at 68 °C, and a final phase of 7 min at 68 °C.

The amplicons were purified with Thermolabile Esonuclease I (NEB), diluted 1:2, and amplified with short cycle with Nextera XT Index. The amplicons were normalised with SequalPrep (Thermo Fisher Scientific, Waltham, MA, USA) and multiplexed. The pool was purified with Agencourt XP 1X magnetic beads, loaded onto MiSeq and sequenced with V3 Chemistry—300 paired-end strategy.

In silico analysis was performed using QIIME2 tools version 2021.4. [43]. The obtained reads were cleaned of primers by using the Cutadapt software (v. 2021.4) and then processed with the denoised paired plugin of the DADA2 software v1.26 [44]. Briefly, sequences have been trimmed at the 3' end and filtered by quality and length. Then, they were de-replicated and merged to obtain unique sequences, and chimeras were eliminated. The Amplicon Sequence Variants (ASVs) with a frequency <0.01% were not considered. All reads from bacterial communities were classified to the lowest possible taxonomic rank using a reference dataset from the SILVA database (v. 138) [45]. Alpha-diversity was calculated by using the Shannon index [46].

The 16S rDNA gene sequences generated in this study have been deposited in the NCBI Sequence Read Archive under the accession number PRJNA1219130.

2.7. Bacterial Consortium and Mesocosm Experiment Setup

A bacterial consortium was assembled by combining the four isolated hydrocarbon-oxidising bacterial strains, following the methodologies described by Yu et al. and Chen et al. [47,48], with modifications, in order to assess the capabilities of the analysed microorganisms to efficiently remove hydrocarbons from freshwater collected from Castel San Vincenzo Lake (Molise region, Southern Italy; Figure 2) and artificially contaminated with diesel oil, and the impact of the bioaugmentation process on the native lake microbial communities. Each strain was individually cultured in LB medium (50 mL) for 16 h; cells were harvested by centrifugation at $7000\times g$ for 10 min, washed, and resuspended in sterilised lake water. Each bacterial suspension was adjusted to an optical density (OD_{600}) of 1.0. Subsequently, equal volumes of the suspensions were mixed to constitute the hydrocarbon-degrading consortium (10 mL), which has been used in the mesocosm experiments.

Water samples (15 L) were collected from Castel San Vincenzo Lake in sterile bottles and used to determine the main physical-chemical features (temperature, pH, conductivity, total hydrocarbon concentration, and major ions) and the mesocosm setup.

In detail, pH and electrical conductivity was measured using multiparameter instruments (Hanna Instruments Edge, Woonsocket, RI, USA). The determination of inorganic anions and cations was carried out through ion chromatography (ICS-1000 Thermo Fisher Scientific, Waltham, MA, USA). For the total hydrocarbon determination, the sample was

acidified (HCl 1:1, $\text{pH} \leq 2$) and extracted with a mixture of n-hexane (80%) and methyl-tert-butyl ether (20%). The extract, obtained via liquid–liquid extraction in a separatory funnel, was passed through a column packed with silica gel to remove any polar substances. The eluate was collected in a container and evaporated, and the residue was weighed gravimetrically (IRSA-APAT, 2004; [49]).

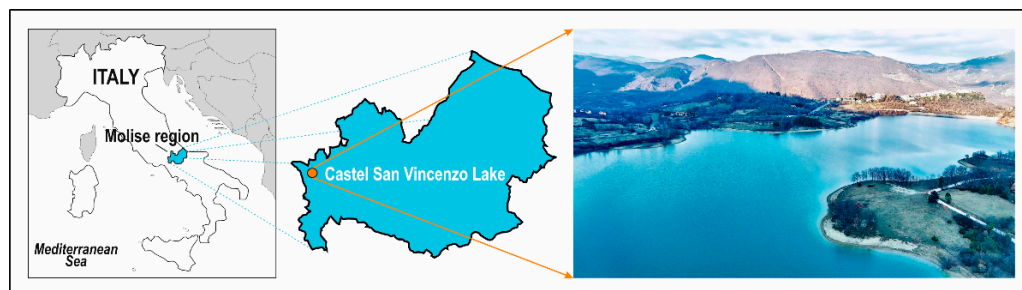


Figure 2. Localisation and aerial view of Castel San Vincenzo Lake (Molise region, Southern Italy).

Mesocosms (1 L) were prepared in 2 L conical flasks (Figure 3), according to the following experimental plan: (1) a “bioaugmentation” mesocosm comprising lake water contaminated with diesel oil (2% *v/v*) and supplemented with the constructed bacterial consortium (2% *v/v*), (2) a “natural attenuation” mesocosm containing only lake water and diesel oil, and (3) a “negative control” mesocosm consisting of sterilised lake water contaminated with diesel oil to account for hydrocarbon leaks due only to abiotic factors. Each mesocosm was prepared in duplicate and incubated under static conditions at 28 °C for 28 days.

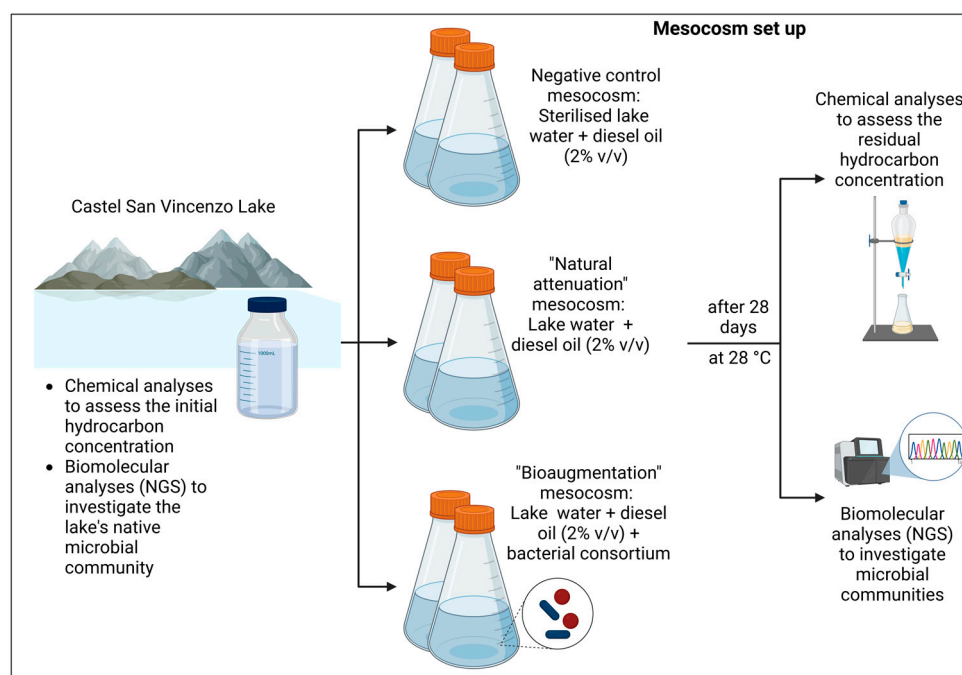


Figure 3. Experimental design for mesocosm experiments (Created in Biorender, <https://www.biorender.com/>).

After the incubation period, molecular analyses were conducted to characterise microbial communities, following the protocol described in Section 2.6.

The biodegradation efficiency of diesel oil was calculated using the following formula (Equation (4)):

$$E = \frac{C_0 - C_t}{C_0} \times 100 \quad (4)$$

where E represents the biodegradation efficiency (%), C_0 is the initial diesel oil concentration (mg/L), and C_t is the concentration at the final time (mg/L) [47].

3. Results

3.1. Isolation, Characterisation, and Genome Sequencing of Hydrocarbonoclastic Bacteria, Along with Microbial Community Analysis of Spring Waters and Surrounding Soils

After four enrichment cycles in Bushnell–Haas (BH) medium supplemented with diesel oil as the sole carbon source, four pure cultures of distinct hydrocarbon-oxidising bacterial strains exhibiting different phenotypic traits were selected and considered for genome sequencing and further investigation. The strains were named S1W1, S2W2, S2S5, and S2S8. Strains S1W1 and S2W2 were isolated from S1 and S2 spring water, respectively, whereas strains S2S5 and S2S8 were isolated from the surrounding soil of the S2 spring.

Strain S1W1 was a Gram-negative bacterium with a coccobacillary morphology, forming translucent colonies on the LB agar plates. Strain S2W2 was a Gram-positive coccobacillus, producing creamy white colonies on LB medium. Strain S2S5 was Gram-positive with rod-shaped cells, producing pink colonies on LB agar plates, while strain S2S8 was a Gram-negative short rod-shaped bacterium, producing light yellow colonies. All the isolates were catalase-positive. The partial sequencing of the 16S rDNA gene, conducted for preliminary taxonomic assignment, revealed that the strains S2S8 and S1W1 belong to the genus *Acinetobacter*, and S2W2 and S2S5 belong to the genera *Rhodococcus* and *Gordonia*, respectively.

The data obtained from the genome sequencing of the four hydrocarbonoclastic bacteria were processed through the MEGAnnotator2 pipeline [31] to reconstruct their genomic sequences. A taxonomic assessment was performed using MEGAnnotator2, which involved a thorough analysis of the complete 16S rDNA gene and whole-genome average nucleotide identity (ANI). This analysis assigned strain S2S5 to *Gordonia amicalis* (ANI score of 98%) and strain S2W2 to *Rhodococcus erythropolis* (ANI score of 99%) (Table 1). Conversely, for strains S2S8 and S1W1, their classification within the genus *Acinetobacter* was established solely through ANI analysis (Table 1). Additionally, a quality control assessment was conducted on the newly assembled genomes using CheckM, revealing a completeness level of 99.8% for *G. amicalis* strain S2S5, 99.9% for *R. erythropolis* strain S2W2, and 99.7% and 100% for *Acinetobacter* strains S2S8 and S1W1, respectively. The genome lengths of these strains were comparable to other strains of the same species available in the NCBI repository. Gene prediction analyses indicated that the genomes of strains S2S5, S2W2, S2S8, and S1W1, encompass 4629 genes (including 5 rRNA and 49 tRNA genes), 5885 genes (including 6 rRNA and 54 tRNA genes), 3269 genes (including 5 rRNA and 67 tRNA genes), and 3625 genes (including six rRNA and 59 tRNA genes), respectively. Detailed genomic features for these strains are summarised in Table 1. Based on these findings, the reconstructed genomes of strains S2S5 and S2W2 met all quality criteria necessary for subsequent large-scale comparative genomic analyses.

The NGS analysis provided detailed information about the composition of microbial communities in spring waters and soil samples.

The rarefaction analysis, a measure used to estimate alpha-diversity in the samples and assess whether sequencing captured microbial diversity, indicated that the soil and water samples from spring S2 exhibited relatively higher biodiversity than those from spring S1 (Supplementary Figure S1). The Shannon index values for S2-water and S2-soil

samples were 6.56 and 7.11, respectively, whereas those for S1-water and S1-soil samples were 4.30 and 5.84, respectively.

Table 1. Comprehensive genomic and taxonomic characterisation of hydrocarbon-degrading bacterial strains S1W1, S2W2, S2S5, and S2S8. The table details sequencing metrics, genome assembly parameters, completeness, contamination levels, average coverage, and key genomic features, including genome length and gene counts.

	S2S5	S2W2	S2S8	S1W1
Sequencing output:	1,172,742	785,928	1,115,851	1,002,674
High quality reads:	1,132,596	756,097	1,085,988	970,118
Filtered reads:	1,132,594	756,087	1,085,987	970,117
Contigs generated at:	K127	K127	K127	K127
16S rRNA gene identity:	<i>Gordonia amicalis</i> JCM 11271 100.000	<i>Pseudomonas viridiflava</i> 100.000	<i>Bacillus mycoides</i> 98.813	<i>Acinetobacter soli</i> 98.046
ANI screening:	<i>Gordonia amicalis</i> 98.4177	<i>Rhodococcus erythropolis</i> 98.6463	<i>Acinetobacter kanungonis</i> 85.9859	<i>Acinetobacter populi</i> 91.6293
Genome completeness:	99.76	99.94	99.73	100.00
Genome contamination:	0.3	0.09	0.96	0
Average coverage:	110,7166	58,0149	152,2452	120,9935
Number of contigs:	110	32	54	83
Genome length:	5,030,312	6,366,085	3,445,643	3,858,766
Number of genes:	4629	5885	3269	3625
Number of rRNA genes:	5	6	5	6
Number of tRNA genes:	49	54	67	59
Certain taxonomical level:	<i>Gordonia amicalis</i>	<i>Rhodococcus erythropolis</i>	<i>Acinetobacter</i> sp.	<i>Acinetobacter</i> sp.

As shown in Figure 4a, the main three bacterial phyla found in S1 spring water were *Campilobacterota* (46.95%), *Bacteroidota* (21.12%), and *Proteobacteria* (18.16%), collectively representing 86.23% of the prokaryotic community. In contrast, the microbial community of the S1 spring's surrounding soil was dominated by *Proteobacteria* (80.6%), followed by *Firmicutes* (5.62%), and *Actinobacteriota* (4.35%). High relative abundance values for the phylum *Proteobacteria* were also observed in the water and soil samples from S2 (65.82% and 41.23%, respectively). In S2 spring water, *Proteobacteria* were followed by *Bacteroidota* and *Firmicutes* (12.46% and 6.55%, respectively), while in the surrounding soil, *Actinobacteriota* (21.42%) and *Bacteroidota* (10.40%) were the second and third dominant phyla. At a lower taxonomic level (Figure 4b), the bacterial genera *Sulfurovum* (44.72%), *Chlorobium* (20.02%), and *Thiovirga* (10.01%) were dominant in S1 spring water, whereas *Malikia* (12.30%), C39 (6.26%), and *Novosphingobium* (5.35%) were the most abundant genera in S2 spring water. In contrast, the top three genera representing microbial communities of soils were *Acidosoma* (21.26%), KCM-B-112 (10.72%), and *Pseudoxanthomonas* (7.40%) for samples collected near the S1 spring, and KCM-B-112 (13.25%), *Mycobacterium* (8.63%), and a genus within the *Thermaceae* family (5.71%) for samples collected near the S2 spring.

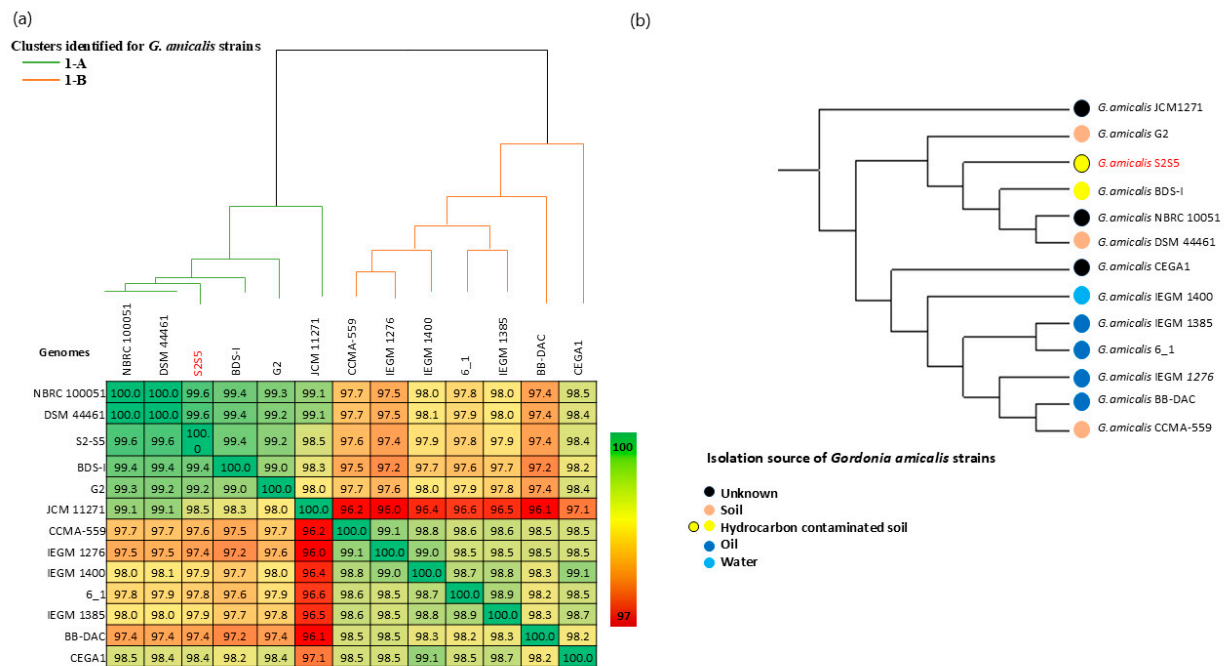


Figure 5. (a) Comparative genomics analysis carried out on *Gordonia amicalis* strains. Panel (a) shows the average nucleotide identity (ANI) values between the genomes of the 12 *Gordonia amicalis* strains obtained from NCBI and the S2S5 strain, along with a corresponding dendrogram. The colour-based scheme highlights two main clusters in green, each consisting of strains with a high percentage of genomic identity between them. Panel (b) shows the phylogenomic tree of the 12 *Gordonia amicalis* strains obtained from NCBI and the S2S5 strain of the same species analysed in this study. The tree was built based on the core genome identified for this species. For each strain, the source of isolation was highlighted with a coloured circle.

On the other hand, the ANI analysis performed on the 52 genomes of the *R. erythropolis* species (including the S2W2 strain isolated from naturally contaminated hydrocarbon spring water) allowed the identification of a larger cluster encompassing 38 strains, in which the newly isolated strain S2W2 is included (cluster 2-A), as well as a smaller cluster comprising 14 strains (cluster 2-B) (Figure 6). Furthermore, the ANI analysis again revealed in the whole a higher percentage of nucleotide identity among strains isolated from similar environments, such as contaminated or uncontaminated soils. However, it is noteworthy mentioning that some strains of *R. erythropolis* isolated from contaminated soils (such as *R. erythropolis* IEGM 1415, KB1, and NRRL B-16352) showed a lower percentage of nucleotide identity compared with those isolated from other environments. This discrepancy may be ascribed to the unspecified chemical contaminant in their isolated source, which may exert different ecological pressures on the evolution of the bacterial strains.

For strains S2S8 and S1W1, whose membership in the bacterial genus *Acinetobacter* had already been determined, ANI analysis was performed by including 91 genomes (Supplementary Table S3), representative of each of the known species of the genus *Acinetobacter*, to understand the species membership of strains S2S8 and S1W1, which were isolated from naturally hydrocarbon-contaminated soil and spring water, respectively (Figure 7). Specifically, strain S2S8 showed a higher percentage of nucleotide identity with strain Y-23 of *Acinetobacter tibetensis* (ANI score of 98%). In comparison, strain S1W1 showed a higher percentage of nucleotide identity (ANI score of 96.7%) with strain ANC 4466 of *Acinetobacter puyangensis*. Consequently, it can be inferred that strain S2S8 belongs to *Acinetobacter tibetensis*, while strain S1W1 belongs to *Acinetobacter puyangensis*.

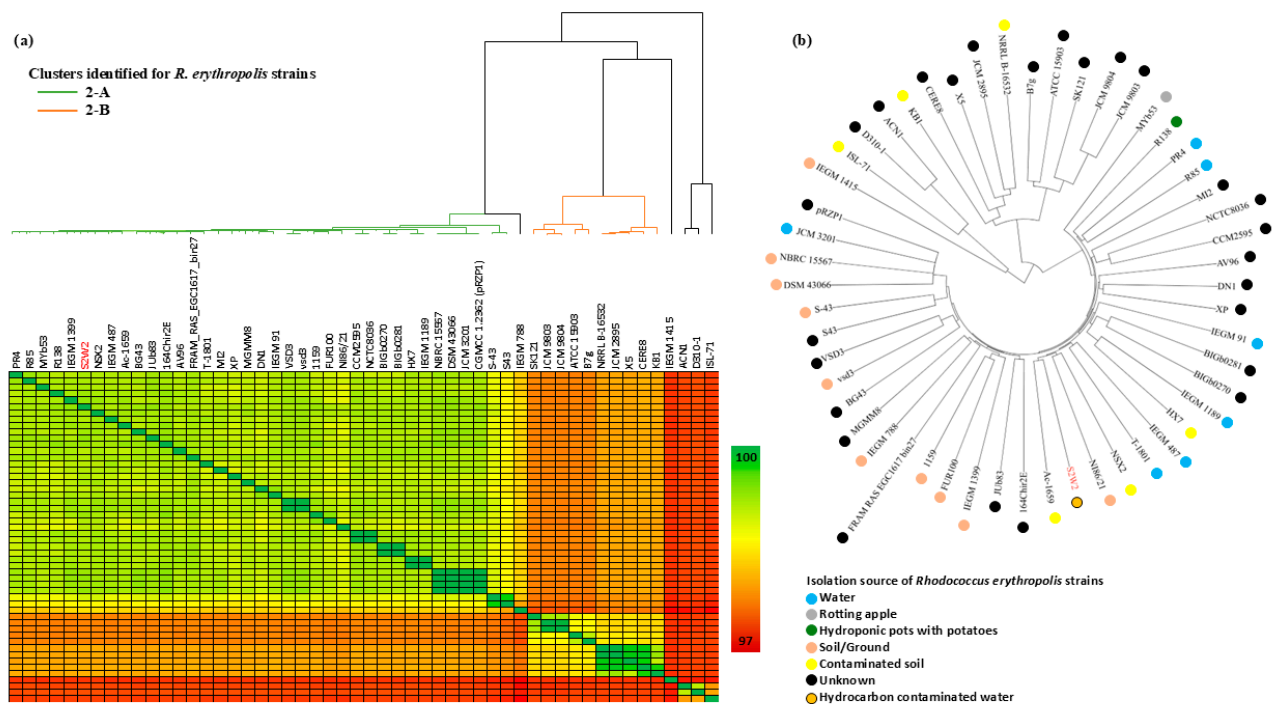


Figure 6. Comparative genomics analysis carried out on *Rhodococcus erythropolis* strains. Panel (a) shows the ANI analysis among the 52 *Rhodococcus erythropolis* genomes and corresponding dendrogram. The colour-based scheme highlights a cluster of high-prevalence strains in green, showing a high internal degree of genomic identity (ANI score of 98.7). Panel (b) shows the phylogenomic tree of the 52 *R. erythropolis* strains (including the S2W2 strain isolated from hydrocarbon contaminated water) analysed in this study. The tree was built based on the core genome identified for this species. For each strain, the source of isolation was highlighted with a coloured circle.

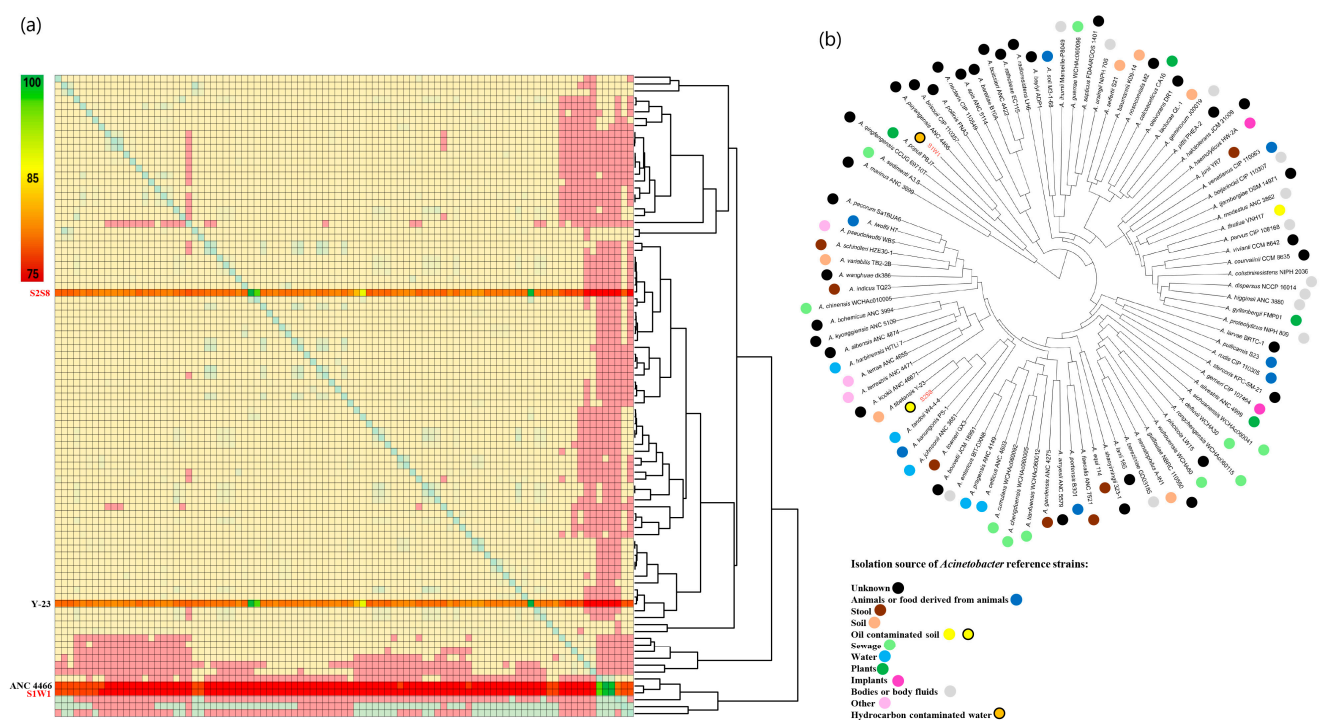


Figure 7. Comparative genomics analysis carried out on *Acinetobacter* reference strains. Panel (a) shows the ANI analysis among the 91 *Acinetobacter* reference genomes, the S2S8 and the S1W1 strains, and the corresponding dendrogram. Panel (b) shows the phylogenomic tree of the 93 *Acinetobacter* strains (including the S2S8 and the S1W1 strains isolated from contaminated soil and water) analysed in this study. For each strain, the source of isolation was highlighted with a coloured circle.

3.3. Assessment of the Unique Genetic Repertoire and Prediction of Genes Involved in Toxic Compound Degradation

The *G. amicalis* and *R. erythropolis* genomes were used to perform a comparative genomics analysis to predict the pangenome and core genome of these species, providing a comprehensive view of the genetic peculiarities of the S2S5 and S2W2 strains isolated in this research [50] (Supplementary Figure S2, Tables S4 and S5).

In contrast, no comparative genomics analysis was conducted on the genomes of *A. tibetensis* (including the S2S8 strain) and *A. puyangensis* (including the S1W1 strain) due to the low number of strains available for these species, which makes comparative genomics investigations unsuitable.

The analysis of the pangenome of *G. amicalis* revealed the presence of 2621 gene sequences encompassing the core genome, in addition to 2700 dispensable genes, which were observed only in a subset of the 13 *G. amicalis* strains. Furthermore, a total of 3645 strain-specific Truly Unique Genes (TUGs) were identified. Of these, 142 were found exclusively in the genome of S2S5, representing the unique genetic features possessed by this newly isolated bacterial strain (Supplementary Figure S2a and Table S4). Thus, the PFAM and InterPro databases were employed to screen the TUGs of strain S2S5 for protein domains with known functions that potentially play a key role in the bacterium's adaptability, colonisation, and survival mechanisms. Moreover, a BLASTp homology analysis was performed to support the assignment of a putative annotation to each of these genes.

In detail, the S2S5 strain of *G. amicalis* showed the presence of several genes related to DNA recombination, bacteriophage infection, and heavy metal transport. These genes could be critical for the bacterium's adaptation to the environment. Specifically, a unique gene in *G. amicalis* S2S5 was predicted to code for an enzyme encompassing a "Heavy metal-associated domain, HMA" (InterPro accession IPR006121), which has been found in bacterial transporter genes involved in resistance to toxic metals. Consequently, it can be suggested that this gene sequence may be involved in the mechanisms by which strain S2S5 can handle resistance to heavy metals.

The analysis was subsequently extended to assess the existence of unique genes common to all *G. amicalis* strains belonging to the same phylogenetic clade (cluster 1-A) as our S2S5 isolate, leading to the identification of thirteen distinctive genes unique to this clade (Supplementary Tables S6 and S7). Amongst the predicted protein functions, the identification of the pyridoxamine 5'-phosphate oxidase (pdxH) gene is intriguing, as it is involved in the pyridoxal 5'-phosphate (PLP) biosynthesis pathway. Several studies have shown that PLP is essential for enzymes that degrade toxic compounds, including train-3-chloro-L-aspartate [51], and that increased PLP biomass is found in environments heavily contaminated with polycyclic aromatic hydrocarbons [52]. These findings support the hypothesis that the presence of the PLP-producing enzyme pdxH in the *G. amicalis* S2S5 strain could play an important role in bioremediation processes.

In parallel, the same analyses were performed on *R. erythropolis*, leading to the identification of 2384 genes constituting the core genome, 10,692 dispensable genes, and 12,409 TUGs (Supplementary Figure S2b and Table S5). Specifically, 25 TUGs with known protein domains were found in strain S2W2. Among them, a gene encoding a sulfatase was identified, suggesting that strain S2W2 may be capable of metabolising sulphates, providing an adaptive advantage in specific habitats. Additionally, a TadE-like domain typical of extracellular structures involved in adhesion to specific substrates, was identified, although the nature of these substrates cannot be definitively predicted. On the other hand, genome analysis of phylogenetically related strains of *R. erythropolis*, clustered with strain S2W2 (cluster 2-A), did not reveal any gene sequences that are characteristic and exclusive to this clade.

To better understand the evolutionary relationships among the bacterial strains included in this study, a phylogenetic analysis was conducted by aligning the core genes previously obtained through pangenomic analysis from the *G. amicalis*, *R. erythropolis*, and *Acinetobacter* reference strains. For the *G. amicalis* species, the strain S2S5 was phylogenetically closely related to strains isolated from contaminated soils. This correlation between isolation sources and evolutionary distance suggests the possible coevolution of these strains, driven by their adaptation to contaminated soil, thus explaining the conserved genomic features related to contaminant degradation and detoxification. Interestingly, *R. erythropolis* strain S2W2, despite being included in a macro-cluster with other strains isolated from different habitats (soil, contaminated soil, and water), was more closely related to a strain found in contaminated soils, even though it was isolated from spring waters.

To determine whether the S2S5 strain of *G. amicalis*, the S2W2 strain of *R. erythropolis*, the S2S8 strain of *A. tibetensis*, and the strain S1W1 of *A. puyangensis* could be used efficiently for bioremediation, a degradative database was created. This database included homologues of genes coding for subunits that constitute enzymes involved in the degradation processes of hydrocarbons, their derivatives, and other toxic compounds that may be present in soils. In addition, gene sequences encoding enzymes involved in the degradation and transport of PCBs and heavy metals were incorporated, as their presence could be crucial for successfully utilising a bacterial strain in bioremediation.

Screening of the strains selected for this study revealed that strain S2S5 of *G. amicalis*, along with other strains of the same species, contains genes encoding subunits that constitute enzymes involved in hydrocarbon degradation (Supplementary Figure S3a and Table S8).

Notably, strain S2S5 has multiple gene sequences coding for the alpha subunit of a hydroxylase involved in the degradation of benzene (an aromatic hydrocarbon). Since the genome of the *G. amicalis* S2S5 strain possesses only the gene sequence encoding the alpha subunit of this hydroxylase, we manually screened the genes surrounding the sequences coding for the alpha subunit. Intriguingly, we found the presence of gene sequences coding for proteins with unknown functions.

It was then evaluated whether the strains of *G. amicalis* selected for this study could degrade alkanes. In this regard, gene sequences encoding the three enzymatic subunits of a hydroxylase involved in alkane degradation (alkB system) and a gene encoding the enzyme belonging to the cytochrome CYP153 family, again involved in alkane oxidation, were identified. The *G. amicalis* strains selected for this study were also found to possess genes encoding components of enzymes involved in detoxification from heavy metals. Notably, strain S2S5 exhibited coding gene sequences related to the detoxification from mercury. Additionally, the genomes of *G. amicalis* strains contained gene sequences involved in the degradation of other toxic compounds that could be present in contaminated soils, such as tetrathionate, cyanide, acetaldehyde, ethylene glycol, and atrazine, as well as plastic compounds and their derivatives such as nylon, polylactic acid (PLA), and dibutylphthalate (DBP).

Performing the same analysis on *R. erythropolis* strains selected for this study revealed that, like *G. amicalis* strains, they encode genes for enzymatic subunits involved in the degradation of benzene, catechols, and 4-nitrobenzoate (Supplementary Figure S3b and Table S9). Additionally, fifty bacterial genomes, including the chromosome sequence of strain S2W2, contain gene sequences encoding one of the components of a reductase complex involved in the degradation process of benzoyl-CoA, an intermediate in the benzene degradation process. This was not detected in the *G. amicalis* genomes analysed (Supplementary Figure S3a). Furthermore, gene sequences encoding the three subunits (alkB, rubB, and alkG) of a hydroxylase involved in alkane degradation, as well as genes

encoding an enzyme from the cytochrome CYP153 family involved in alkane oxidation, were also identified.

Moreover, we also observed that all the genomes of the *R. erythropolis* strains possess genes encoding a reductase involved in mercury detoxification, and the chromosome sequences of forty-six strains, including strain S2W2, possess genes encoding a cadmium transporter. In addition, genes encoding components of enzymes potentially involved in the degradation of other toxic compounds were also identified in the genomes of the *R. erythropolis* strains. Specifically, gene sequences encoding components of a reductase involved in the degradation of tetrathionate were identified (whereas in the *G. amicalis* strains, only the gene sequences coding for the catalytic alpha subunit and gamma subunit were detected). Additionally, gene sequences predicted to encode enzymes involved in the degradation of cyanuric acid, commonly used in the production of herbicides like atrazine [53], and plastic compounds and their derivatives, such as nylon, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), PLA, DBP, polyethylene terephthalate (PET), polyethylene (PE), and polybutylene succinate (PBS), were identified.

The same screening was also performed on the genomes of the *Acinetobacter tibetensis* and *Acinetobacter puyangensis* strains selected for this study (Supplementary Figure S3c and Table S10).

This analysis showed that the genome of the *A. tibetensis* S2S8 strain possesses specific gene sequences predicted to encode components and subunits of enzymes involved in benzene degradation, such as the alpha subunit of a hydroxylase deputed to the degradation of this aromatic hydrocarbon. Moreover, the genome of strain S2S8 contains gene sequences predicted to code enzymes involved in the degradation of benzene derivatives, such as catechol and 4-nitrobenzoate, as well as two components of a reductase involved in the degradation of benzoyl-CoA.

The chromosome sequence of the *A. tibetensis* S2S8 strain also appears to possess genes predicted to encode enzymes involved in detoxification from heavy metals such as mercury and nickel.

Further screening revealed the presence of genes in the genome of strain S2S8 predicted to code enzymes involved in the degradation of additional toxic compounds, including the gene sequence for one of the subunits of a reductase involved in the degradation of tetrathionate, as well as genes for enzymes involved in the degradation of acetaldehyde, atrazine, ethylene glycol, and plastic compounds and their derivatives (PE, PLA, PHBV, and nylon).

In parallel, strain S1W1 of *A. puyangensis* has been shown to possess specific genomic sequences predicted to synthesise enzymes involved in the degradation of catechol and benzoyl-CoA. Additionally, the S1W1 strain appears capable of reducing the bioavailability of mercury and nickel in the environment, as well as degrading toxic compounds, such as tetrathionate, acetaldehyde, atrazine, ethylene glycol, and plastic compounds like PE, PHBV, and nylon.

3.4. Assessment of Emulsification Properties and Microbial Adhesion to Hydrocarbons

The assessment of the emulsification capacity (EC) of the four bacterial strains isolated in this research, conducted in both Bushnell–Haas (BH) and Luria–Bertani (LB) growth media, revealed the formation of emulsions in all cases. Notably, *Acinetobacter puyangensis* strain S1W1 and *Gordonia amicalis* strain S2S5 achieved EC values approaching 50% in the BH medium. In the LB medium, *G. amicalis* strain S2S5 exhibited an even higher EC value, reaching 80%. Emulsion stability (EI) in BH medium was observed exclusively for *G. amicalis* strain S2S5, although a slight decline was noted after 48 h. In the LB

medium, all four bacterial strains maintained emulsion stability for 24 h (EI_{24}), with values exceeding 50%.

Interestingly, after 48 h in LB medium, high values of this parameter were still recorded for *A. puyangensis* strain S1W1 and *G. amicalis* strain S2S5. In addition to emulsification tests, the ability of the four bacterial isolates to adhere to hydrocarbons was also evaluated. The test of microbial adhesion to hydrocarbons was not performed on *Acinetobacter puyangensis* strain S1W1, as strong cell aggregation in the form of flocs was observed after the resuspension in the buffer solution used. *Acinetobacter tibetensis* strain S2S8 demonstrated low hydrophobicity, resulting in minimal adhesion to diesel oil. In contrast, *Rhodococcus erythropolis* strain S2W2 and *Gordonia amicalis* strain S2S5 exhibited high cellular hydrophobicity values of 90.62% and 76.00%, respectively, demonstrating a strong capacity to adhere to diesel oil. The results of the different tests are summarised in Table 2.

Table 2. The table provides an indirect evaluation of the hydrocarbon-oxidising activities of bacterial strains isolated from hydrocarbon-contaminated environments. The assessment includes the following parameters measured in both BH and LB growth media: emulsification capacity (EC), emulsification index at 24 h (EI_{24}), emulsification index at 48 h (EI_{48}), and microbial adhesion to hydrocarbons (MATH).

Hydrocarbon-Oxidising Bacterial Strains	EC % in BH	EI_{24} % in BH	EI_{48} % in BH	EC % in LB	EI_{24} % in LB	EI_{48} % in LB	MATH %
<i>Acinetobacter puyangensis</i> S1W1	50.00	-	-	30.00	66.67	66.67	
<i>Rhodococcus erythropolis</i> S2W2	26.67	-	-	20.00	55.55	-	90.62
<i>Gordonia amicalis</i> S2S5	53.33	53.33	46.67	83.33	100.00	90.48	76.00
<i>Acinetobacter tibetensis</i> S2S8	13.33	-	-	40.00	93.33	20.00	11.11

3.5. Construction of a Microbial Consortium and Bioaugmentation of Artificially Hydrocarbon-Contaminated Lake Waters to Assess the Degradative Capacity of the Four Hydrocarbonoclastic Strains in a Different Environmental Context

To assess the potential of the four strains to metabolise hydrocarbons when inoculated into an environment different from that of their isolation, a consortium was established and used to conduct a bioaugmentation in lake waters collected from Castel San Vincenzo Lake, artificially contaminated with diesel, through mesocosm experiments. Before setting up the mesocosms, waters of the Castel San Vincenzo Lake were analysed to determine their main chemical-physical characteristics, assess whether hydrocarbons were present, and investigate the native microbial communities. The results of these investigations are reported in Table 3 and showed that the concentration of total hydrocarbons in the lake waters fell below the detection limit of the method used (10 mg/L).

As previously described, three different mesocosms were prepared: (1) a “bioaugmentation” mesocosm comprising lake water and supplemented with the constructed bacterial consortium; (2) a “natural attenuation” mesocosm containing only lake water; and (3) a “negative control” mesocosm consisting of sterilised lake water. All the mesocosms were artificially contaminated with diesel oil (concentration of 2%). After 28 days of incubation at 28 °C, the total hydrocarbon concentration in each mesocosm was determined, and microbial community composition was analysed in the “natural attenuation” and “bioaugmentation” experiments.

Table 3. Physico-chemical parameters of Castel San Vincenzo Lake water. The table outlines key environmental factors, including pH, temperature, conductivity, total hydrocarbon concentration, and the concentrations of major anions (Cl^- , SO_4^{2-}) and cations (Na^+ , K^+ , Mg^{2+} , Ca^{2+}).

Parameters	Values
pH	7.89
Temperature ($^{\circ}\text{C}$)	23.50
Conductivity ($\mu\text{S}/\text{cm}$)	246.30
Total hydrocarbons (mg/L)	<10.00
Cl^- (mg/L)	2.06
SO_4^{2-} (mg/L)	7.34
Na^+ (mg/L)	1.69
K^+ (mg/L)	0.68
Mg^{2+} (mg/L)	4.17
Ca^{2+} (mg/L)	49.75

Chemical investigations revealed a loss of 11.84% of hydrocarbons in the control mesocosm, likely due to abiotic processes. In mesocosms augmented with the microbial consortium, a decrease of 30.95% of contaminants was detected. Furthermore, the “natural attenuation” mesocosm demonstrated a 17.85% loss of hydrocarbons, attributable to both abiotic processes and the contribution of native lake water microbial communities. These results suggested that, although the abiotic processes may partially account for the recorded loss of hydrocarbons, native microbial communities can play a key role in contaminant removal. Furthermore, the enrichment with the four hydrocarbonoclastic strains isolated from the Tramutola study site can enhance the degradation efficiency.

The results obtained from the molecular investigations showed, already at the phylum level, notable variations in mesocosm microbial communities compared to non-treated lake waters, likely induced by both the artificial diesel contamination and bioaugmentation, as well as by the experimental conditions applied (Figure 8a). The top three phyla retrieved from Castel San Vincenzo Lake were *Proteobacteria*, *Cyanobacteria*, and *Bacteroidota*, which accounted for 81.02% of the prokaryotic community. A drastic reduction in *Cyanobacteria* was observed in the “natural attenuation” and “bioaugmentation” mesocosms, as well as a decrease in *Bacteroidota*, accompanied by an increase in *Proteobacteria*, with relative abundance values of 81.91% and 66.49%, respectively.

Actinobacteriota represented the second most abundant phylum in the “bioaugmentation” mesocosm, with a relative abundance of 18.43%, higher than that detected in the lake waters and “natural attenuation” mesocosm. *Verrucomicrobiota* values ranged from 3.54% in the “natural attenuation” mesocosm to 6.19% in lake waters and 7.96% in the “bioaugmentation” experiment. No *Archaea* were detected.

At the taxonomic level of genus (Figure 8b), the main known taxa found in the lake waters were *Cyanobium_PCC-6307* (24.19%), *Limnobacter* (6.97%), and *Dinghuibacter* (6.88%). On the other hand, *Niveispirillum*, *Caulobacter*, and *Arcicella* were found among the most represented genera in the “natural attenuation” mesocosm. In the “bioaugmentation” experiment, the genus *Azospirillum* predominated over the others with a relative abundance value of 42.37%, followed by *Rhodococcus* (9.21%), *Gordonia* (7.91%) (genera to which two of the strains constituting the microbial consortium belonged), and *Comamonas* (7.27%). Interestingly, the genus *Acinetobacter* (to which the strains S1W1 and S2S8 belonged) that was not detected in the lake water, was recovered with a very low relative abundance percentage (<1%) in the “bioaugmentation” mesocosm. This observation supported the

hypothesis that, among the four strains used for the consortium construction, only S2W2 and S2S5 were able to thrive in Castel San Vincenzo Lake water and were responsible for the observed hydrocarbon loss.

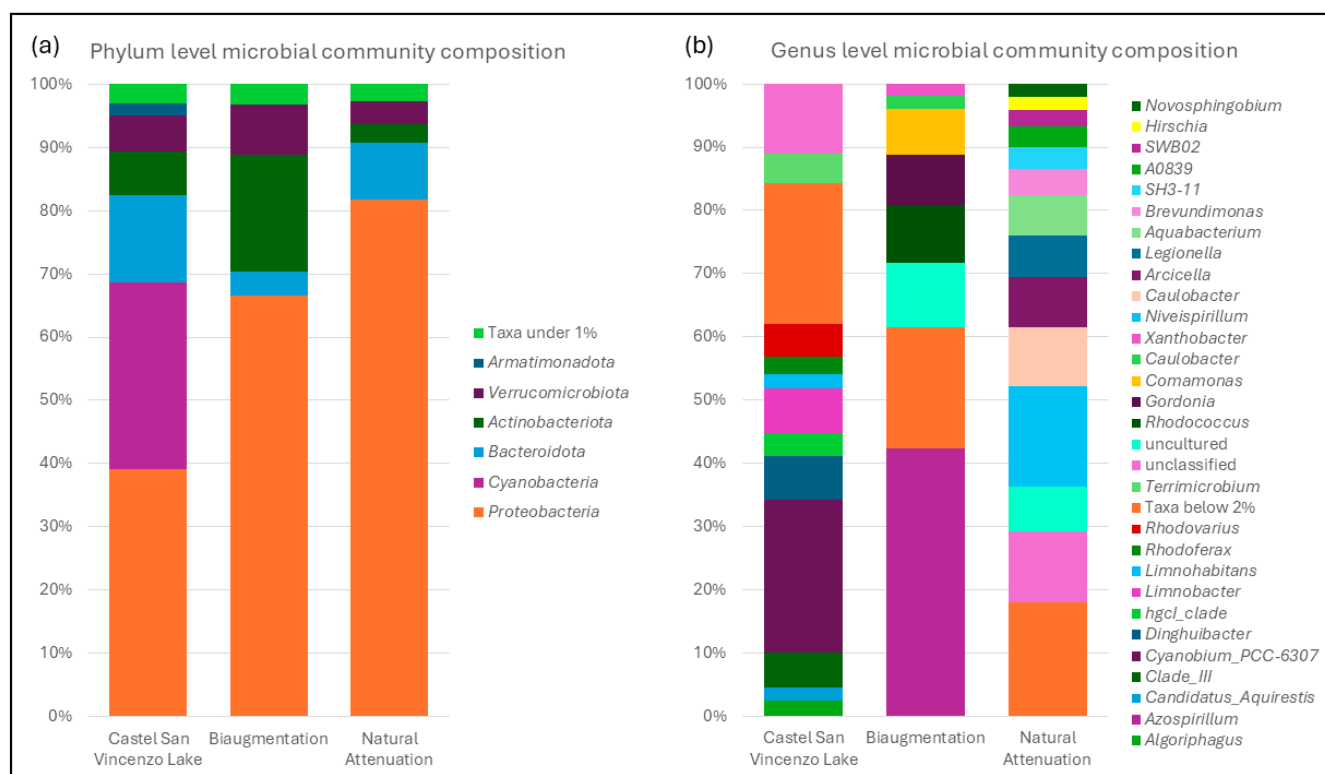


Figure 8. Microbial community composition at (a) phylum level and (b) genus level of Castel San Vincenzo Lake waters, and “Bioaugmentation” and “Natural Attenuation” mesocosms.

The rarefaction analysis revealed a reduction in microbial diversity in the “bioaugmentation” mesocosm (Shannon index value 4.53) compared to lake water (Shannon index value 5.44) (Supplementary Figure S4). In contrast, a Shannon index value (5.48) close to that of lake water was observed for the “natural attenuation” experiment.

4. Discussion and Conclusions

The growing significance of global oil pollution and oil-based products has become a pressing issue in recent decades, driven mainly by rapid population growth, industrialisation, and increased human activities. These determinants have led to the build-up of toxic substances in various environmental systems, posing a significant challenge due to their harmful effects on ecosystems, human health, and economic stability [54]. Remediating hydrocarbon-contaminated sites is particularly difficult and often requires substantial financial resources and time [55].

Biological remediation techniques, which harness the metabolic abilities of microorganisms, have emerged as effective alternatives for addressing hydrocarbon pollution. However, the success of bacterial strains in degrading petroleum hydrocarbons is influenced by a range of interrelated elements across ecological, biochemical, and genetic variables [15]. Understanding these factors is crucial for optimising bioremediation strategies to mitigate the harmful effects of pollutants. This study aimed to isolate and characterise novel bacterial strains from naturally hydrocarbon-contaminated springs (S1 and S2) and the surrounding soils in the Agri Valley (Southern Italy). This site is known for its natural outcrops of hydrocarbons, which have been recognised since the late 19th cen-

tury [29,56]. Specifically, the hydrocarbon springs S1 and S2 emerge along a fault zone that promotes fluid up-flow, facilitating the mixing of hydrocarbons and gas (mainly CH₄ and H₂S) [29] with the local groundwater, which is primarily fed by the nearby carbonate aquifer. Previous studies [57] have identified several different PAHs, including Naphthalene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(g,h,i)perylene, Indene(1,2,3-c,d)pyrene, Chrysene, Anthracene, Phenanthrene, Fluoranthene, benzo(j)fluoranthene, Dibenzo(a,h)anthracene, Pyrene, and Dibenzo(a,l)pyrene in S1 and S2 spring waters and the surrounding soils. Moreover, the presence of H₂S has been confirmed in spring S1. Continuous exposures to hydrocarbons and H₂S have shaped microbial communities, naturally selecting strains capable of biodegrading these pollutants or utilising hydrogen sulphide. NGS analyses provided evidence of this, revealing that in S1 spring water, microorganisms predominantly belonged to the genera *Sulfurovum*, *Thiovirga*, and *Chlorobium*—taxa that include strict chemolithoautotrophs sulphur-oxidising bacteria (SOB) [58,59], as well as microorganisms capable of using reduced sulphur compounds as electron donors for anoxygenic photosynthesis [60]. In the S2 spring and the surrounding soils of the S1 and S2 springs, microorganisms of the genera *Malikia*, C39, *Novosphingobium*, *Acidisoma*, KCM-B-112, *Pseudoxanthomonas*, and *Mycobacterium*, including species known for their ability to degrade contaminants/hydrocarbons, were found.

Following a traditional cultivation-based approach, which involved applying selective pressure through diesel oil supplementation, the attention has been focused on four different bacterial strains identified as *Gordonia amicalis* S2S5, *Rhodococcus erythropolis* S2W2, *Acinetobacter tibetensis* S2S8, and *Acinetobacter puyangensis* S1W1. Members of the genera *Gordonia* and *Rhodococcus* are widely distributed in nature across various environments and are known for their remarkable catabolic abilities, allowing them to utilise a broad range of hydrocarbons with diverse chemical structures [61,62]. For instance, Hao et al. [63] showed that *Gordonia amicalis* strain LH3 could degrade long-chain n-alkanes under both aerobic and anaerobic conditions, while Sowani et al. [64] demonstrated that *Gordonia amicalis* strain HS-11, a tropical soil isolate, degraded 92.85% of the provided diesel oil after 16 days of aerobic incubation. Additionally, Thi-Mo et al. [65] found that strains X5 and S67 of *Rhodococcus erythropolis* are capable of degrading hydrocarbons (hexadecane) even in their solid form, achieving 30–40% degradation at low temperatures (10 °C) within 18 days.

Similarly, the genus *Acinetobacter* includes both biotechnologically relevant species and nosocomial pathogens, with some members capable of degrading diesel oil components [66–68], n-alkanes [69–72], and aromatics [72–74].

A study by Dohare et al. [75] demonstrated that the *Acinetobacter pittii* strain ED1 degraded 68.61% of diesel oil within 7 days, increasing to 90% after 21 days, when grown at 30 °C, pH 7.0, and 1% diesel concentration.

All the strains isolated in this study could grow in BH medium with diesel oil as the sole carbon source, a finding further supported by genome analyses that revealed various genes predicted to be involved in hydrocarbon biodegradation. Additionally, the presence of several other genes encoding putative proteins responsible for heavy metal detoxification and the breakdown of other environmental pollutants suggested that these microorganisms have promising bioremediation potential, not only in environments contaminated by hydrocarbons but also in sites affected by multiple pollutants.

Furthermore, experimental trials conducted on the novel selected bacterial strains unequivocally demonstrated the ability of some of them to enhance the bioavailability of hydrocarbons. This capacity may be attributable to the production of biosurfactants, which are naturally derived surfactants produced by biological entities, particularly microorganisms. These biosurfactants could be utilised as a cost-effective and environmentally friendly method to enhance the bioremediation of oil components [76]. Additionally, the

high cellular hydrophobicity exhibited by these strains suggests a significant capacity to adhere to diesel, further contributing to their bioremediation efficacy [77].

The mesocosm experiments—aimed at assessing the biodegradation efficiency of a microbial consortium encompassing the four newly isolated hydrocarbon-oxidising bacterial strains—were performed using water samples collected from Castel San Vincenzo Lake, artificially contaminated with diesel oil. The results of microbiological investigations revealed significant changes in the composition of native microbial communities, driven by the addition of contaminants and the bioaugmentation process. Initially, the native microbial communities were dominated by picocyanobacteria of the genus *Cyanobium*, small photoautotrophs inhabiting aquatic systems, which play a crucial role in global oxygen production and CO₂ fixation on the planet [78]. However, after 28 days of incubation at 28 °C, the sole artificial contamination with diesel oil reshaped the communities, with a shift towards genera such as *Niveispirillum* (including nitrogen-fixing bacteria) [79], *Caulobacter*, and *Arcicella*, which include species isolated from various sources. The alterations in the composition of the microbial communities were linked to an approximately 6% reduction in total hydrocarbons in the “natural attenuation” mesocosm (excluding the hydrocarbon loss due to abiotic processes, as measured in the control experiment), indicating the presence of hydrocarbon-oxidising strains in the lake microbiota. On the other side, bioaugmentation with the microbial consortium and the addition of diesel oil promoted the increase in microorganisms from the genera *Gordonia* and *Rhodococcus*, but particularly in the genus *Azospirillum* (mainly represented by *Azospirillum picis*), which includes species isolated from various niches [80], such as the roots of wild plants [81,82], cultured plants [83,84], aquatic environments [85–87], and contaminated areas [83,85,88–90]. Several studies have shown that *Azospirillum* strains can degrade xenobiotics and hydrocarbons and tolerate different heavy metals [80]. In our case, a reduction of approximately 19% of total hydrocarbons was observed in the “bioaugmentation” mesocosm (attributable to both the activities of the newly isolated strains and some components of the native lake microbial community), demonstrating that the addition of the microbial consortium enhanced degradation efficiency. Interestingly, NGS analyses revealed very low relative abundance values of representatives of the *Acinetobacter* genus, indicating that the experimental conditions used to carry out the experimentation may have inhibited the proliferation of microorganisms belonging to this taxon and their degradation activity. However, the exact factors underlying this observation would need to be explored further.

In conclusion, this study has clearly shown that the natural presence of hydrocarbon in the environment can serve as an invaluable reservoir of microbial biodiversity, providing a precious source of new microorganisms with unique biodegradative potentials that can be harnessed for biotechnological applications, such as bioremediation. However, the results also emphasised that, despite the favourable genetic traits and metabolic capabilities, the actual effectiveness of individual strains, as well as microbial consortia applied in environments different from their origin, requires significant efforts to fully understand the factors that influence their proliferation and can promote the contaminant removal process. Furthermore, a careful and detailed analysis of the impact of introducing allochthonous microorganisms on native microbial communities is critical, as these communities may undergo significant variations, and the potential effects on ecosystem equilibrium should be carefully considered.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/su17073083/s1>, Figure S1: Rarefaction curves of water and soil samples collected at the Tramutola study site, Figure S2: Genomic analyses of members of the species *Gordonia amicalis* and *Rhodococcus erythropolis*, Figure S3: Comparative genomic analysis of hydrocarbon degradation and heavy metal transport genes in environmental bacterial strains, Figure S4: Rarefaction curves of water samples from Lake Castel San Vincenzo and mesocosms of “Bioaugmentation” and “Natural Attenuation”. Tables S1–S10: Results of genomic analyses of isolated bacterial strains.

Author Contributions: Conceptualisation, A.B. and F.C.; Methodology, A.B. and F.C.; Validation, C.C., C.T., S.P. and C.M.; Formal Analysis, C.C., C.T., S.P. and C.M.; Investigation, C.C., P.M., F.F., C.T., S.P. and C.M.; Resources, C.C. and C.M.; Writing—Original Draft Preparation, C.C., P.M., F.F., P.R., C.T., M.V., S.P., C.M., G.N., F.C. and A.B.; Writing—Review and Editing, C.C., P.M., F.F., P.R., C.T., M.V., S.P., C.M., G.N., F.C. and A.B.; Visualisation, C.C., P.M., F.F., P.R., C.T., S.P. and C.M.; Supervision, A.B., G.N. and F.C.; Project Administration, A.B. and F.C.; Funding Acquisition, A.B. and F.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded through a collaboration agreement between the University of Molise and the University of Parma for the project “Advances in Remediation Solutions: the case of Heterogeneous Contaminated Aquifers”. Scientific Coordinators: Antonio Bucci and Fulvio Celico. CUP: H49C21000320005.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The NGS data for microbial community analyses and whole-genome sequences presented in this study are openly available in the Sequence Read Archive (SRA) database, under the BioProject accession numbers PRJNA1219130 and PRJNA1223154.

Conflicts of Interest: The authors declare no conflict of interest.

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4 CHAPTER II

APPLICATION OF HYDROCARBON-OXIDISING BACTERIAL STRAINS IN BIOREMEDIATION

PREFACE

The second chapter of this thesis extends beyond the isolation and characterisation of hydrocarbon-oxidising bacteria to examine their application in complex, multi-contaminated soils. Specifically, it focused on integrating an artificial bacterial consortium with a plant-assisted bioremediation strategy, using *Lavandula angustifolia* to remediate a historical industrial soil contaminated with heavy metals (HMs) and polychlorinated biphenyls (PCBs).

The experiment was conducted under controlled conditions in a growth chamber for a period of 90 days. Four different microcosms treatments were established: contaminated and untreated soil (HCS), soil cultivated with *L. angustifolia* (PLANT), soil amended with the selected microbial consortium (BIOAUG), and soil receiving both lavender and the consortium (PLANT+BIOAUG). This experimental design was based on the premise that individual remediation strategies are often insufficient in soils simultaneously contaminated by organic and inorganic pollutants. The integration of bioaugmentation and plant-assisted remediation was therefore adopted to exploit the metabolic versatility of hydrocarbon-oxidizing bacteria together with the rhizosphere-mediated effects of lavender, a resilient aromatic species with economic value and a capacity to accumulate metals in its biomass.

The experimental plan followed two complementary lines of investigation. The first study, published in *Plants*, reported the results obtained for physicochemical parameters, metal concentrations, plant growth, soil enzymatic activity, and microbial abundance across the different microcosms.

The second study built upon these findings by applying high-throughput sequencing and functional prediction approaches to elucidate how the different treatments reshaped soil microbial communities and affected gene functions involved in metal transformation and plant-microbe interactions. A schematic overview of the experimental design and analytical workflow adopted in this chapter is provided in Figure 8. Taken together, these studies provided

a comprehensive evaluation of plant-microbe synergies in heavy metal remediation, thereby supporting the translation of laboratory-scale evidence into field-scale applications.

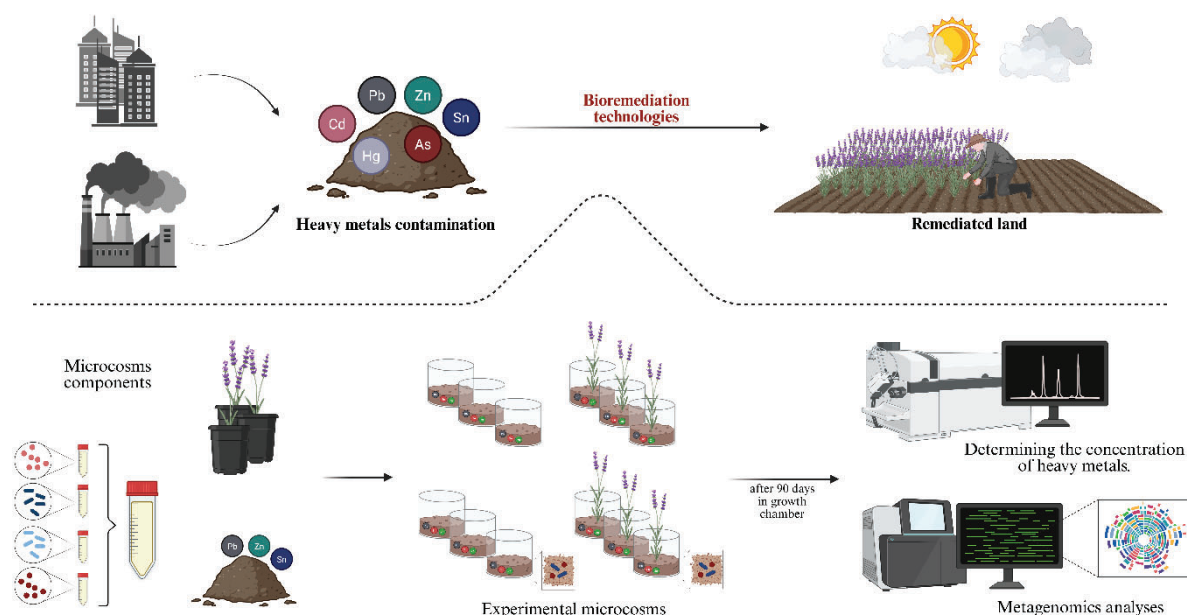


Figure 8 - The graphical abstract of the work carried out, illustrating the origin of heavy metal contamination in industrial soils, the implementation of plant-assisted bioremediation combined with microbial bioaugmentation using *Lavandula angustifolia*, and the multi-level analytical approach applied to assess remediation outcomes, including physicochemical analyses, metal quantification and high-throughput sequencing-based characterisation of soil microbial communities.

4.1. Synergistic Effects of *Lavandula angustifolia* and a Bacterial Consortium on Bioremediation of a Heavy Metal- Contaminated Soil

4.2. Metagenomic Insights into Plant–Microbe Synergies for heavy metals contaminated soil remediation

Article

Synergistic Effects of *Lavandula angustifolia* and a Bacterial Consortium on Bioremediation of a Heavy Metal-Contaminated Soil

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Abstract

Heavy metal (HM) contamination represents a significant threat to soil functionality and ecosystem health. The present study aims to assess the efficacy of an integrated bioremediation strategy combining *Lavandula angustifolia* (lavender) and a four-strain bacterial consortium in restoring a multi-contaminated soil collected from a former industrial site in Southern Italy. Microcosm experiments were conducted over a period of 90 days, including three treatments and a control: a planted condition (PLANT), a bioaugmented condition (BIOAUG), and a combined plant and bioaugmentation condition (PLANT+BIOAUG). The control (HCS) consisted of unplanted and non-bioaugmented soil. Soil physico-chemical parameters (e.g., pH, electrical conductivity, and heavy metal concentrations), plant growth, microbial abundance, and dehydrogenase activity (DHA) were measured at the initial and final experimental time. Finally, a Soil Quality Index (SQI) was applied. The combined treatment (PLANT+BIOAUG) promoted a significant reduction in total Pb and Sn concentrations by 44.7% and 66.9%, respectively. Moreover, a significant increase in soil pH and microbial abundance was observed. Applying the SQI to integrate overall soil data made it possible to highlight the highest quality score (0.73) for this condition. These findings suggest the potential effectiveness of lavender-assisted bioaugmentation as a scalable and multifunctional strategy for remediating heavy metal-contaminated soils, in line with ecological restoration principles.

Keywords: plant-assisted bioremediation; lavender; microbial consortium; soil quality index



Academic Editors: Anas Raklami and Ahmed Nafis

Received: 6 August 2025

Revised: 25 August 2025

Accepted: 30 August 2025

Published: 2 September 2025

Citation: Cavone, C.; Rutigliano, A.; Cotugno, P.; Rolando, L.; De Carolis, C.; Barra Caracciolo, A.; Grenni, P.; Savino, I.; Bucci, A.; Naclerio, G.; et al. Synergistic Effects of *Lavandula angustifolia* and a Bacterial Consortium on Bioremediation of a Heavy Metal-Contaminated Soil. *Plants* **2025**, *14*, 2734. <https://doi.org/10.3390/plants14172734>

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1. Introduction

Heavy metal (HMs) pollution is one of the most widespread and hazardous threats to the environment, food security, and ecosystem functioning [1,2], representing a substantial threat to plant growth and productivity [3]. High levels of these elements primarily originated from anthropogenic activities such as metallurgy, mining, industrial production, vehicular traffic, and intensive use of fertilisers and pesticides, and they tend to accumulate in soils. Moreover, they can enter food chains through plant uptake, raising concerns for both environmental and public health [4–6]. Unlike organic pollutants, HMs are not biodegradable and can persist in environmental compartments over long periods [7,8], altering chemical and physical properties of soils and compromising biogeochemical cycles [8]. Heavy metals are typically classified as either essential or non-essential nutrients. For example, copper (Cu), zinc (Zn), iron (Fe), manganese (Mn), and molybdenum (Mo) are essential micronutrients required in minimal quantities by plants to facilitate proper growth and maintain overall health and well-being. Indeed, they fulfil a pivotal role as cofactors for a variety of enzymes, thereby playing a crucial part in fundamental physiological processes [9]. The most critical elements include lead (Pb), zinc (Zn), tin (Sn), cadmium (Cd), nickel (Ni), and chromium (Cr), which are known for their toxicity, mutagenicity, and ability to interfere with essential cellular processes such as photosynthesis, respiration, and DNA replication [5,10]. Traditional soil remediation technologies—such as excavation, chemical washing, and solidification/stabilisation—can be effective, but at the same time, they are costly, invasive, and unsustainable, particularly for large or multi-source contaminated sites [4,11]. These techniques frequently decrease soil quality, reducing microbial biodiversity and hindering long-term ecological recovery [8,12]. Consequently, scientific interest has increasingly shifted towards bioremediation technologies, which utilise plants and microorganisms for removing, detoxifying, or stabilising contaminants. Among these, plant-assisted remediation (including rhizoremediation) has emerged as a promising approach with a low environmental impact, combining purification efficiency with soil regeneration [5,7,13]. This technology employs selected plant species which can absorb, accumulate, stabilise or transform contaminants through differentiated physiological and biochemical mechanisms [14,15], which also involve their associated microbiota [16]. Aromatic and medicinal plants (MAPs), such as *Lavandula angustifolia*, have been identified as being particularly effective in remediation of contaminated soils [17,18]. *L. angustifolia* is an aromatic, perennial shrub native of the Mediterranean area. It is widely cultivated for extraction of its essential oil [19] and it is known for combining several advantageous traits [20]: tolerance to abiotic stress, low translocation of heavy metals to the epigeal part and to oil fractions, and stimulation of beneficial rhizo-microbiota through root exudates [21] when cultivated on polluted soils [20,22]. Recent studies have shown that when lavender is combined with soil amendments such as biochar [23] or with the bioaugmentation [24], its ability to accumulate and stabilise lead (Pb), cadmium (Cd), nickel (Ni), and zinc (Zn) is enhanced.

Overall characteristics make lavender a promising candidate for integrated phytoremediation approaches. Beyond its remediation capacity, the utilisation of lavender in urban landscapes can be further supported by its aesthetic appeal and drought tolerance, making it well suited for incorporation into green infrastructure in contaminated or degraded peri-urban areas. Furthermore, its cultivation makes it possible biomass valorisation through extraction of essential oils or production of compost, biochar, and hydrolats, thereby reducing risks of contaminants entering food chains [25,26].

Nevertheless, phytoremediation can be less effective in heavily contaminated soils, where metal bioavailability is low and microbial activity is low. To address these limitations, microbial bioaugmentation—introducing metal-tolerant bacterial consortia—has

emerged as a complementary strategy [27]. Microorganisms can transform and immobilise metals through biosorption, bioaccumulation, biotransformation, and redox-mediated precipitation [6,10,28]. Bacterial resistance to heavy metals typically involves a suite of adaptive mechanisms including active efflux through P-type ATPases and RND-type transporters, intracellular sequestration via metallothionein and glutathione, and enzymatic redox transformation of toxic ions [29]. Such mechanisms not only ensure microbial survival in polluted environments but also increase bioavailability and mobility of metals, facilitating their uptake or immobilisation in the rhizosphere [30].

In this context, a consortium comprising *Gordonia amicalis* (Actinomycetota), *Rhodococcus erythropolis* (Actinomycetota), *Acinetobacter puyangensis* and *A. tibetensis* (Pseudomonadota, γ -Proteobacteria) strains was tested for improving lavender remediation capacity in removing HMs. These genera are recognised for their biodegradation capacity, including reductive dechlorination and co-metabolic transformation [8]. Additionally, they produce siderophores and biosurfactants which enhance metal chelation and solubilisation of hydrophobic organic compounds, respectively [31,32]. Bacteria belonging to these genera have demonstrated strong biofunctional properties in contaminated environments, particularly in synergy with plant roots [33–35]. Positive interactions between plants and rhizo-microbiota have shown to stimulate microbial activity [12], improve soil physico-chemical properties (e.g., pH and electrical conductivity), and promote adaptation to metal stress [12,30,36].

Bacterial consortia are generally more robust than single strains in complex matrices, as they facilitate functional cooperation, extend the range of degradable substrates, and display higher resilience under variable environmental conditions [37,38]. Moreover, biosurfactant-producing bacteria can improve organic and inorganic pollutant bioavailability [39] by lowering surface tension, which is essential for effective breakdown of recalcitrant compounds.

The present study aims to evaluate the combined efficacy of phytoremediation with *Lavandula angustifolia* and bioaugmentation with the microbial consortium (*Gordonia amicalis*, *Rhodococcus erythropolis*, *Acinetobacter puyangensis*, and *Acinetobacter tibetensis* strains) applied to a soil multi-contaminated with Zn, Sn, and Pb from the highly impacted site of Taranto—Cimino Manganeccia. Chemical (pH, electrical conductivity, metal concentrations) and microbiological (total microbial abundance and dehydrogenase activity) investigations were performed and tested with multivariate statistical analyses (ANOVA and PCA) to evaluate treatment effectiveness. Moreover, to assess overall soil improvements, a Soil Quality Index (SQI) was applied, integrating these parameters. The SQI considered heavy metals (Zn, Sn, and Pb) as negative indicators and pH, electrical conductivity (EC), microbial abundance, and dehydrogenase activity as positive indicators, supporting a comprehensive evaluation of soil functionality and remediation effectiveness over time [40]. This approach ensured that both the reduction in pollutants and the restoration of ecological balance were considered in assessing treatment performance.

The overarching objective was to produce empirical data which may support the development of eco-sustainable, scalable, and replicable strategies for remediation of contaminated soils, aligned with bioeconomy principles and long-term restoration of terrestrial ecosystems.

2. Results

2.1. Plant Growth, Soil pH, and EC

All lavender that was cultivated in the soil microcosms demonstrated robust vitality and exhibited normal development over a period of 90 days. Comparing the PLANT and PLANT+BIOAUG conditions, no significant differences were found in terms of biomass and leaf number. A significant increase in soil pH ($p < 0.05$) compared to the control

soil (HCS) was observed in all treated conditions. At 90 days, the PLANT+BIOAUG had the highest value (pH: 7.38 ± 0.07), followed by BIOAUG (pH: 7.28 ± 0.04) and PLANT (pH: 7.13 ± 0.00) ones. The study revealed no statistically significant differences in EC among the treatments examined. The values ranged from 1.03 ± 0.01 to 1.50 ± 0.08 dS/cm. All data are reported in Table 1.

Table 1. Soil pH and electrical conductivity (EC, dS/m) were measured at the commencement of the experiment and after 90 days under different treatment conditions. The values are expressed as the mean $\pm$ standard deviation among treatments and times. For pH results, superscript letters (*a*, *b*, *c*), indicating statistical differences in the application of Tukey's post hoc test ($p < 0.05$), are reported.

Treatments	Days	pH	EC
		Mean $\pm$ st.dv	Mean $\pm$ st.dv
HCS	0	6.64 ± 0.23^a	0.99 ± 0.01
	90	7.35 ± 0.08^c	1.20 ± 0.04
PLANT	0	6.64 ± 0.23^a	0.99 ± 0.01
	90	7.13 ± 0.00^b	1.38 ± 0.18
BIOAUG	0	7.31 ± 0.06^c	1.22 ± 0.01
	90	$7.28 \pm 0.04^{b,c}$	1.50 ± 0.08
PLANT+BIOAUG	0	7.31 ± 0.06^c	1.22 ± 0.01
	90	$7.38 \pm 0.07^{b,c}$	1.39 ± 0.20

2.2. Contaminant Removal and Plant Uptake

Inductively coupled plasma mass spectrometry (ICP-MS) analysis revealed the presence of various heavy metals (Be, Al, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Sn, Sb, Cd, Pb) in soil samples from the various microcosms. The concentrations of Sn, Zn, and Pb exceeded the Italian legal limits (Italian Legislative Decree 152/06), which are 1 mg/kg for Sn, 150 mg/kg for Zn, and 100 mg/kg for Pb, respectively (Figure 1). However, at 90 days, significant ($p < 0.05$) decreases in Sn concentrations were observed. Specifically, Sn concentrations decreased from 81.01 mg/kg to 26.83 mg/kg in the PLANT+BIOAUG, to 68.89 mg/kg in the PLANT, and to 78.46 mg/kg in the BIOAUG conditions. Moreover, in HCS microcosms, the average Sn concentration observed at 90 days (48.09 mg/kg) was lower than the initial one (81.01 mg/kg). Due to the high variability in Sn concentrations, statistical analysis revealed no significant differences from 0 and 90 days.

No significant decrease in Zn concentration was recorded in all the experimental conditions. Lead (Pb) showed a significant overall decrease (Kruskal–Wallis $p = 0.02$), and concentrations decreased from 1328.98 mg/kg to 841.80 mg/kg in the PLANT, to 1071.99 mg/kg in the BIOAUG, and to 734.31 mg/kg in the PLANT+BIOAUG conditions. As observed for Sn in HCS microcosms, the high variability of Pb values at 90 days did not reveal any significant reduction in Pb concentrations in this experimental condition.

The concentrations of Zn, Sn, and Pb observed in plant tissues (leaves and roots) and the translocation factor (calculated as the ratio of metal concentration in leaves to that in roots) are reported in Table 2. Zn was the most abundant heavy metal found (39.88 ± 16.67 mg/kg).

Table 2. Zn, Sn, and Pb concentrations (average values and standard deviation) observed in plant tissues (leaves, roots) and TF (translocation factor) calculated for the different experimental conditions (PLANT, PLANT+BIOAUG).

		Zn	Sn	Pb
PLANT	Leaves	37.15 ± 5.38	0.10 ± 0.03	5.74 ± 2.51
	Roots	39.88 ± 16.67	0.50 ± 0.02	26.49 ± 6.59
	TF	0.93	0.20	0.22

Table 2. Cont.

PLANT+BIOAUG				
		Zn	Sn	Pb
	Leaves	11.26 ± 2.96	0.10 ± 0.04	15.46 ± 5.20
	Roots	20.33 ± 11.53	0.51 ± 0.03	20.39 ± 6.21
	TF	0.55	0.20	0.76

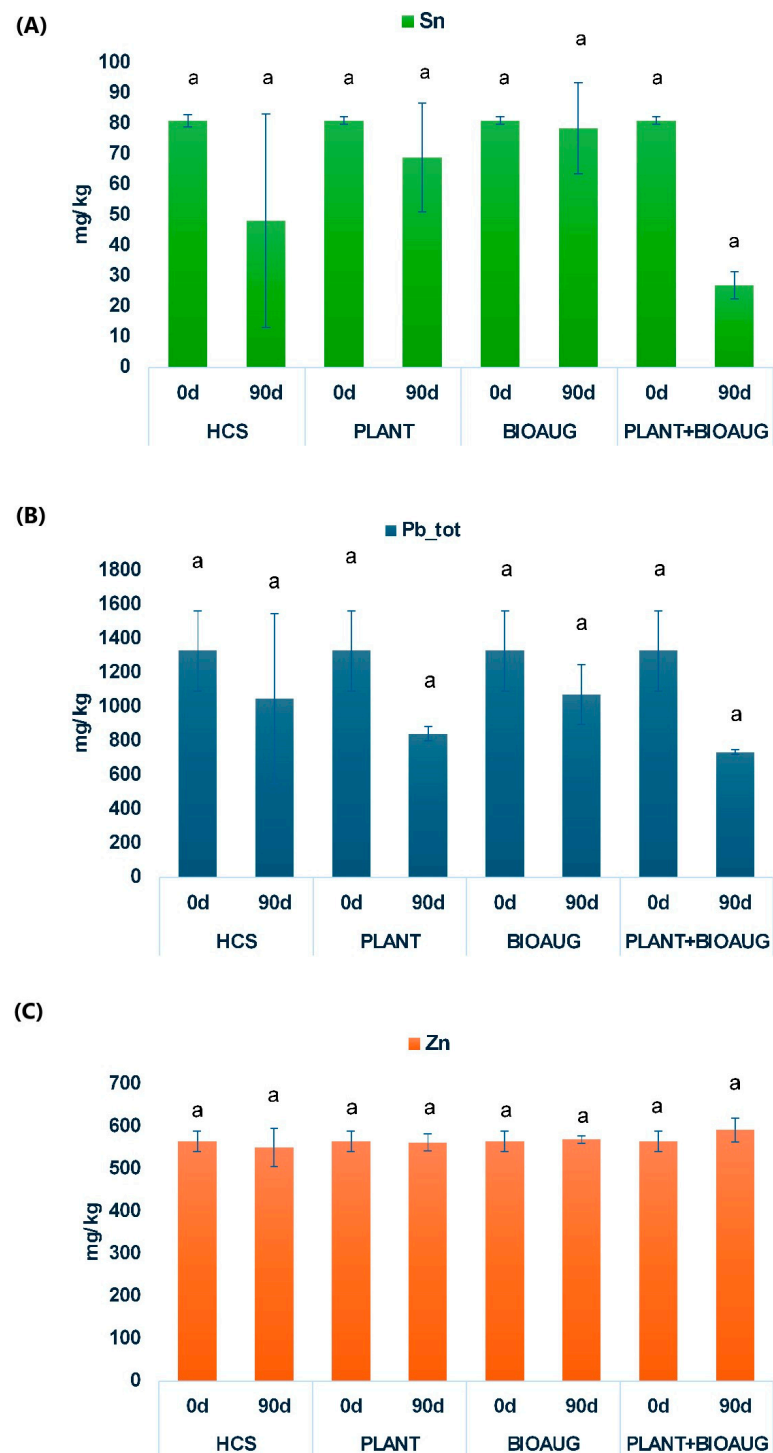


Figure 1. Average concentrations (mg/kg) of Sn (A), Pb (B), and Zn (C) in soils samples collected at 0 and 90 days in the various experimental microcosms (HCS, PLANT, BIOAUG, and PLANT+BIOAUG). Values are shown as mean $\pm$ standard error. Lowercase letter (a) is employed to evidence no statistical difference (Tukey's post hoc test) among the experimental conditions.

2.3. Microbial Abundance and Activity

The presence of lavender plants (PLANT and PLANT+BIOAUG) in the microcosms resulted in a significant ($p < 0.001$) increase in microbial abundance at 90 days. Differently, in BIOAUG, a sharp decrease from day 0 to 90 d ($p < 0.05$) was observed (Figure 2A). Indeed, the PLANT+BIOAUG condition showed the highest abundance values (up to 1.40×10^8 cells/g), suggesting that the co-presence of the lavender with the bacterial consortium was the best condition for increasing the overall microbial community number and making bacterial consortium survival possible. Conversely, the decline observed in BIOAUG alone may be indicative of limited persistence of the inoculated strains and potential competition with autochthonous microorganisms, which could have hindered consortium establishment.

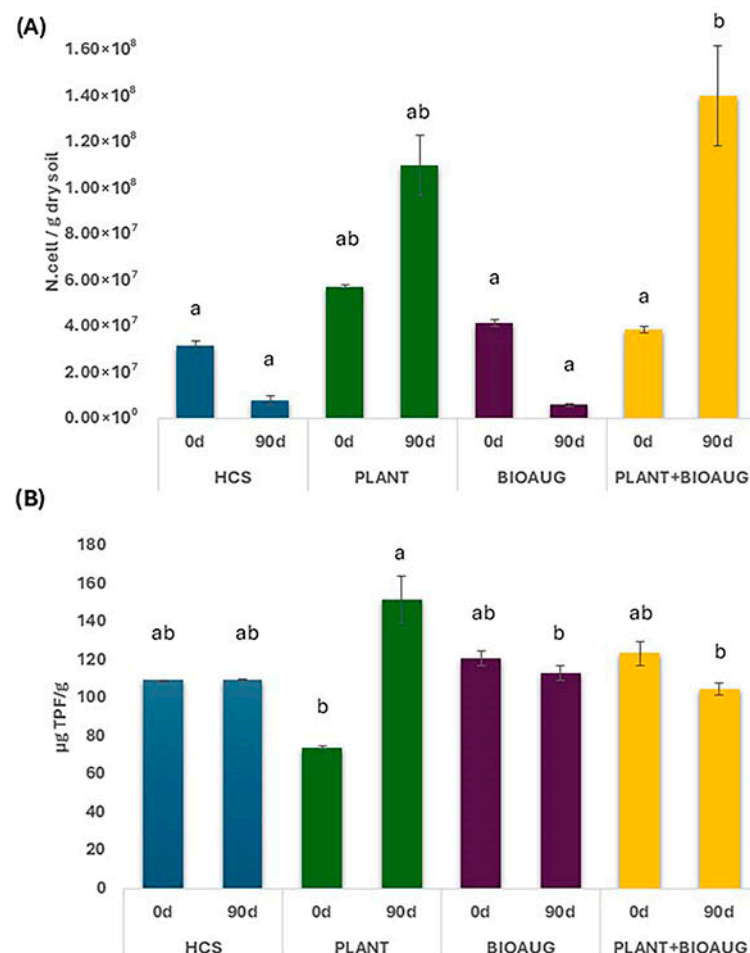


Figure 2. Average values of (A) total microbial abundance and (B) dehydrogenase activity in soils sampled after 0 and 90 days in the various experimental conditions (HCS, PLANT, BIOAUG, and PLANT+BIOAUG). Values are shown as mean $\pm$ standard deviation. Lowercase letters indicate statistically significant differences among the treatments.

The PLANT treatment showed the highest dehydrogenase value (DHA: 151.92 μ g TPF/g), underscoring the role of plant roots in supporting natural soil microbial communities. DHA values were lower in the PLANT+BIOAUG and in the BIOAUG treatments (Figure 2B).

2.4. Soil Quality Index

Figure 3 reports the soil quality index (SQI), which integrated pH, electrical conductivity (EC), microbial abundance (DAPI), and dehydrogenase activity (DHA) values as

positive indicators and heavy metal (Zn, Sn, and Pbtot) concentrations as negative ones. The SQI after 90 days shows the highest value in PLANT+BIOAUG (SQI = 0.73), followed by PLANT (SQI = 0.58) and BIOAUG (SQI = 0.48). Interestingly, the control soil (HCS) remained at a very low value (SQI = 0.32). The possibility to use all parameters measured in the SQI made it possible to rank the soil improvement and to give a value of its quality in response to the remediation strategies applied. All parameters, except EC, because its variations were not significant, gave a contribute to the index value. Overall parameters integrated in the SQI demonstrated that the PLANT+BIOAUG condition attained a substantial enhancement in soil quality. The PLANT condition demonstrated a moderate improvement. Differently, bioaugmentation alone was not effective for improving soil quality, presumably because the application of bacterial species which outcompete the natural ones can be negative from an ecological point of view.

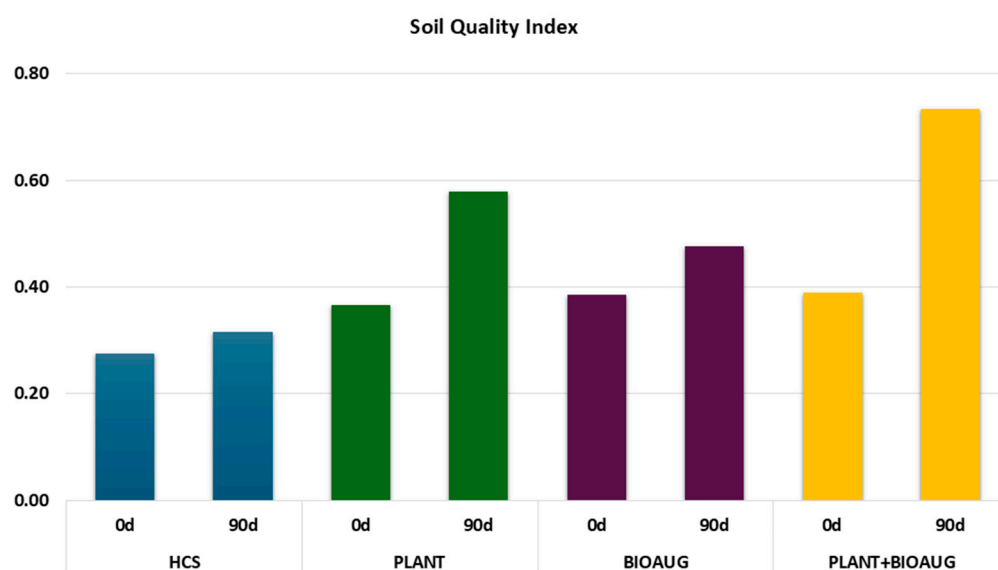


Figure 3. Soil Quality Index (SQI) values calculated by integrating pH, electrical conductivity, microbial abundance, dehydrogenase activity, and heavy metal parameters.

2.5. Multivariate Analysis

A principal component analysis (PCA) was conducted on the chemical and biological parameters (Figure 4). The first two components, PC1 and PC2, explained 34.1% and 19.9% of the total variance, respectively, for a cumulative contribution of 54.0%. The PC1 shows how heavy metal contamination (Sn and Pb) is in the opposite position of pH and microbial abundance (DAPI), indicating that reductions in metal concentrations were closely associated with improvements in soil microbial growth and neutralisation of acidity. The PC2 shows how DHA and EC are positively correlated, reflecting the influence of root–microbe interactions and enzymatic activity on soil ionic balance. The biplot (Figure 4) demonstrates a clear separation of the treatments over time. Indeed, HCS (control) data exhibited a negative association with PC1, suggesting the presence of residual contamination and lower microbial activity. Considering the treatments after 90 days, PLANT and PLANT+BIOAUG are mainly influenced by overall positive factors (DAPI, DHA, and pH), indicating the collaborative impact of lavender rhizosphere activity and microbial interactions in enhancing soil quality recovery. Furthermore, BIOAUG is not grouped in a cohesive cluster, suggesting the low effectiveness of microbial bioaugmentation alone.

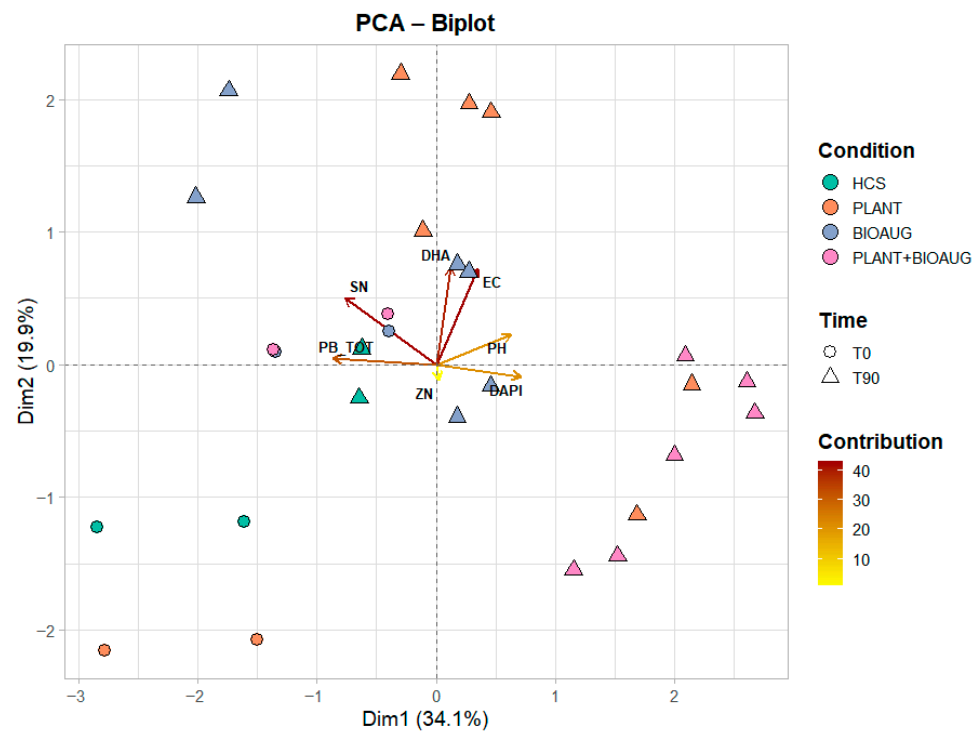


Figure 4. Principal component analysis (PCA) of physico-chemical and microbiological parameters (EC, pH, DHA, DAPI, and metal concentrations). The length and colour of each vector represent the distribution of the corresponding variable on the principal component (Dim1 and Dim2).

The statistical robustness of the treatment effects on the multivariate soil profiles was confirmed by PERMANOVA, which indicates a significant difference between PLANT+BIOAUG ($p < 0.001$) and PLANT ($p < 0.05$) from the other treatments.

3. Discussion

The combined strategy of bioremediation with *Lavandula angustifolia* and bioaugmentation with a selected microbial consortium (PLANT+BIOAUG) has been demonstrated to be effective in decreasing HM contamination (Sn and Pb) and increasing overall soil quality, considering both physical–chemical and microbiological parameters. The increase in soil pH observed across all treatments (particularly in PLANT+BIOAUG) can be attributed to synergistic biological and chemical processes. As demonstrated in the study by Sharma et al. [41], root exudates from *L. angustifolia* interacting with metal ions (e.g., Pb and Sn) reduce proton displacement (H^+) and increase pH levels. Concurrently, a microbial ammonification process is initiated by the bacterial consortium (*Gordonia*, *Rhodococcus*, *Acinetobacter*), resulting in the release of NH_3 and the concomitant consumption of H^+ ions [42]. The production of biosurfactants has been demonstrated to enhance metal solubility, thereby promoting precipitation as hydroxides or carbonates at higher pH [43]. In addition, the degradation of acidic metabolites via microbial activity has been shown to contribute to alkalinisation [44]. The interconnected mechanisms underpinning the optimal pH in PLANT+BIOAUG are such that plant–microbe synergies are maximised, thereby facilitating metal immobilisation.

The PLANT treatment showed the highest microbial dehydrogenase value, confirming that lavender presence promoted a general increase in number and activity of natural soil microbial communities, thanks to the positive interactions established in the rhizosphere [12,16,45]. However, DHA values were lower in the bioaugmented conditions (PLANT+BIOAUG and BIOAUG treatments), suggesting competition phenomena between autochthonous and allochthonous bacterial populations. Interestingly, in BIOAUG, the

lowest microbial abundance, associated with relatively high microbial activity, can be ascribed to the competitive exclusion of the bacterial consortium. Indeed, the dead bacterial biomass of the inoculants could be a carbon and nutrient source for the autochthonous soil bacteria, stimulating their overall activity [46]. A recent study [47] has demonstrated that the processes of microbial necromass and cell lysis result in the release of dissolved organic carbon and labile nutrients. These nutrients are then rapidly metabolised by native microbial communities. This phenomenon can also explain the low effectiveness of bioaugmentation (BIOAUG) in this experiment, as found in another works [48].

Supporting our results, a recent study on the same consortium used in this experiment has shown that these bacteria are potentially able to tolerate and remove heavy metals and to produce biosurfactants [31]. Other authors have also found that *Acinetobacter* [43] and *Gordonia* [49] genera interact with heavy metals in soil, facilitating their immobilisation and sequestration through various mechanisms such as biosorption, precipitation, and intracellular accumulation, also modulating local metal concentrations in the rhizosphere [29,50]. *Rhodococcus* and *Acinetobacter* have also been reported to produce exopolysaccharides (EPSs) and biofilm matrices which contribute to HM adsorption and immobilisation through bonds with functional groups such as carboxyls, phosphates, and hydroxyl groups [51]. Moreover, the rhizosphere of *L. angustifolia* has been demonstrated to secrete chelating compounds, including organic acids and phenolic metabolites. These compounds have the capacity to bind HMs and reducing their mobility in soil [52]. Overall, the metabolic characteristics of the genera in the consortium mentioned above, with the natural chelating capacity of lavender root exudates, can explain the best performance of the PLANT+BIOAUG condition, thanks to their active role in HM removal. However, this fact does not exclude a possible contribution of the rhizosphere natural microbial community in HM reduction in the PLANT condition [30].

Our results suggest a pivotal role of *L. angustifolia* in shaping the composition and functionality of the rhizospheric microbial community, which made possible the survival of the microbial consortium and its coexistence with the natural soil microbial populations. This was possible through a combination of chemical and physical modifications, through signalling (e.g., root exudates) and symbiotic interactions, which have defined new rhizosphere microenvironments [53]. Indeed, root exudates comprise a wide variety of chemicals (e.g., simple sugars, organic acids, and amino acids) which enhance microbial activity [41], modify soil chemistry, and contribute to nutrient cycling and acid–base equilibrium [54]. Consequently, lavender favoured Sn and Pb removal, both absorbing/stabilising them, in line with recent work [55] and favouring the microbial role. Any significant reduction in Zn was observed in all the experimental pots. At these Zn values (about 500 mg/kg), lavender with or without the microbial consortium was enabled to promote the decrease in this metal, which is an essential micronutrient for all plant species. In a previous work [20], phytoremediation capability of lavender has been observed at higher concentrations (>2000 mg/kg) than those obtained in the present experiment. In the treatment without lavender and/or bioaugmentation (HCS), the HM results did not show any statistically significant reduction. These results are in line with those obtained in a recent pot experimental trial performed using soil collected from the same survey site of the present work and different plant species (*Brassica juncea* and *Sorghum bicolor*) [56].

The analysis of HMs in leaves and roots evidenced that HM absorption occurred primarily via the roots. Zn concentrations observed in plant tissues of the planted microcosms were not in line with the results shown by Ancona et al. [57,58] in previous field phytoremediation experiments. In fact, in this study, any significant Zn uptake was observed in lavender leaves, as evidenced by TF values < 1.

4. Materials and Methods

4.1. Study Area and Sample Collection

In November 2023, soil samples were collected from a historically contaminated area, located close to Taranto (Apulia Region, Southern Italy; GPS coordinates: 40°28'04.92" N, 17°18'12.68" E). The soil was degraded not only in terms of pollution but also in terms of low nutrient content (organic carbon content: 15.02 ± 3.21 g/kg, nitrogen: 0.17 ± 0.07 g/kg and phosphorous: 8.12 ± 1.81 mg/kg), with a pH of 6.64 and EC of 0.99 dS/cm.

This area was classified as a Contaminated Sites of National Interest (SIN) by the Italian government due to long-standing emissions from metallurgical and petrochemical industries. The area has been the subject of plant-assisted bioremediation (PABR) strategies for approximately a decade, with the application of the Monviso poplar clone (*Populus generosa* × *Populus nigra*). A preliminary investigation, conducted by Ancona et al., 2017 [57], on the chemical contamination revealed a non-uniform distribution of polychlorinated biphenyls (PCBs) across various locations within the studied area. The concentrations of PCBs often exceeded the national legal limit (60 ng/g for gardens, parks and residential areas, as outlined by the Italian Legislative Decree 152/06). Additionally, high concentrations of several heavy metals (especially Sn, Pb and Zn) were detected, requiring specific attention.

Approximately 10 kg of a surface a composite soil (0–20 cm) was collected for performing the microcosm experiments. Prior to utilisation, stones, larger clods and residues of plant materials were removed; the soil was then dried at room temperature (18–20 °C) and sieved (2 mm mesh). The soil was put in a cylindrical container and placed on a roller for 3 days for homogenising it.

4.2. Plant, Bacterial Consortium, and Experimental Design

Lavandula angustifolia (Lamiaceae family) is a Mediterranean perennial shrub widely used in phytoremediation for its tolerance to metal-contaminated soils and its ability to support rhizospheric microbial activity [20,24]. *L. angustifolia* plants were obtained from a commercial nursery (Mondo Piante, Terlizzi, Puglia). Healthy and uniform plants (6–7 cm in height) were transplanted into microcosms at the start of the experiment.

The bacterial consortium (previously tested for removal of organic pollutants such as diesel) consisted of the following strains: *Gordonia amicalis* (strain S2S5), *Rhodococcus erythropolis* (strain S2W2), *Acinetobacter puyangensis* (strain S1W1), and *Acinetobacter tibetensis* (strain S2S8). It was obtained as described in Cavone et al. [31], with some modifications. The bacterial cultures were grown separately in 50 mL of LB medium at 28 °C for 24 h until they reached the mid-logarithmic phase. Bacterial cells were then harvested by centrifugation at 3500 rpm for 10 min, washed twice with sterile water, and resuspended in water (10 mL). Each bacterial suspension was adjusted to an optical density (OD 600 nm) of 1.0. Subsequently, equal volumes of the suspensions were mixed to constitute the microbial consortium (10 mL), which has been used in the mesocosm experiments.

The microcosm experiment consisted in plant pots (1 L capacity) containing HM-contaminated soil samples (500 g each). Three different conditions were tested: plant-assisted bioremediation (PLANT), with only a lavender plant, bioaugmentation (BIOAUG) with only the bacterial consortium, and a combination of plant-assisted bioremediation and bioaugmentation (PLANT+BIOAUG). The inoculum was incorporated into the BIOAUG and PLANT+BIOAUG treatments at a final concentration of 2% v/w, with the consortium population being introduced at a concentration of 1×10^8 CFU/g soil. Finally, untreated control microcosms (HCS) consisted exclusively in the historically contaminated soil. Each microcosm was tested in triplicate and maintained in a growth chamber (Climate chamber Binder KBF 720, GmbH, Wetzlar, Germany) at environmental temperature (25 ± 1 °C);

photoperiod 16/8 h light/dark; and relative humidity reaching 65/80% light/dark. Microbiological and chemical analyses were performed at the start and at the end of the experiment (90 days). The overall experimental design is illustrated in Figure 5.

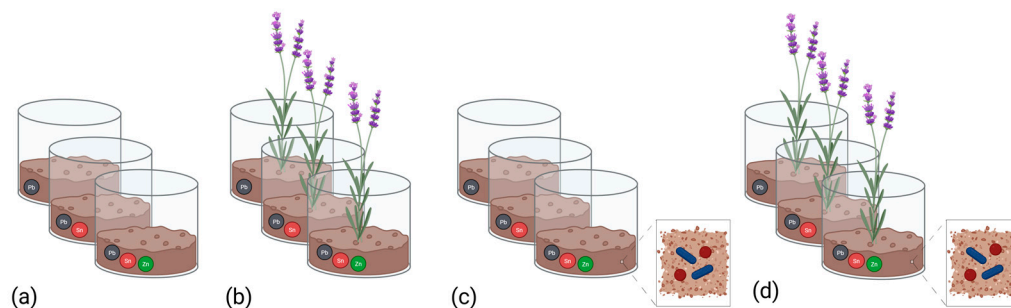


Figure 5. Experimental design of soil microcosms. (a): untreated contaminated soil (HCS), (b) soil planted with *Lavandula angustifolia* (PLANT), (c) soil inoculated with bacterial consortium (BIOAUG), and (d) combined treatment (PLANT+BIOAUG).

4.3. Plant Growth Analysis

Plant growth was evaluated at the end of the experiment (90 days). The plant height (cm) and the number of leaves (N° leaves) were measured immediately. For the biomass, lavender plants (shoot and roots) were extracted from the soil and weighed. The dry weight was obtained by placing the roots and leaves in an oven at 40 °C until a constant mass was reached.

4.4. Physico-Chemical Analysis of the Soil and Pollutant Plant Uptake Assessment

The soil chemical properties, including pH and soil electrical conductivity (EC), were determined in accordance with the Italian Official Methods of Soil Chemistry (MUACS), approved by the Italian Ministry for Agricultural Policies [59]. The pH was determined potentiometrically, following the Method III.1 protocol, on a suspension of soil-neutral salt solution (potassium chloride, KCl) containing 10 g of soil. The electrical conductivity (EC) was carried out following the Method IV.1 protocol, using aqueous soil extracts (5:1 water/soil ratio). The soil extract was subjected to mechanical shaking for 2 h, followed by an equal time of undisturbed rest. Then, it was filtrated (Whatman No. 42 paper filter). A single drop (0.1%) of a sodium hexametaphosphate solution ($\text{NaPO}_3)_6$ was added to each extract. Conductivity was measured at 25 °C using a multi-parameter probe (Hanna Instruments Edge, Woonsocket, RI, USA).

Determination of heavy metals in soil samples was performed by inductively coupled plasma mass spectrometry analysis (ICP-MS, Agilent 7700 Series ICP-MS, Agilent Technologies, Tokyo, Japan). Firstly, dried soil was milled to a fine powder (granulometric fraction with diameter less than 2 mm) and then accurately weighed and mineralised. Mineralisation was performed by treating 500 mg of powdered samples with 9 mL of concentrated HCl and 3 mL of HNO_3 and by heating (Ethos Touch Control, Milestone, Microwave Laboratory Systems) with a two-step procedure: 10 min to reach 200 °C followed by 15 min at 200 °C. Following cooling, the mineralised samples were transferred into vials, adjusted to a volume of 50 mL with MilliQ water, and diluted 25 times prior to ICP-MS analysis. This procedure was undertaken to ensure a maximum content of 5% acids and 0.2% dissolved solids. Quantification of all mineral elements was performed by ICP-MS. Standard reference materials (multi-element calibration standards 2A, Agilent) were used for precision, quality assurance, and checking the measurements. The average values of two replicates were taken for each analysis [58]. Total lead content was calculated as the mean of isotopic concentrations (^{206}Pb , ^{207}Pb , ^{208}Pb). In order to evaluate pollutant plant

uptake capabilities, heavy metal analyses on plant biomass (leaves and roots) samples have been performed according to the method reported in Angelini et al., 2022 [60].

4.5. Analysis of the Microbiological Soil Community

The total microbial abundance (N. cells/g soil) was determined by the epifluorescence direct count method with DAPI (4',6-diamidino-2-phenylindole) as the DNA fluorescent dye. The DAPI staining method enables the detection of microbial cells in a sample, regardless their physiological state or metabolic activity. Soil samples (1 g each) were collected from each replicate microcosms and immediately transferred to a test tube containing 9 mL of a fixing solution (composed of phosphate-buffered saline: 130 mM NaCl; 7 mM Na₂HPO₄, 3 mM NaH₂PO₄; 2% formaldehyde (v/v); 0.5% Tween 20 (v/v) and 100 mM sodium pyrophosphate). After shaking, the suspensions were left to settle for 24 h to allow larger particles to precipitate. An aliquot of the supernatant was then treated with DAPI incubated 30 min in the dark at 4 °C and subsequently filtered through a 0.2 µm polycarbonate filter. Microbial cells were counted using a Leica DM 4000 B fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) [45].

The dehydrogenase activity (DHA), which reflects microbial respiration rate and provides information on the active portion of the soil microbial community [61,62], was evaluated in soil samples (6 g each) using a colourimetric method based on the quantification of 2,3,5-triphenyl formazan (TPF, red) produced from the reduction of 2,3,5-triphenyltetrazoliumchloride (TTC, uncoloured). Soil samples were incubated with TTC for 24 h at 37 °C in the dark. The soil microbial activity (expressed as TPF/g soil) was measured using a Multiskan Sky Microplate Spectrophotometer (Thermo Scientific, Waltham, MA, USA) [63]. All measurements were performed at the start (day 0) and at the end of the experiment (90 days).

4.6. Soil Quality Index (SQI)

To provide an integrated measure of soil functionality, a Soil Quality Index (SQI) was calculated integrating seven selected physicochemical and microbiological parameters: specifically, heavy metal concentrations (Zn, Sn, Pb), pH, electrical conductivity (EC), microbial abundance (DAPI counts), and dehydrogenase activity (DHA).

In accordance with [64], for each soil parameter we used, we calculated a parameter score (S) from 0 to 1 for each experimental condition, reflecting low and high quality, respectively. The two criteria we used are as follows:

More is better: $S = (x - x_{\min}) / (x_{\max} - x_{\min})$.

Less is better: $S = 1 - (x - x_{\min}) / (x_{\max} - x_{\min})$.

The more-is-better criterion was applied to pH, electrical conductivity, microbial abundance, and dehydrogenase activity for their positive influence for soil fertility. The less-is-better criterion was applied to heavy metal concentrations, because high concentrations can be toxic to soil organisms [65].

The overall parameter score (SQI) was calculated as reported by [66] as follows:

$$SQI = \sum_{i=1}^n Si / n$$

In which *S* is each parameter score obtained from each parameter; *n*: number of parameters used (in our case, *n* = 7). The SQI can have values from 1 (the maximum quality) to 0 (the lowest quality).

4.7. Statistical Analysis

Statistical analyses were performed using R (v.4.3.0). The Shapiro–Wilk test was performed to verify the normality of the data distribution. Parametric (one-way or two-way ANOVA followed by Tukey HSD) or non-parametric (Kruskal–Wallis test followed by Wilcoxon post hoc tests) tests were applied on chemical and microbiological data.

For the multivariate analysis, a principal component analysis (PCA) was conducted on the standardised data (centred and scaled) to explore the relationships between chemical and microbiological variables and to visualise the influence of the treatments (presence of plant and/or microbial consortium) on the data distribution. The results of the PCA were represented graphically with confidence ellipses and group hulls. PERMANOVA was also performed to statistically test the effect of the experimental variables.

5. Conclusions

Overall, PLANT+BIOAUG was the most effective condition in promoting significant HM removal, and this was presumably due to both bioaugmented and natural soil microbial populations. This was possible thanks to the lavender rhizosphere, which favoured the co-existence of both bioaugmented and natural microbial populations, increasing both physical (through root development) and chemical (e.g., root exudate production) soil characteristics and its quality, as evidenced by the highest SQI value.

The combined application of *L. angustifolia* and a selected bacterial consortium represents a highly promising and functional approach to the sustainable management of contaminated soils, reducing heavy metals and stimulating overall soil microbial activity at the same time. These results were obtained with real contaminated matrices in microcosm conditions, and field applications are necessary in order to better evaluate the stability of the microbial consortium for a longer time and under variable conditions. Above all, competition with the native microbiota adapted to the variable site-specific conditions needs to be investigated. Knowing climate variability, water availability, root penetration, and pollutant amount and distribution in field conditions can be very useful for planning optimal inoculation strategies in actual environmental conditions and for stimulating bacterial consortium survival over time.

This approach is particularly effective because it aligns with the principles of agroecology and the circular economy. These principles combine environmental remediation with the utilisation of commercially valuable aromatic crops, providing a multifunctional solution for environmentally friendly contaminated site management.

Author Contributions: Conceptualisation, V.A. and C.C.; methodology, V.A.; validation, C.C., A.R., P.C., and L.R.; formal analysis, C.C., A.R., P.C., L.R., and C.D.C.; investigation, C.C., A.R., I.S., L.R., and C.D.C.; data curation, C.C., L.R., and C.D.C.; writing—original draft preparation, C.C., V.A., A.R., P.C., P.G., L.R., C.D.C., I.S., A.B., G.N., F.C., V.F.U., and A.B.C.; writing—review and editing, C.C., V.A., C.D.C., and A.B.C.; visualisation, C.C., V.A., A.R., C.D.C., A.B., G.N., F.C., P.G., and A.B.C.; supervision, V.A. and V.F.U. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

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4.2. METAGENOMIC INSIGHTS INTO PLANT–MICROBE SYNERGIES FOR HEAVY METALS CONTAMINATED SOIL REMEDIATION

This chapter is based on a manuscript submitted to *New Biotechnology* (Elsevier) and is currently under review (Manuscript Number NBIOT-D-26-00013). The study builds on and improves the research framework that was previously published in *Plants*. This new work goes beyond the previous investigation by using metagenomic analyses. These data, which have not been previously published, enable an in-depth assessment of microbial community structure, diversity patterns and predicted functional traits. This, in turn, strengthens the ecological interpretation and mechanistic framework of the bioremediation process.

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ABSTRACT

Heavy metal contamination poses a serious threat to soil ecosystems and requires sustainable remediation approaches capable of restoring both chemical quality and microbial functionality. This study evaluates the effectiveness of plant-assisted bioremediation (*Lavandula angustifolia*) and bioaugmentation with a selected bacterial consortium of four strains (*Gordonia amicalis*, *Rhodococcus erythropolis*, *Acinetobacter puyangensis*, and *A. tibetensis*)

in soils that have been historically contaminated with multiple pollutants. Microcosms were created with four treatments, i.e. HCS, PLANT, BIOAUG and PLANT+BIOAUG, and monitored for a period of 90-days through chemical analyses, quantification of eDNA, 16S rDNA sequencing, diversity metrics, differential abundance tests and functional prediction. The PLANT+BIOAUG combination demonstrated the most effective removal of Pb (44.75%) and Sn (66.87%), suggesting a robust synergistic interaction between plant and microbial inoculum. Microbial α -diversity remained stable across treatments, while β -diversity analyses (Bray–Curtis, PERMANOVA $p = 0.001$) revealed significant community restructuring. Taxonomic analyses highlighted shifts in key genera and an enrichment of bacterial families associated with metal transformation, redox processes, and stress tolerance. The functional prediction identified 7,959 KEGG functions, with the combined treatment showing the highest functional redundancy in metal efflux systems, siderophore production, electron transport pathways, and EPS/biofilm formation. Overall, integrating *L. angustifolia* with a metal-resistant microbial consortium could improve both contaminant removal and microbial functional potential, supporting a robust and sustainable strategy for the remediation of multi-contaminated soils. These results provide valuable insights into synergistic plant-microbe processes and offer practical guidelines for *in situ* bioremediation within the framework of the circular economy and nature-based models.

KEYWORDS:

metagenomics, microbial communities, 16S rDNA, bioremediation, heavy metals contamination.

1. INTRODUCTION

Rapid population growth, socioeconomic progress, and intense urbanisation and industrialisation are causing an exponential increase in soil contamination, generating a serious global environmental crisis [1]. Among the most critical contaminants are persistent organic pollutants (POPs) and inorganic pollutants, particularly heavy metals (HMs). Some heavy metals play essential roles as enzyme cofactors, while others are considered non-essential [2]; the latter, which have no essential biological functions, are highly toxic at low concentrations

and pose a threat to living organisms [3]. Their non-biodegradability and tendency to bioaccumulate result in ongoing ecological damage [4,5], contributing to a decline in soil quality through alterations to the composition and functionality of the microbiome [6–8], as well as through toxic effects on plants [9], and causing serious damage to vital organs, cognitive deficits and cancer-causing diseases even at low levels of exposure or chronic exposure [10,11].

The development of effective strategies to mitigate heavy metal contamination is a key objective in the field of contemporary environmental protection [12]. This process involves the removal of various classes of metal contaminants, as well as the selection of the most suitable remediation technologies in relation to the specific characteristics of the contaminated site [13]. Traditionally, soil remediation has relied on physical methods, such as soil replacement and thermal desorption, and chemical methods, such as solidification/stabilisation, soil washing, oxidation-reduction and electrokinetic remediation. While these approaches are rapid and effective, they have several drawbacks, including high energy costs, invasiveness, the potential to alter soil properties and the risk of generating secondary pollution [13–15]. In recent years, bioremediation methods have gained increasing attention due to their economic and environmental sustainability [16,17]. These approaches align with international policies, including those championed by the Environmental Protection Agency (EPA) and the European Commission through Nature-Based Solutions (NbS) [18]. NbS aim to enhance the homeostatic capacities of ecosystems while providing social and economic benefits.

Microbial bioremediation of heavy metals is a complex process involving several biochemical mechanisms, such as chelation and the transformation of toxic forms into non-toxic intermediates. Other mechanisms include ion exchange, precipitation and metal efflux [19]. Furthermore, it is crucial to understand the interactions between metals, soil, microbiota and plants [20], as these systems modulate adaptation to contaminated environments and influence the effectiveness of remediation strategies [3]. Plant-assisted bioremediation (PABR) strategies exploit the synergistic interactions between plants and rhizosphere microorganisms, combining the stabilising or phytoextractive action of plants with the ability of these microorganisms to transform, mobilise or immobilise heavy metals [21,22]. Plants have a significant impact on the bioavailability of metals [23]. They absorb metals through their roots, accumulate them in their tissues, precipitate them in the rhizosphere and exude organic

compounds [24]. Meanwhile, the microbial community contributes to processes such as biosorption, bioaccumulation, bioprecipitation and redox transformations [25]. Aromatic plants are showing great promise as tools for PABR due to their high tolerance to heavy metals [26], extensive root systems [27], and production of secondary metabolites capable of modulating the microbial community [28]. *Lavandula angustifolia* Mill., belonging to the *Lamiaceae* family, is notable for its capacity to enhance the structure and functionality of the rhizosphere microbiome, tolerate metal-contaminated soils, promote metal stabilisation [29,30], and increase beneficial microbial activity [31]. This contributes to better soil quality in the long term. In addition, the production of valuable essential oils and bioactive extracts [32] makes these species interesting from a circular economy perspective, as it reduces the costs associated with managing contaminated biomass.

The next step in the development of bioremediation strategies will be the use of microbial consortia [33]. These consist of different microbial species or strains combining complementary metabolic capabilities [34]. This results in synergistic activities and greater resilience to environmental stresses [35,36]. Concurrently, the development of omic technologies, notably metagenomics, has transformed the approach to studying bioremediation [37], overcoming the limitations of culture-based methods. Shotgun metagenomic sequencing and 16S rRNA gene sequencing are culture-independent approaches that facilitate exploration of the complete taxonomic and functional diversity of microbial communities [38]. This technology facilitates the quantification of changes in microbial composition in response to remediation treatments [39], the identification of genes putatively involved in pollutant metabolism, and the analysis of ecological dynamics and functional capacities of soil microbial communities in an integrated manner [16].

The aim of this study is to evaluate the responses of soil microbial communities to environmental factors during the remediation process. This will be achieved by analysing community structure, inferring enzymatic activity, and functional genes involved in the mobilisation and increased bioavailability of heavy metals. The results obtained contribute to clarifying the mechanisms underlying advanced bioremediation strategies and offer new operational guidelines for *in situ* interventions on degraded soils.

2. MATERIALS AND METHODS

2.1. SAMPLE COLLECTION AND MICROCOSM SET UP

Soil applied in microcosms were collected from a historically contaminated area [22], located close to Taranto (Apulia Region, Southern Italy; GPS coordinates: 40. 28 N, 17. 18 E). This area was recognised by the Italian government as a Contaminated Site of National Interest (SIN) due to long-term emissions from the metallurgical and petrochemical industries. The soil was characterised by chemical contamination and low nutrient availability. In November 2023, a composite sample (0–20 cm depth) was collected, air-dried at room temperature, sieved through a 2 mm mesh and homogenised prior to its use in experimental microcosms.

As reported in Cavone et al., 2025 [21,40], the microcosms were composed in pot (size 40x37 cm) containing 500 g of the homogenised contaminated soil. Four experimental treatments (three replicate pots per treatment) were established: (i) historically contaminated soil (HCS), (ii) soil inoculated with a hydrocarbon-degrading bacterial consortium (BIOAUG), (iii) soil planted with *L. angustifolia* (PLANT), and (iv) combined treatment consisting in bacterial consortium and *L. angustifolia* plants (PLANT+BIOAUG). The overall experimental design is illustrated in Fig. 1 below.

The *L. angustifolia* plants were obtained from a Mondo Piante plant shop in Terlizzi, Puglia.

Bacterial consortium, composed of *Gordonia amicalis*, *Rhodococcus erythropolis*, *Acinetobacter puyangensis*, and *Acinetobacter tibetensis*, was prepared from mid-log phase cultures grown in LB medium at 28 °C for 24 hours. The cultures were then centrifugally washed twice with sterile water and adjusted to an optical density (OD₆₀₀) of 1.0 before being mixed in equal volumes. The inoculum was incorporated into BIOAUG and PLANT+BIOAUG treatments at a final concentration of 2% (v/w), corresponding to 1×10^8 CFU g⁻¹ of soil.

All microcosms were maintained in a controlled environment chamber at 25 ± 1 °C, with a 16/8 h light/dark cycle and relative humidity of 65–80%. The experiment ran for a period of 90 days.

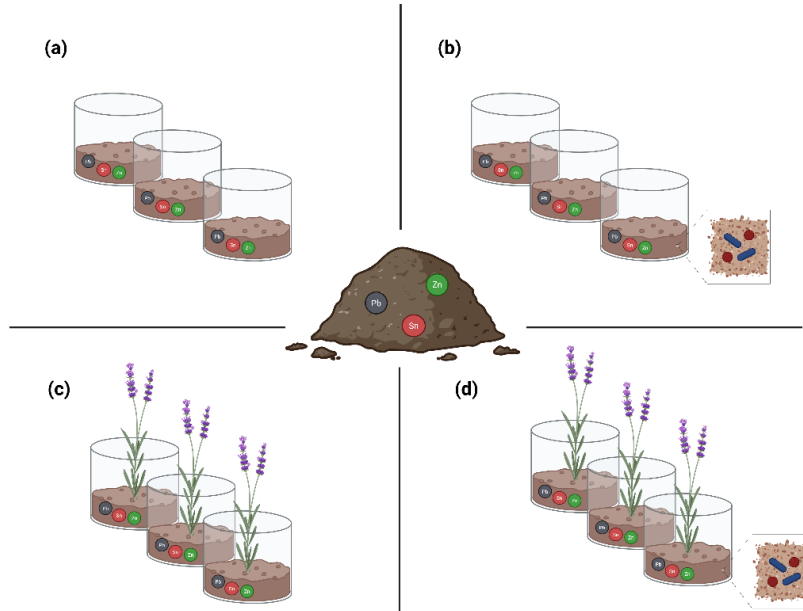


Figure 1. Experimental design of soil microcosms. (a): historically contaminated soil (HCS), (b) soil inoculated with bacterial consortium (BIOAUG), (c) soil planted with *L. angustifolia* (PLANT), and (d) combined treatment (PLANT+BIOAUG).

2.2. DETERMINING OF HEAVY METALS AND EDNA CONCENTRATION IN MICROCOSMS' SOILS

Determination of heavy metals (Be, Al, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Sn, Sb, Pb, Cd) in the experimental microcosms were performed at 0 and 90 days of incubation in growing chamber, sampling 500 mg of powdered soils from each microcosms, by inductively coupled plasma mass spectrometry analysis (ICP-MS) according to Cavone et al., 2025 [21].

The percentage of heavy metal removal (R%) was calculated as follow:

$$R\% = \frac{C_i - C_f}{C_i} \cdot 100$$

where C_i correspond to the initial concentration, while C_f is the concentration observed at 90 days.

In parallel with the heavy metal determinations, the eDNA was extracted from 0.250 g microcosms' soils, in accordance with the manufacturer's instruction of the DNeasy PowerSoil

kit (QIAGEN, Germantown, MD). The concentration of extracted genomic materials was measured using Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. 16S rDNA AMPLICON SEQUENCING

Universal primers named Pro341F/Pro805R were used for the amplification of the V3-V4 hypervariable region of the prokaryotic 16S rDNA in accordance with [41]. PCR products were checked on 1.5% agarose gel. The amplicons were purified by enzymatic treatment with Thermolabile Exonuclease I (NEB), diluted 1:2, and amplified with a short cycle using Nextera XT Indexes (Illumina). PCR products were then normalised using SequalPrep (Thermo Fisher) and multiplexed. Pooled amplicons were purified and concentrated using magnetic beads Agencourt AMPure XP 1X (Beckman Coulter Inc.). High-throughput sequencing was performed, on illumina MiSeq platform (V3 chemistry - 300 bp paired-end), at BMR Genomics Srl, Padova, Italy. Raw sequence reads have been submitted in the Sequence Read Archive (SRA) under the BioProject accession number PRJNA1400311.

The reads of the 21 samples (9 samples for the 0-day and 12 samples for the 90-day) were cleaned of primers using Cutadapt software (v. 2023.7) and then processed with the denoised-paired plugin of the DADA2 software [42]. Briefly, the sequences have been trimmed at the 3' end (Forward: 275 bp; Reverse: 200 bp), filtered for quality and length. The sequences were then dereplicated and merged to obtain unique sequences. Finally, the chimeras were removed. The Amplicon Sequence Variants (ASVs) with a frequency <0.005% were not considered. All reads from bacterial communities were classified to the lowest possible taxonomic rank using a reference dataset from the SILVA database (v.1 38) and GreenGenes2 database (v. 2022.10).

2.4. DATA ANALYSIS

Data analyses were performed using R (version 4.4.2) with the packages phyloseq, microeco, and microbiome.

Alpha-diversity analyses were performed on all samples using a set of metrics, including Observed Features, Chao1, Shannon, Simpson, Pielou's evenness, and Faith's PD. For each index, the Kruskal–Wallis test was used to verify the significance of the comparisons between the experimental conditions.

Beta-diversity analyses were performed on all samples using Bray-Curtis metrics. Statistical analyses were performed to compare the experimental conditions using the PERMANOVA test.

Differential abundance between sample groups was tested with DESeq2 [43,44]. Heatmaps were generated using R package ComplexHeatmap [45].

The Prediction of functional capabilities in the soil microbial communities was carried out using TAX4FUN2 tool [46], an open-source R package that generate functionals profiles, based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [47], and calculate the redundancy of specific functions by the functional redundancy index (FRI).

3. RESULTS

3.1. HMs AND EDNA MONITORING IN MICROCOSMS' SOILS

ICP-MS analyses performed on microcosms soils at 0 and 90 days evidenced that only Sn, Zn, and Pb concentrations exceeded the Italian legal limit (Italian Legislative Decree 152/06), equal to 1mg/kg, 150 mg/kg, and 100 mg/kg respectively (Figure S1).

As defined in equation 1, the percentage of removal of these metals was calculated for each experimental treatment (Table 1). After 90 days of incubation, metal removal efficiency varied considerably between the different treatments. High apparent removal rates for Pb and Sn were observed in HCS microcosms. However, these values were associated with very high standard deviations, indicating substantial variability, probably due to an hot spot in one replicate. This prevents the interpretation of these reductions as a consistent or reliable removal effect. Zinc removal was minimal in PLANT ($0.49 \pm 5.50\%$) and negative in BIOAUG ($-0.78 \pm 4.55\%$) and PLANT+BIOAUG ($-4.78 \pm 6.67\%$), suggesting a slight mobilisation of this metal in the presence of the *L. angustifolia*, the microbial consortium, or their combination. Although a positive Zn removal was observed in HCS, the large associated variability similarly indicates that this apparent decrease is not robust.

In contrast, lead removal was consistently higher in treatments involving *L. angustifolia*, reaching values of $19.34 \pm 19.45\%$ in BIOAUG, $36.66 \pm 11.62\%$ in PLANT and $44.75 \pm 9.84\%$ in PLANT+BIOAUG. It is noteworthy that the PLANT+BIOAUG treatment demonstrated the

highest Pb removal efficiency and exhibited a lower level of variability, thereby underscoring the reliability of this outcome.

Tin showed a limited removal percentage in the BIOAUG condition ($3.14 \pm 18.41\%$) and moderate removal in PLANT treatment ($14.95 \pm 22.23\%$). However, the combined PLANT+BIOAUG treatment resulted in a significantly higher Sn removal rate ($66.87 \pm 5.58\%$), with minimal variability. These results indicate that the simultaneous presence of *L. angustifolia* and the microbial consortium enhanced the removal of Pb and Sn, suggesting a synergistic effect between the two components. However, the apparent reductions observed in HCS should be interpreted with caution due to their high standard deviations.

The DNA concentration (ng/μl) in the soil samples of the microcosms showed an increasing trend from 0 to 90 days (Table 1). This concentration increased from 7.16 ± 1.48 to 15.86 ± 3.30 ng/μl in PLANT. The BIOAUG condition revealed a moderate increase (from 13.38 ± 1.26 to 15.72 ± 3.78 ng/μl). In contrast, the PLANT+BIOAUG treatment maintained stable DNA levels over time (from 12.53 ± 2.46 to 12.50 ± 2.87 ng/μl), suggesting potential microbial competition or community stabilisation in the presence of the plant and the microbial consortium.

Table 1. Percentage of Zn, Pb and Sn removal and eDNA concentration after 90-days of incubation per treatment.

Treatment	Zn removal (%)	Pb removal (%)	Sn removal (%)	eDNA (ng/μL) 0 days	eDNA (ng/μL) 90 days
HCS	2.56 ± 8.95	21.08 ± 40.13	40.63 ± 43.22	7.16 ± 1.48	12.82 ± 2.31
PLANT	0.49 ± 5.50	36.66 ± 11.62	14.95 ± 22.23	7.16 ± 1.48	15.86 ± 3.30
BIOAUG	-0.78 ± 4.55	19.34 ± 19.45	3.14 ± 18.41	13.38 ± 1.26	15.72 ± 3.78
PLANT+BIOAUG	-4.78 ± 6.67	44.75 ± 9.84	66.87 ± 5.58	12.53 ± 2.46	12.50 ± 2.87

3.2. MICROBIAL DIVERSITY ANALYSES

In order to study the effect of different bioremediation techniques on the bacterial community, 16S rDNA amplicon sequences were used to determine microbial diversity within and between treatments. ASVs were filtered at a frequency of 0.005%, and subsequent rarefaction analysis resulted in 710189 reads, corresponding to 2129 ASVs acquired from 21 samples (HCS, PLANT, BIOAUG, and PLANT+BIOAUG in triplicate at 0 days and at 90

days). The alpha rarefaction curves produced on the rarefied sequences revealed that the sequencing depth was sufficient to study microbial diversity in all samples (Fig. 2.A and B).

The selected ASVs were then used to measure both α and β diversity, as well as for taxonomic assignment according to the SILVA database. Furthermore, analyses of the differential abundance of taxa were performed.

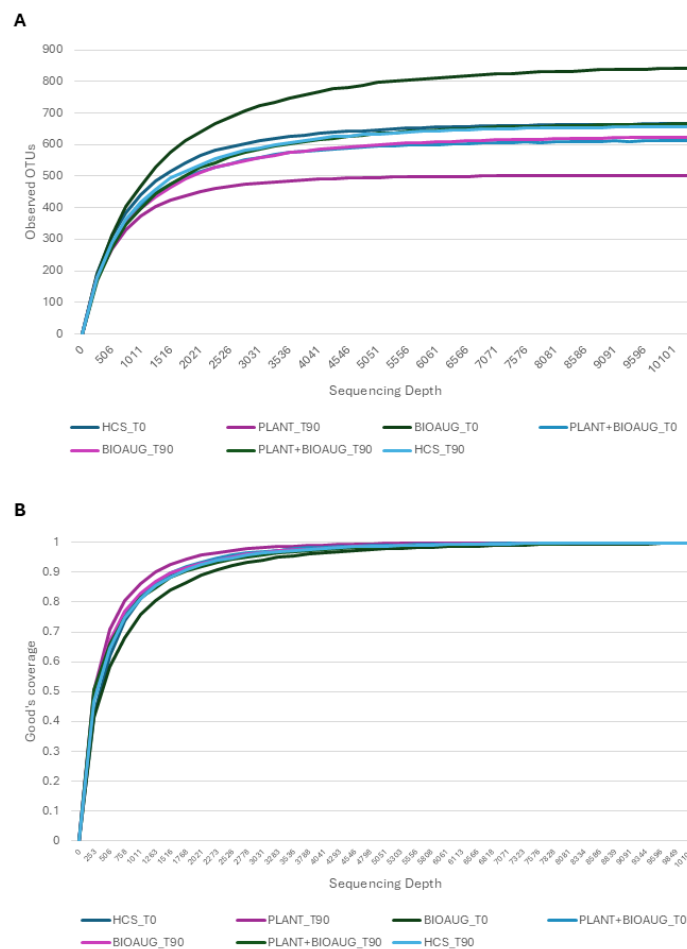


Figure 2. Alpha rarefaction curves produced on the rarefied sequences showing observed OTUs (A) and Good's coverage (B).

3.2.1. α -diversity indexes

The α -diversity was assessed using ecological indices (observed feature, Chao1, Shannon, Simpson, Evenness, and Faith's Phylogenetic Diversity) to evaluate the effects of designated conditions and time on microbial community diversity (the Shannon and Chao1 box plots are shown in Fig.3). The Kruskal-Wallis's method was used to test for differences in diversity per sample across conditions. No significant differences were detected between the treatment conditions or sampling times for any of the diversity metrics (all $p > 0.05$; data available in

Table 2). A marginally non-significant interaction effect ($p = 0.089$) was observed for Faith phylogenetic diversity, especially in PLANT+BIOAUG condition at 90 days, suggesting a potential time-dependent response in phylogenetic diversity across treatments. Overall, the results indicate that neither the treatment nor the incubation time had a significant impact on microbial alpha diversity overtime.

Table 2. Alpha-diversity indices measured in microcosm soils across treatments (HCS, PLANT, BIOAUG, PLANT+BIOAUG) at 0 and 90 days. Values represent mean diversity estimates per treatment. No significant differences were detected across treatments or timepoints (Kruskal–Wallis test, $p > 0.05$).

	Time	Observed	Chao1	Shannon	Simpson	Evenness	Faith'sPD
HCS	0d	667.00	667.64	6.17	1.00	0.95	39.70
	90d	658.00	659.63	6.01	1.00	0.93	36.89
PLANT	0d	667.00	667.64	6.17	1.00	0.95	39.70
	90d	502.00	502.18	5.87	1.00	0.95	38.58
BIOAUG	0d	845.00	852.65	6.22	1.00	0.92	43.79
	90d	625.00	627.38	5.95	1.00	0.93	35.13
PLANT+BIOAUG	0d	612.67	613.33	5.98	1.00	0.93	36.34
	90d	667.67	670.71	5.85	0.99	0.90	42.74

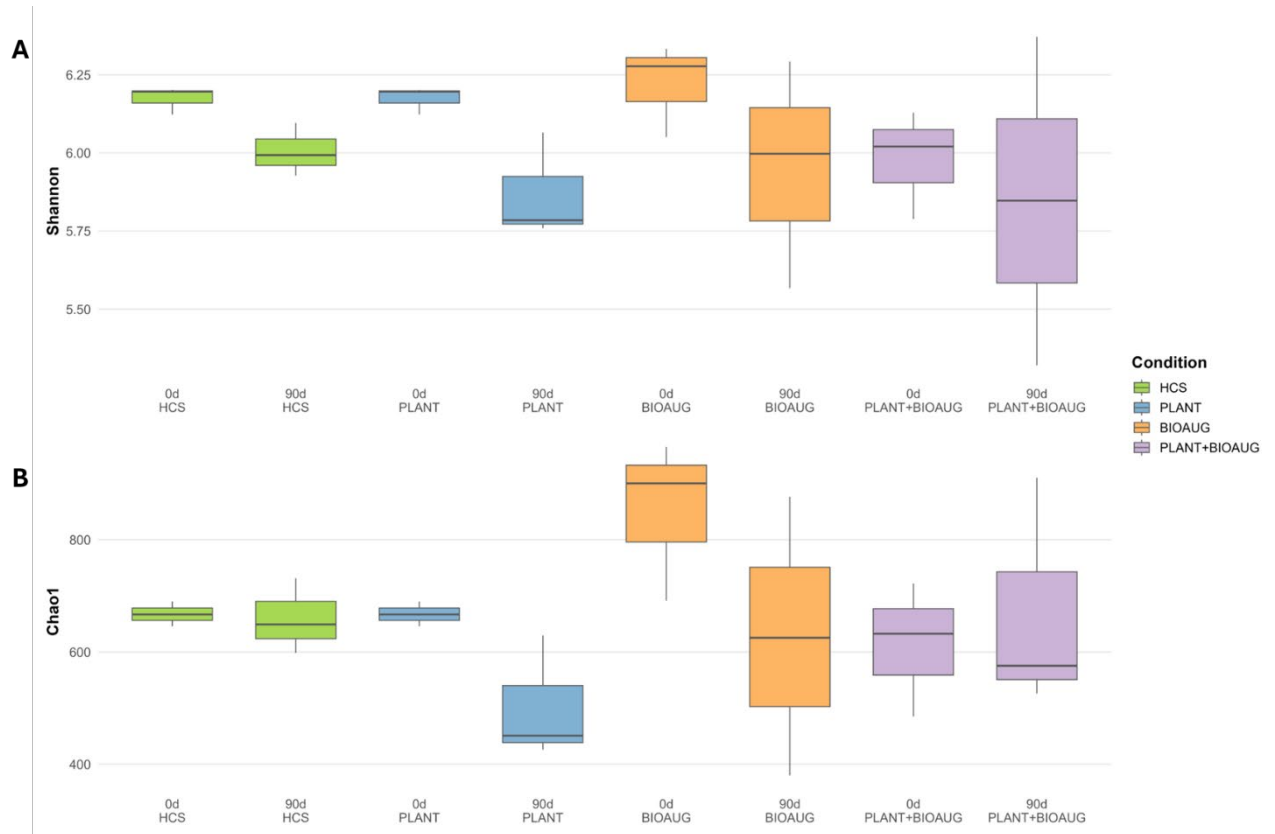


Figure 3. α -diversity within treatments on the 0- and 90-day, according to the indexes Shannon and Chao1. No significant differences were detected according to the Kruskal-Wallis test.

3.2.2. β -diversity indexes

A study of compositional changes (β -diversity) was conducted using the abundance-based Bray-Curtis metric. As illustrated in Figure 4, the principal coordinate analysis (PCoA) was performed based on the distance matrix obtained with this index. The PCoA based on the Bray-Curtis matrix (Fig. 4.A) explained 39.85% of the total variance (PC1 22.7%, PC2 17.01%), PERMANOVA test demonstrated significant variations in community structure ($p = 0.001^{**}$), both between treatments and over time. This indicates that different bioremediation strategies induce a compositional remodelling of microbial communities, while maintaining a similar overall richness.

Venn diagram analysis (Fig. 4B), based on presence/absence of ASVs after merging replicates within each condition, highlighted a large persistent core microbiome shared among the baseline soil (HCS_T0) and all treatments at 90 days (381 ASVs, 56.6%), supporting the resilience of a substantial portion of the community. The PLANT+BIOAUG combination showed the highest number of condition-specific taxa (241 unique ASVs, 7.1%), which

exceeded the numbers observed in PLANT_T90 (72 unique ASVs, 1%) and BIOAUG_T90 (187 unique ASVs, 2.2%). This is consistent with the emergence of treatment-specific microbial niches driven by the combined effects of plant presence and bioaugmentation. Furthermore, the two conditions with *L. angustifolia* (PLANT_T90 and PLANT+BIOAUG_T90) shared 182 ASVs not detected in the other groups, indicating a consistent plant-associated component of the community at 90 days.

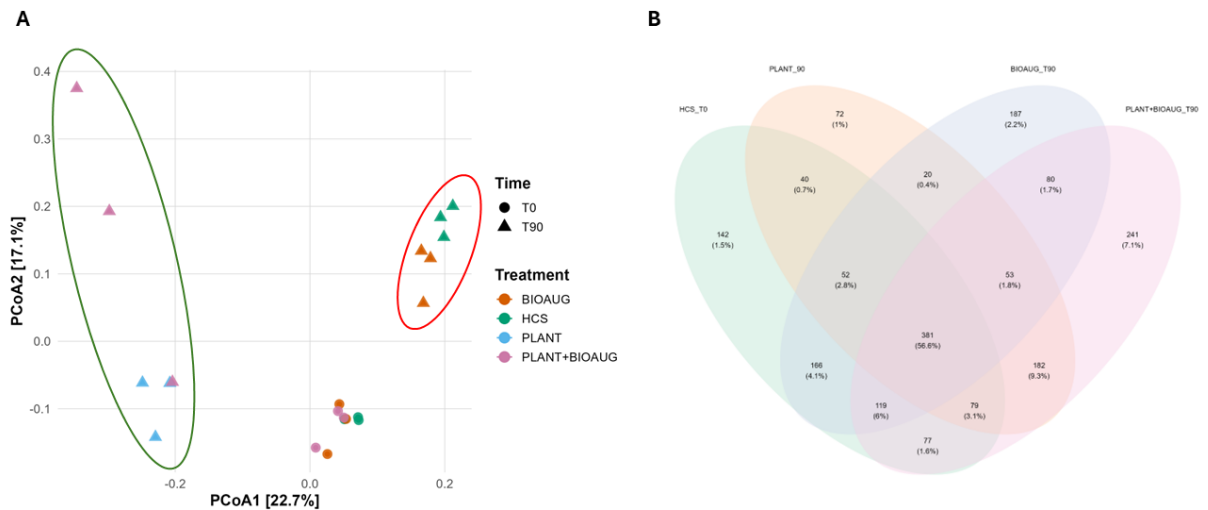


Figure 4. (A) Principal Coordinate Analysis (PCoA) of soil bacterial communities based on the Bray–Curtis dissimilarity index, showing the distribution of samples at the beginning of the experiment (T0) and after 90 days (T90) under the different treatments. At T90, the green ellipse groups samples collected in the presence of *Lavandula angustifolia* (PLANT and PLANT+BIOAUG), whereas the red ellipse groups samples collected under plant-free conditions (HCS and BIOAUG). (B) Venn diagram illustrating the number of shared and unique amplicon sequence variants (ASVs) between the initial contaminated soil (HCS at T0) and the soils subjected to the different treatments after 90 days.

3.3. ANALYSIS OF THE MICROBIAL COMMUNITY IN SOILS

Taxonomy was assigned to 738 ASVs at 99% similarity using SILVA database (v.138), achieving the genus level for the most of ASVs with few exceptions for which family or order belonging to was annotated, providing detailed information on the composition of microbial communities in soil samples across treatments. At the phylum level (Fig. 5.A), microbial communities were consistently dominated by *Proteobacteria*. However, their relative abundance decreased over time in soils subjected to microbial inoculation, particularly in the combined PLANT+BIOAUG treatment.

Bacteroidota and *Acidobacteriota*, which were initially among the most abundant phyla, generally declined across treatments. This suggests that there was selective pressure imposed by both the presence of plants and bioaugmentation strategies. *Actinobacteria* demonstrated contrasting trends, with an increase observed under bioaugmentation conditions and a decrease in planted systems. This highlights differential responses of major phyla to plant- and microbial-mediated factors. Minor phyla, including *Cyanobacteria* and *Desulfobacteriota*, increased primarily in the combined treatment, suggesting the establishment of synergistic interactions capable of promoting specific microbial consortia.

At a lower taxonomic level (Fig. 5.B), community composition showed greater variability and treatment-specific patterns. Bioaugmentation promoted the persistence and enrichment of several introduced or functionally relevant taxa, while planted treatments showed a more selective pattern. In particular, *Microvirga* shows an increase over time in BIOAUG, indicating resilience and the ability to compete in both native and bioaugmentation conditions. In the PLANT+BIOAUG combination, however, its abundance decreases after 90 days, suggesting unfavourable competition with other genera or adaptation to the microenvironment. *Bacillus* levels increased significantly in all treatments, peaking in PLANT+BIOAUG after 90 days, highlighting the ability of this spore-forming genus to colonize disturbed and enriched environments and provide key functions for bioremediation. *Vicinamibacteraceae* is stable in PLANT and BIOAUG, while it disappears in PLANT+BIOAUG at 90 days, indicating reduced competitiveness in systems with high microbial diversity. *Steroidobacter* and *Sphingomonas* are generally absent or present in low numbers in most treatments, except for BIOAUG, where they emerge following microbial inoculation, indicating strong artificial selection towards genera with specific functions (degradation of organic contaminants/metals). *Bryobacter*, *Rubrobacter*, and *Flavisolibacter* only emerge after 90 days in unplanted BIOAUG systems. These results underscore the adaptability of the community in response to bioaugmentation stimuli. Research findings indicate that *Comamonas* and *Acinetobacter* are present in BIOAUG and PLANT+BIOAUG at 0 days. This observation underscores the integration of functional taxa that are imported through the microbial consortium. Genera such as *WCHB1-32*, *Gracilibacter*, and *Pantalinema* appeared only in the combined PLANT+BIOAUG treatment after 90 days, suggesting the possible creation of selective ecological niches.

Across treatments, a significant proportion of the community consisted of "uncultured," "unclassified," and "taxa less than 1%," particularly in the combined PLANT+BIOAUG treatment at 90 days. The significant contribution of low-abundance taxa suggests that the microbial community has diversified and developed into a more complex assemblage. It is important to note that the transition from *phylum* taxonomic levels to *genus* levels resulted in a reduction in apparent variability, enabling the identification of more stable and treatment-specific microbial patterns.

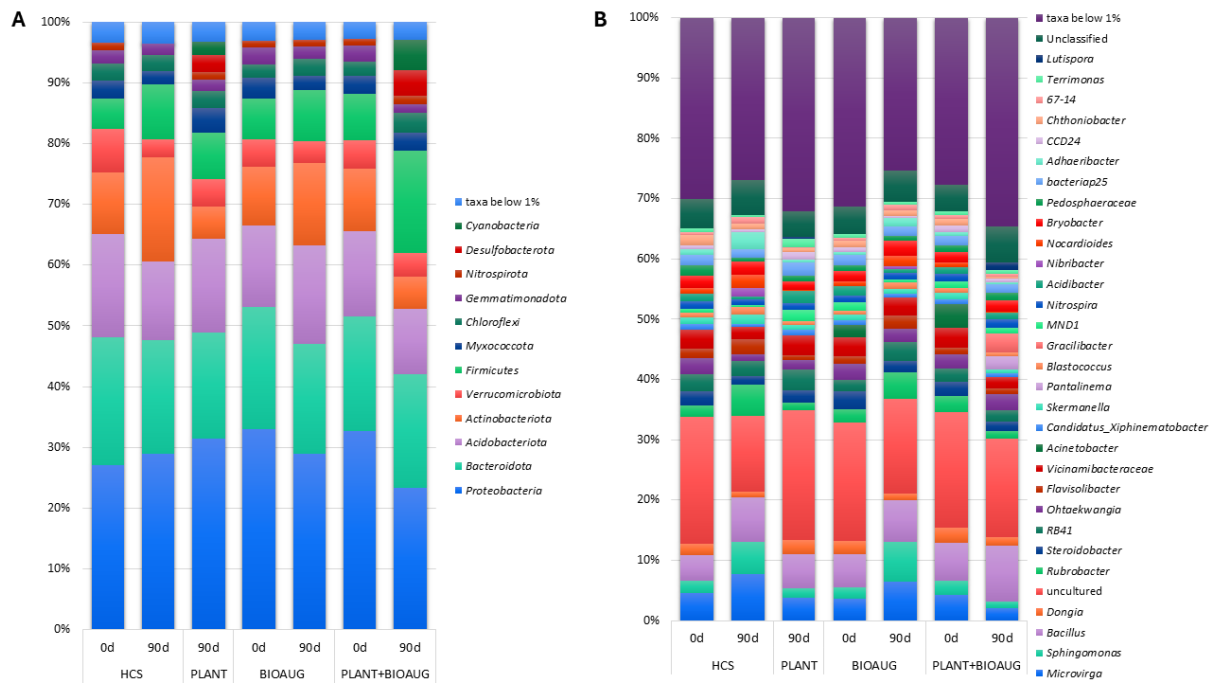


Figure 5. Community composition at (A) phylum level and (B) genus level of different treatments at 0- and 90-days.

3.4. TAXA DIFFERENTIAL ABUNDANCE BY DESEQ2 ANALYSIS

Differential taxa abundance analyses were performed using DESeq2. Treatment comparisons 90 days with the HCS control (0-day) are shown in Fig. 6.

Not all ASVs were annotated to the *genus* level, and in the reported analysis, the taxa were decoded at *family* taxonomic level.

In the BIOAUG treatment at 90-days, an enrichment of families typical of aerobic and stress-resistant environments was observed, including *Sphingomonadaceae* ($\log_2FC = +1.86$), *Hymenobacteraceae* (+1.42), *Moraxellaceae* (+1.84) and *Rubrobacteriaceae* (+1.42), and *Beijerinckiaceae* (+0.76). Concurrently, families such as *Parachlamydiaceae* (−3.92) and

Legionellaceae (−2.68) are significantly reduced, delineating a community oriented towards degraders and residual phototrophs, with a loss of groups not characteristic of the inoculum. The PLANT treatment determines the largest shift, with significant increases in families associated with rhizospheric micro-niches or biogeochemical processes, such as *Nitrosomonadaceae* (+1.75) *Saprospiraceae* (+3.29) and *Geobacteraceae* (+2.15). The heatmap confirms a high representation of these groups in the plant samples. Concurrently, the treatment has been shown to induce a substantial reduction in various *Actinobacteria*, including *Sphingomonadaceae* (−0.17), *Micrococcaceae* (−1.04) and *Nocardiaceae* (−2.93), as well as a decrease in *Hymenobacteraceae* (−0.79). Taken together, these changes suggest the establishment of a structured rhizosphere community, with a strong contribution from taxa involved in redox processes and carbon metabolism. The combined treatment (PLANT+BIOAUG) shows a blended profile, characterised by the simultaneous enrichment of taxa associated with both the rhizosphere and microbial degradation induced by inoculation. Specifically, there are notable increases in *Comamonadaceae* (+3.03), *Geobacteraceae* (+2.91), *Bacillaceae* (+1.83) and *Gracilibacteraceae* (+2.80), which the heatmap highlights as distinctive of the combined group. Concurrently, the presence of taxa characteristic of oligotrophic environments, including *Blastocatellaceae* (−0.38), *Caulobacteraceae* (−3.09) and *Nocardioidaceae* (−2.07), was identified.

The results show that the three treatments shape the microbial community differently: BIOAUG favours aerobic and degrading taxa, PLANT induces a restructuring linked to the rhizosphere and biogeochemical cycles, while PLANT+BIOAUG produces a synergistic profile integrating both factors.

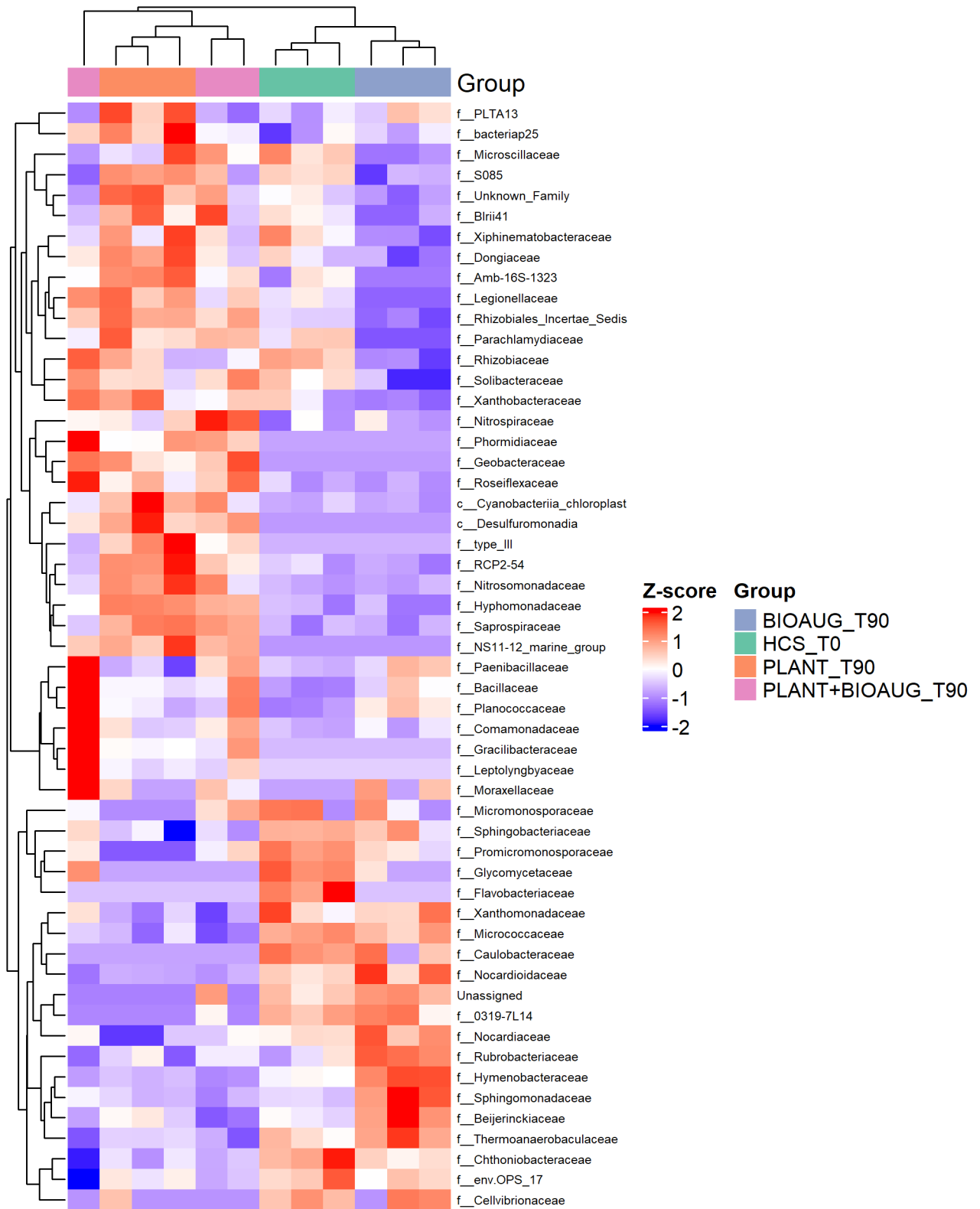


Figure 6. Heatmaps representative of the differential abundance at Family level obtained by comparing the PLANT, BIOAUG and PLANT+BIOAUG treatments after 90 days with the soil (HCS) at the initial time point.

3.5. PREDICTION OF THE NATIVE MICROBES' FUNCTIONAL CAPABILITIES

Functional prediction analysis was performed on the ASVs with more than ten counts per sample in at least two technical replicates, using the TAX4FUN2 tool; overall, 7959 KEGG functions were predicted in the microcosms. The information related to KO terms were screened and 190 functional annotations of genes related to reduction and resistance to heavy metals, degradation of organic pollutants, redox metabolism, the electron transport system (ETS), response to oxidative stress, DNA repair mechanisms, organic acid metabolism, and the production of EPS, biosurfactants, and siderophores were selected. The most representative absolute functional redundancy index (FRI) calculated by treatment at baseline and final time points is reported in Table 3 and Fig. 7.

In the control (HCS), both at 0- and 90-days, the distribution of KOs was relatively homogeneous and not characterised by particularly dominant groups, suggesting a functional state typical of an untreated soil. In BIOAUG at 0 days, the introduction of the microbial consortium generated an immediate enrichment of functions related to flavoprotein-based energy metabolism and metal efflux, indicating a direct contribution of the inoculated strains to cellular resistance mechanisms and intracellular metal management. After 90 days (BIOAUG_T90), these groups were further consolidated and distributed across generally higher FRI values. The PLANT_T90 treatment showed a different functional profile, with a more marked increase in functions associated with chelation via siderophores, consistent with the potential of *L. angustifolia* plants to modulate metal availability through the release of root exudates and the selection of siderophore-producing bacterial genera. In these treatments (PLANT and BIOAUG at 90 days) consistent values were also observed for genes associated with the response to oxidative stress, in line with the selective pressure exerted by heavy metals and the need to maintain an efficient redox balance.

The combined treatment (PLANT+BIOAUG_T90) showed the most structured and redundant functional pattern, with concomitant high values of electron transport system (ETS) annotations, metal efflux and siderophore chelation, suggesting a synergistic effect between plants and microbial consortium. This combination highlighted not only an enhancement of the functions directly involved in detoxification (efflux, siderophores), but also a stable contribution of the biofilm and EPS group, which can facilitate the immobilization of metals

and cellular protection in localized micro-niches of the rhizosphere. The results indicate that the functional redundancy associated with key processes for heavy metal resistance and mitigation is not only modulated by the type of treatment but is significantly amplified by the inoculated microbial consortium and further enhanced by plant–microbe interaction. These findings are consistent with a combined improvement in the capacity of the soil–rhizosphere system to support functions relevant to bioremediation under conditions of persistent contamination.

Table 3. Functional groups selected for the bioremediation of heavy metals, with reference to lead (Pb), zinc (Zn) and tin (Sn).

Group	KEGG	Functional annotation
Electron transfer	K00239	Succinate dehydrogenase / fumarate reductase, flavoprotein subunit
	K00240	Succinate dehydrogenase / fumarate reductase, iron-sulfur subunit
	K00330- K00331; K00342	NADH-quinone oxidoreductase (subunit A-B and M)
	K00425	Cytochrome bd ubiquinol oxidase subunit I
	K02300	Cytochrome o ubiquinol oxidase subunit IV
	K03612- K03617	Rnf electron transport complex (A–G subunits)
Pb resistance	K01533	Cu ²⁺ -exporting ATPase
	K01534	Cd ²⁺ /Zn ²⁺ -exporting ATPase
	K02654	leader peptidase (prepilin peptidase) / N-methyltransferase
Zn/Cd/Co resistance	K02074	zinc/manganese transport system ATP-binding protein
	K07263	zinc protease
	K09817	zinc transport system ATP-binding protein
	K15726	cobalt-zinc-cadmium resistance protein CzcA
	K16264	cobalt-zinc-cadmium efflux system protein
Sn resistance	K02115	F-type H ⁺ -transporting ATPase
	K00027	malate dehydrogenase (oxaloacetate-decarboxylating)
	K00029	malate dehydrogenase (oxaloacetate-decarboxylating) (NADP ⁺)
	K00767	nicotinate-nucleotide pyrophosphorylase (carboxylating)
	K00969	nicotinate-nucleotide adenylyltransferase
	K17686	Cu ⁺ -exporting ATPase
Oxidative stress response	K03386	Peroxiredoxin (alkyl hydroperoxide reductase C)
	K03387	Alkyl hydroperoxide reductase F
	K03671- K03672	Thioredoxin 1 and Thioredoxin 2
	K03781	Catalase
	K03782	Catalase-Peroxidase
	K04564	Superoxide dismutase (Fe–Mn family)
	K04565	Superoxide dismutase (Cu–Zn family)
Biofilm, EPS and Siderophores	K00381	Sulfite reductase (β-component); Fe-S metabolism
	K00806	Undecaprenyl diphosphate synthase
	K00627	Pyruvate dehydrogenase E2 component
	K00658	2-oxoglutarate dehydrogenase E2 component
	K01993	HlyD secretion protein
	K01000	Phospho-N-acetylmuramoyl-pentapeptide-transferase
	K20997	PslA polysaccharide biosynthesis protein



Figure 7. FRI of the functional activity of genes related to the reduction and resistance to heavy metals, degradation of organic pollutants, redox metabolism, electron transport system (ETS), response to oxidative stress, DNA repair mechanisms, organic acid metabolism and production of EPS, biosurfactants and siderophores in the 0- and 90-day treatments. The five highlighted functional groups are marked as follows: response to oxidative stress (asterisk), reduction and resistance to heavy metals (square, triangle, circle), electron transport system (cross), production of EPS, biosurfactants and siderophores (diamond).

4. DISCUSSIONS

The presence of heavy metals in soil systems has been shown to alter the chemical and physical characteristics of the soil, resulting in direct toxic pressure on flora and fauna and even causing a lack of development [48]. This study provides a deeper understanding of the ecological and functional mechanisms underlying the effectiveness of bioremediation treatments against heavy metals (Zn, Pb, and Sn) from historically contaminated soil. The combination of *L. angustifolia* and the selected hydrocarbon-oxidising bacterial consortium produced the most effective outcome in terms of contaminant removal and microbial functional enhancement. After 90 days, the PLANT+BIOAUG treatment achieved the highest reductions in Pb (44.75%) and Sn (66.87%) concentrations. This confirms the robust synergistic effect between plant roots and the inoculated microbial community.

The metagenomic data, resulted from 16S rDNA Next-Generation Sequencing, extend and refine those findings, showing that the observed contaminant removal corresponds to a shift in microbial community structure and function. The stability of α -diversity across treatments indicates that remediation did not compromise species richness [49]. Instead, it appears to have reorganised the existing community towards functionally relevant taxa adapted to the new rhizospheric environment. No differences were found in α -diversity, but there were clear variations in β -diversity, demonstrating a structural change in the microbial communities in response to the different bioremediation approaches [16]. The analysis of samples in PCoA plots demonstrated a clear distinction between the PLANT+BIOAUG cluster and the other conditions, indicating that the synergistic effect of plant-microbe interaction acted as the predominant ecological factor [8].

Taxonomic analysis showed that *Proteobacteria*, *Bacteroidota* and *Acidobacteriota* were dominant in all treatments, as reported in previous studies [50,51]. Bioaugmented soils showed a reduction in *Proteobacteria*, suggesting a selection effect [52]. The phylum *Actinobacteria* increased in BIOAUG conditions and was also dominant in other Pb and Zn polluted soils [53]. A decrease was seen in the lavender treatments. The genus *Microvirga* had increased abundance in the BIOAUG condition and decreased abundance in PLANT+BIOAUG, suggesting competition or adverse adaptation to the rhizosphere [37]. *Bacillus* had increased substantially in all treatments, peaking in PLANT+BIOAUG after 90 days, showing the genus's capacity to

inhabit disturbed, enriched environments and perform key functions in bioremediation [1,54]. *Steroidobacter* and *Sphingomonas* mainly gave rise to the BIOAUG condition, indicating strong artificial selection for taxa that degrade organic contaminants and manage heavy metals [39].

The distinct taxonomic profile in PLANT+BIOAUG treatment confirms that the interplay between the plant and inoculated bacteria is the principal ecological force in shaping community composition [55], favouring the enrichment of genera with documented roles in heavy metal tolerance, redox transformations [56], siderophore and biosurfactant production [57], and EPS-mediated protection [58]. BIOAUG is less effective than expected, which is consistent with previous results [59]. In nature, native microbial populations tend to outcompete inoculated strains. When introduced without the modifying influence of the rhizosphere, these inoculated strains exhibit reduced persistence [33]. This competitive exclusion may also provide a rationale for the discrepancy between microbial abundance and activity observed in the complementary microbiological dataset.

Functional predictions derived from the metagenomic data further substantiate the superiority of the combined treatment. The PLANT+BIOAUG system exhibited the highest degree of functional redundancy in metabolic pathways directly implicated in heavy metal mitigation. This functional enrichment is indicative of a community that is metabolically versatile and highly resilient [60]. It can withstand toxic pressure while maintaining biogeochemical processes that are essential for immobilising and transforming metals [19]. Functional redundancy is likely to play a stabilising role, thereby enabling the system to maintain its functional integrity despite taxonomic fluctuations. It is evident that the pollutant removal, taxonomic reorganisation and functional enrichment observed in PLANT+BIOAUG are indicative of the synergy between *L. angustifolia* and the selected microbial consortium being central to the remediation performance. Plants alone promote microbial abundance and activity, improve soil quality, and modulate contaminant availability, whereas the consortium alone struggles to persist and contribute meaningfully [12]. However, when combined, plant roots create an ecological niche that supports both native and inoculated microbes [61]. This facilitates metabolic complementarity and enhances the overall capacity of the system to

tolerate and sequester heavy metals [23]. This cooperative dynamic represents a robust and sustainable approach for the recovery of contaminated soils.

The presence of certain taxa and functions related to efflux supports the occurrence of enzymatic transformations and biosorption phenomena that reduce the bioavailability of toxic metals [62]. The plant-microbe system acts as an integrated biocatalytic unit, where *L. angustifolia* acts as an integrated biocatalytic unit, directly controlling metal dynamics through its own rhizosphere processes, and indirectly by modulating the microbial community composition and metabolism in the root zone. This allows the plant-microbe system to collectively enhance the phytoremediation of metal(loid) contaminants [63].

The combined treatment PLANT+BIOAUG showed high capabilities in recovering Sn and Pb contaminated soils evidencing how these bioremediation techniques (PABR and Bioaugmentation) are in line with the goals of Nature-Based Solutions and the circular economy framework promoted by the European Commission [18]. By reinforcing functional redundancy and stability, this system also supports the long-term recovery of soil ecological services [31].

5. CONCLUSIONS

This study evidenced that the combination of *L. angustifolia* and the bacterial consortium represents a promising and sustainable biotechnological solution for the remediation of historically Pb and Sn contaminated soils. The combined use of these bioremediation technologies reshapes soil microbial community structure promoting HM stabilisation and functional resilience through coordinated plant-microbe mechanisms, including metal chelation, efflux, biofilm formation, and redox-driven transformations. These findings emphasise the ecological and technological value of plant-assisted bioaugmentation as a scalable, low-impact strategy for environmental restoration and soil quality improvement.

Further studies should extend the monitoring period, assess the persistence of inoculated strains under natural conditions, and apply metatranscriptomic or metabolomic analyses to validate functional predictions [64].

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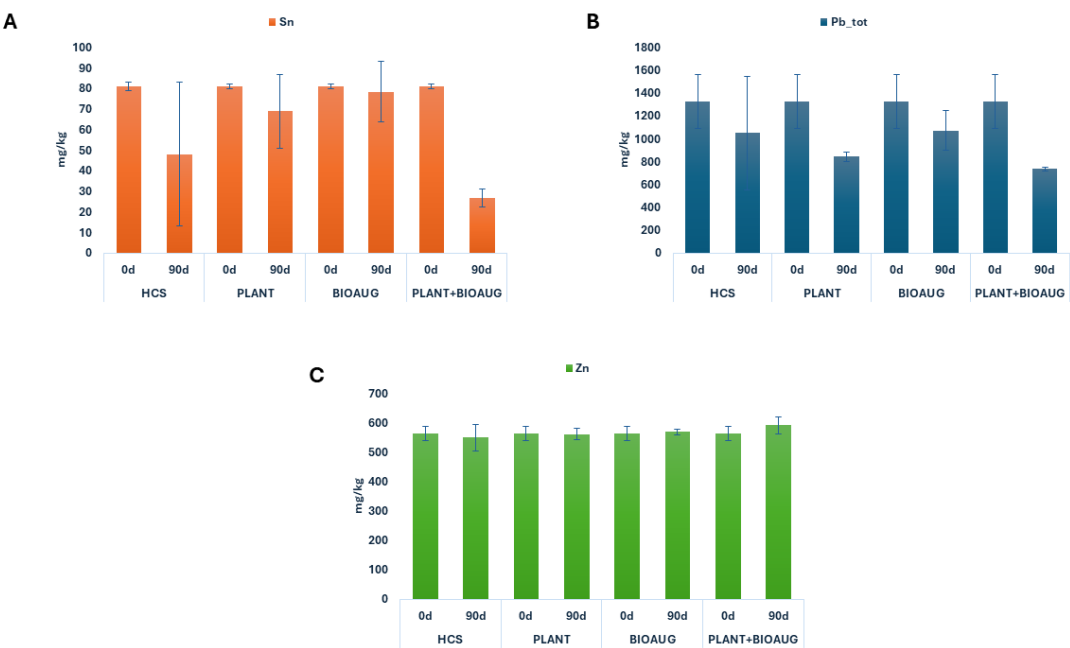
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Supplementary Figure 1 - Average concentrations (mg/kg) of Sn (A), Pb (B), and Zn (C) in microcosm soils samples collected at 0 and 90 days in the various experimental microcosms (HCS, PLANT, BIOAUG, and PLANT+BIOAUG), modified by Cavone et al., 2025 [21].

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The present thesis demonstrates that naturally hydrocarbon-contaminated springs represent a valuable reservoir of microbial biodiversity and a starting point for the development of bioremediation strategies. The isolation of four hydrocarbon-oxidising strains – *G. amicalis* S2S5, *R. erythropolis* S2W2, *A. puyangensis* S1W1, and *A. tibetensis* S2S8 – provided evidence that prolonged exposure to hydrocarbons and other pollutants selects for microorganisms with high catabolic capabilities. These isolates were found to be capable of using diesel oil as a sole carbon source and to produce biosurfactants, which could favour the solubilisation of hydrophobic compounds. Genomic analysis revealed the presence of genes involved in the degradation of aromatic and aliphatic hydrocarbons, as well as the detoxification of heavy metals. This suggests that these strains may exhibit an advantage in environments that are multi-contaminated.

The utilisation of these strains within a consortium facilitated the evaluation of bioaugmentation efficacy under controlled conditions. In artificially diesel contaminated lake water mesocosms, the inoculation resulted in a greater reduction in hydrocarbons than that achieved by natural attenuation. These results indicate that, at least in a laboratory setting, bioaugmentation with selected microbial strains can indeed accelerate degradation processes. However, the observed decrease in alpha-diversity indicates that the introduction of external consortia would alter the native microbial communities of the remediated matrices. Consequently, further investigation should focus on ecosystem resilience and the long-term sustainability of such interventions. Furthermore, lab-scale conditions do not replicate the complexity of real sites, where abiotic factors (temperature, humidity, nutrients), the presence of multiple contaminants, and competition with native microbiota can influence the persistence and activity of inoculated strains.

The extension of this approach to a multi-contaminated industrial soil demonstrated the efficacy of a combination of bioaugmentation and phytoremediation. In mesocosms, the supplementation of the bacterial consortium with *L. angustifolia* resulted in significant reductions in lead and tin concentrations, and concomitant improvements in soil pH and quality index. The plant demonstrated notable resilience in the contaminated environment, with the

inoculation process showing no discernible impact on plant growth patterns. The results showed that the amount of environmental DNA remained comparable across the different treatments, indicating that the introduction of a bacterial consortium did not cause uncontrolled microbial growth or biomass decline, but rather led to a dynamic equilibrium between microbial reproduction and turnover. This observation suggests that bioaugmentation did not compromise the overall microbial functional capacity of the soil. Furthermore, community analysis through 16S rRNA gene sequencing revealed a microbiota characterised by taxa associated with detoxification and redox cycling functions, thereby confirming the potential for plant-microbe synergy. The persistence of a core of shared taxa across treatments and the absence of negative effects on overall diversity indicate that the combination with *L. angustifolia* could mitigate the ecological impact of bioaugmentation, while favouring the selection of desired functions.

The integration of native hydrocarbon-oxidising strains with tolerant plant species is proposed as a promising strategy for addressing the complexity of multi-contaminated matrices. The multidisciplinary approach adopted in this study combines genomic isolation and characterisation, microcosm and mesocosm testing, and metabarcoding analyses, thus offering a comprehensive overview of the potential and limitations of bioremediation. However, it is imperative to acknowledge that the evidence is derived from controlled experimental conditions; consequently, field application will necessitate the adaptation and optimisation of protocols based on site specifics. This process involves evaluating the persistence of inoculated consortia, interactions with native vegetation, and the ability to maintain long-term catabolic functions.

While the evidence obtained is encouraging, further investigation is required before its translation to a full-scale can be undertaken. It is therefore appropriate to: (i) validate the consortia in semi-real and field conditions, by experimenting with variable climatic and structural conditions, monitoring the persistence and activity of the inoculated strains and interactions with the native microbiota; (ii) integrate advanced omics analyses, such as the study of the transcriptome, proteome, and metabolome of the consortia and rhizosphere communities, in order to verify the effective expression of catabolic genes and identify key metabolites in detoxification processes; (iii) optimise inoculation formulations and species combinations; (iv)

analyse interactions with emerging contaminants (micro- and nanoplastics); and (v) consider socio-economic aspects.

In summary, the thesis provides an experimental basis for the use of native strains of hydrocarbon-oxidising bacteria in consortium, in combination with aromatic plants, as an integrated approach to the remediation of contaminated soils and waters. However, the transition from laboratory to applied scale will require a multidisciplinary research programme integrating microbiology, ecology, environmental engineering and economic analyses to develop effective, sustainable and replicable solutions.

ACTIVITY DURING THE PH.D.

WORKSHOP PARTICIPATION:

- Participation in the workshop entitled “Environmental DNA: a new powerful tool for monitoring biodiversity” at University of Molise, Department of Biosciences and Territory (Pesche, Italy; 26-29/04/2022).
- Participation in the workshop entitled “Metabolomics Approaches for One Health” at University of Milano-Bicocca (Milan, Italy; 30-31/05/2024).
- Participation in the workshop entitled “Profiling of microbial communities using targeted and shotgun metagenomics” at University of Bari “Aldo Moro” (Bari, Italy; 30/06-04/07/2025)

CONFERENCE PARTICIPATION:

- XXXIV SIMGBM Congress (Cagliari, Italy; 21-25/09/2023). Participation with the submission of abstract entitled “Isolation and characterization of novel hydrocarbon-oxidizing bacterial strains for the environmental remediation” by Cavone C., Monaco P., Naclerio G., Rizzo P., Celico F., Bucci A.

Atti XXXIV Congresso SIMGBM Cagliari 2023, p.92.

- RemTech EUROPE (Ferrara, Italy; 16-20/09/2024). Participation with the submission of an abstract and talk presentation entitled “From the isolation and characterization of hydrocarbon-oxidizing bacterial strains to the assessment of their capabilities for bioremediation” by Cavone C., Monaco P., Fantasma F., Rizzo P., Celico F., Naclerio G., Bucci A.

Book of Abstract RemTech Expo, CNR Edizioni, 2024, p. 541; ISBN 978-88-8080-662-2 (stampa), 978-88-8080-663-9 (elettronico).

- SETAC Europe 35th Annual Meeting (Vien, Austria; 11-15/05/2025). Participation with the submission of an abstract entitled “Assessment of plant-assisted bioremediation and bioaugmentation strategies effectiveness for multi-contaminated soil rehabilitation” by

Cavone C., Naclerio G., Bucci A., Barra Caracciolo A., Rutigliano A., Cotugno P., Rolando L., Savino I., Grenni P., Celico F., Uricchio V.F., Ancona V.

Book of Abstracts, SETAC Europe 2025, pp. 850–851; PRINT ISSN 2309-8031, ONLINE ISSN 2310-3043.

- IX European Bioremediation Conference (Chania, Crete; 15-19/06/2025). Participation with the submission of an abstract entitled “Plant-assisted bioremediation with *Lavandula angustifolia* and microbial consortium: evidence from microcosm studies” by Cavone C., Bucci A., Naclerio G., Grenni P., Savino I., Rolando L., Uricchio V.F., Ancona V.

Book of Abstracts – EBC IX; ISBN 978-618-5558-13-0.

- Giornata della ricerca 2023 (Isernia, IS, Italy, 18/12/2023). Achieved findings and new research frontiers at the University of Molise. Participation in the conference with the submission of an abstract and poster presentation entitled: “Exploring the synergistic potential of hydrocarbonoclastic bacteria for bioremediation” by Cavone C., Monaco P., Fantasma F., Rizzo P., Celico F., Naclerio G., Bucci A.

- Giornata della ricerca 2022 (Isernia, Italy, 20/12/2022). Achieved findings and new research frontiers at the University of Molise. Participation with the submission of an abstract and poster presentation entitled “Isolation and characterization of hydrocarbonoclastic bacteria for bioremediation” by Cavone C., Naclerio G., Bucci A. ISBN 9788836231584

- UniMol PhD EXPO 2022 (Campobasso, Italy, 19/12/2022). Participation in the UniMol PhD EXPO 2022 with the submission of an abstract and poster entitled: “Hydrocarbonoclastic bacteria: breakthrough in environmental recovery” by Cavone C., Naclerio G., Bucci A. ISBN 9788896394434

LIST OF PUBLICATIONS

The publications resulting from the research conducted during the PhD program are listed below:

1. Natural Hydrocarbon-Contaminated Springs as a Reservoir of Microorganisms Useful for Bioremediation: Isolation and Multilevel Analysis of Hydrocarbonoclastic Bacteria from the Agri Valley (Southern Italy).

Cavone C., Monaco P., Fantasma F., Rizzo P., Tarracchini C., Petraro S., Ventura M., Milani C., Celico F., Naclerio G., Bucci A. (2025).

Sustainability, 17(7), 3083. <https://doi.org/10.3390/su17073083>

2. Synergistic Effects of *Lavandula angustifolia* and a Bacterial Consortium on Bioremediation of a Heavy Metal-Contaminated Soil.

Cavone C., Rutigliano A., Cotugno P., Rolando L., De Carolis C., Barra Caracciolo A., Grenni P., Savino I., Bucci A., Naclerio G., Celico F., Uricchio V.F., Ancona V. (2025).

Plants, 14(17), 2734. <https://doi.org/10.3390/plants14172734>

3. Metagenomic Insights into Plant–Microbe Synergies for heavy metals contaminated soil remediation.

Cavone C., Rutigliano A., Cotugno P., Rolando L., De Carolis C., Barra Caracciolo A., Grenni P., Savino I., Bucci A., Naclerio G., Celico F., Uricchio V.F., Ancona V.

The paper is under submission to a peer-review journal (*New Biotechnology Journal* – Elsevier).

OTHER CONTRIBUTIONS

The following list includes additional publications from the same period:

1. Effects of micro and nanoplastics on plant-assisted bioremediation for contaminated soil recovery: A review.

Savino I., Campanale C., Grenni P., Cavone C., Garganese F., Barra Caracciolo A., Uricchio V.F., Ancona V. (2025)

Science of The Total Environment, 1007, 180905, ISSN 0048-9697,
<https://doi.org/10.1016/j.scitotenv.2025.180905>

2. High Purity Grade Phycocyanin Recovery by Decoupling Cell Lysis from the Pigment Extraction: an Innovative Approach.

Lauceri R., Cavone C., Chini Zitelli G., Kamburska L., Musazzi S., Torzillo G. (2023).
Food and Bioprocess Technology, 16, 111–121. <https://doi.org/10.1007/s11947-022-02926-w>

ACKNOWLEDGEMENTS

Se ripenso a questi anni, li vedo come un puzzle sparso su un tavolo troppo piccolo per contenerlo. Complesso, irregolare, ostinato. Un'immagine che ancora non esisteva, che non sapevo nominare, ma che sentivo — in un punto silenzioso dentro di me — meritasse di essere cercata.

Pezzo dopo pezzo, tra incastri che non tornavano, angoli che sembravano non appartenere a nessun posto, tentativi abbandonati sul tavolo e poi ripresi con mani più pazienti. E poi, all'improvviso, quella sensazione rara e preziosa di vedere qualcosa prendere forma.

Ogni puzzle, però, ha i suoi pezzi difficili. Quelli che si girano e si rigirano, che sembrano sbagliati anche quando sono giusti. Questo capitolo è stato il mio pezzo più difficile — e forse, proprio per questo, il più importante.

Tutto è iniziato durante una lezione di Zoologia alla triennale. In quell'aula qualcosa si è acceso — una luce piccola ma tenace. Non è stata una decisione rumorosa, né una certezza improvvisa. È stata una scelta quieta, nata dalla curiosità. Ho scelto la ricerca come si sceglie un puzzle impegnativo: sapendo che sarà lungo, ma intuendo che l'immagine finale varrà l'attesa.

Il primo pezzo di questo puzzle è stato messo dal mio supervisore, il Prof. Naclerio, e al mio co-supervisor, il Prof. Bucci, a loro il più sincero ringraziamento per la guida scientifica, la costante disponibilità e la fiducia dimostrate nel corso di questi anni. Il loro supporto è stato fondamentale non solo per la mia formazione come giovane ricercatrice, ma anche per aiutarmi a trovare sicurezza e direzione in un ambito di studio nuovo e complesso. Mi hanno stimolata trasmettendomi entusiasmo ed emozione e permettendomi di scoprire e osservare con occhi nuovi ciò che studiavo. Un ringraziamento speciale va a Pamela, il cui contributo è stato determinante nel farmi appassionare alla microbiologia e nel trasmettermi curiosità e rigore scientifico. Mi hanno acceso l'amore per la microbiologia: mi hanno mostrato che anche l'invisibile può raccontare storie complesse, se si impara ad ascoltarle.

Un ringraziamento speciale è rivolto al Coordinatore del Dottorato di Ricerca in Biologia e Scienze Applicate, il Prof. Santucci de Magistris, e alla Prof.ssa Scippa per il supporto costante,

per i saggi consigli e per aver creato un ambiente stimolante che ha favorito la mia crescita professionale e accademica.

Il mio puzzle si è arricchito ulteriormente durante il periodo trascorso al CNR di Bari: desidero ringraziare i supervisor del CNR, la Dott.ssa Ancona e il Dott. Uricchio, presso cui ho svolto il terzo e il quarto anno di Dottorato, per avermi offerto l'opportunità di portare avanti una sperimentazione importante ed impegnativa per il mio percorso di ricerca e per la mia crescita professionale.

Un ringraziamento particolarmente sentito va alla Dott.ssa Raffaella Mattarese pezzo indispensabile di questo percorso: con il suo sorriso, la sua forza e il suo supporto costante ha creduto in me quando ne avevo più bisogno, lasciandomi spazio e respiro soprattutto nei mesi più intensi della stesura. Ha saputo creare un ambiente caldo e stimolante, in cui il confronto e le idee hanno nutrito la mia crescita, non solo come ricercatrice, ma come persona.

Poi c'è il cuore del puzzle: i pezzi silenziosi che fanno da cornice e da sfondo, che tengono insieme l'immagine. Sono la struttura invisibile che rende possibile ogni incastro.

Ai miei genitori: voi siete la cornice, quella solida e silenziosa che tiene tutto insieme e rende possibile ricostruire l'immagine anche quando i pezzi sembrano sparsi ovunque. Il vostro supporto, la vostra fiducia e la pazienza nei miei momenti di nervosismo, mi hanno permesso di guardare avanti con coraggio. A mio fratello: anche se i nostri percorsi ci hanno spesso condotti lontani, sapere che l'uno esiste per l'altro è stato una costante fondamentale. Siete la base su cui tutto il resto ha potuto poggiarsi.

Un grazie va ai miei zii e cugini: grazie per essere le radici a cui tornare. Mi avete sempre fatta sentire il vostro affetto, anche a distanza. Ogni parola di incoraggiamento, ogni interesse dimostrato per il mio percorso, ha contribuito a rendere questo puzzle più luminoso.

Ad Anna, Giorgia, Enrico e i Daniele: altri pezzi hanno dato colore al quadro; mi avete accolta in un ambiente che inizialmente sentivo estraneo, troppo calmo e troppo freddo, contribuendo a trasformarlo in un luogo familiare. Con loro ho imparato a muovermi tra nuove discipline, a ridere degli errori e a celebrare le piccole vittorie quotidiane. Alessandra merita un posto speciale: siamo entrate in questo dottorato come colleghe e ne usciamo come qualcosa di più, sorelle. Ci siamo trovate nei momenti in cui le parole erano poche e il peso era tanto, e ci

siamo anche regalate risate e leggerezza quando ne avevamo bisogno. Un'amicizia nata tra scadenze e scontri, che ha imparato a fiorire anche fuori da lì.

A Marianna e Lollo, presenze incrollabili, colore e respiro anche nei giorni più densi. Con pazienza e dedizione avete attraversato con me i momenti più faticosi, sapendo alleggerire quando serviva distrazione e restare in ascolto quando il peso diventava troppo. Mi avete aiutata a mettere ordine nei pensieri e a ritrovare lucidità quando tutto sembrava confuso. Maria, il tuo spirito e la tua tenacia sono stati un esempio silenzioso ma potentissimo: grazie per avermi insegnato che non si crolla quando il vento soffia contro, si impara a restare in piedi. Silvia, Mario e Massimo, grazie per la presenza costante e per i sorrisi che hanno reso più leggeri anche i giorni più tesi. Giuseppe e Barbara, le piccole “mascotte”, inconsapevoli della forza che sanno dare: in un abbraccio, in una risata improvvisa, in uno sguardo curioso su ciò che circonda, capace di rimettere tutto nella giusta prospettiva. Con tutti voi ho ricordato il significato di amore, sacrificio, dedizione e condivisione, che la vita è molto più di una scadenza o un paper da scrivere.

Alessandra, il pezzo che non ti aspetti. Siamo l'opposto l'una dell'altra, e forse è proprio per questo che funziona. La sua energia dirompente e la mia calma si sono sempre bilanciate, senza bisogno di spiegazioni. Con lei ho la certezza di essere capita, e quella di non essere mai sola.

Roberta, amicizia che resiste alle distanze, agli allontanamenti, ai cambi di vita. Un legame fatto di risate, complicità e quella sana follia che rende tutto più leggero: dalle telefonate infinite, alla ricerca di una serata a Milano, e ai rientri senza specchietto.

A Sara, devo la pazienza con cui hai ascoltato i miei sfoghi: nonostante ci conosciamo da poco, hai saputo essere una presenza preziosa su cui contare.

Il ringraziamento si estende all'IRSA-CNR: più che un luogo di lavoro, è uno spazio in cui ci si ritrova davvero. Un'atmosfera serena che, nei mesi più intensi, è stata un regalo inaspettato.

Ai bambini dell'ACR, grazie per avermi insegnato la pazienza e la capacità di rimettermi in gioco, riportandomi ogni volta alla semplicità dei gesti e delle emozioni. Anche nelle giornate più complicate siete stati voi a ricordarmi cosa significa dare il massimo.

A Gio, Ambe, Vera, Robi e al resto del gruppo: in pochi mesi siete riusciti a farmi sentire parte di qualcosa di bello e autentico. Grazie per l'accoglienza, per le risate e per aver reso più leggeri giorni che a volte pesavano molto più di quanto mostrassi.

A Fabri, il pezzo di puzzle trovato nel momento più fragile, quasi alla fine di questo percorso, quando la stanchezza e i dubbi sembravano avere il sopravvento e tutto sembrava sul punto di sgretolarsi. In poco tempo sei riuscito a rimettere i pezzi al loro posto, hai ricomposto ciò che dentro di me traballava. Mi sostieni, mi incoraggi, e con forte determinazione mi ricordi che posso andare avanti, anche quando mi sento fragile, quando penso di non essere abbastanza, e di non potercela fare. Credi in me con una forza che a volte supera la mia. Sei presenza, ascolto, pazienza. Se oggi questo puzzle ha un'immagine compiuta è anche perché tu vedevi un disegno quando io vedevo frammenti e caos.

Infine, un pensiero delicato va ai pezzi mancanti ma che mancanti non sono: a chi mi ha spinto a iniziare questo dottorato e a crederci fino in fondo, ai miei nonni, che sono stati la radice silenziosa di questa scelta. Questo Dottorato è anche vostro.

Oggi, mentre osservo questo puzzle, capisco che ogni pezzo era indispensabile: anche quelli che sembravano insignificanti hanno contribuito a disegnare l'immagine finale. Alcuni si sono incastrati facilmente. Altri ho dovuto girarli più volte tra le mani, con pazienza. Alcuni li ho persi e ritrovati. Ma alla fine, guardando l'immagine completa, so che non è solo il risultato di un lavoro di ricerca, è la somma di legami, scelte, cadute e ripartenze.

A ciascuno di voi, che ha lasciato un segno nel mio cammino, va il mio più sincero e profondo grazie.