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## 1 Technical references

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## 2 Abstract

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Bioremediation represents one of the most sustainable and cost-effective approaches for the remediation of contaminated environmental matrices, including soil, sediments, and groundwater impacted by hydrocarbons and chlorinated solvents. Within the RETURN project (multi-Risk sciEnce for resilienT commUnities undeR a changiNg climate), Spoke VS4 – Environmental Degradation, Work Package 4.5 (Prevention and Remediation), research activities have focused on the development and experimental validation of innovative and eco-friendly bioremediation processes under both aerobic and anaerobic conditions. The objective of Deliverable 4.5.4 is to provide guidelines for the implementation of bioremediation strategies based on the multidisciplinary experimental results obtained by the involved research units.

The activities addressed three main environmental matrices: contaminated soils, groundwater affected by chlorinated solvents, and marine sediments polluted by petroleum hydrocarbons. Laboratory and pilot-scale experiments were carried out to investigate microbial degradation pathways, optimize operational conditions, and evaluate innovative technological solutions to enhance remediation performance.

For groundwater contaminated by chlorinated solvents, experimental investigations focused on the biodegradation of 1,2-dichloroethane (1,2-DCA), a persistent compound frequently detected in aquifers. Laboratory-scale column systems simulating permeable reactive barriers were developed to evaluate the effectiveness of biologically mediated dechlorination processes. These systems were tested under anaerobic conditions using polyhydroxybutyrate (PHB) as a slow-release electron donor and under aerobic conditions using oxygen-release compounds to stimulate oxidative degradation pathways. Microcosm and column experiments demonstrated significant removal efficiencies, confirming the potential of biologically based permeable reactive barriers as a sustainable solution for the treatment of chlorinated solvent-contaminated aquifers.

Complementary microbiological studies focused on the characterization and enhancement of microbial communities capable of degrading hydrocarbons and chlorinated compounds. High-performing bacterial strains and consortia were isolated and immobilized on biodegradable polymeric matrices such as PLA, PCL, and PHB. These biofilm-based systems showed improved stability and biodegradation performance compared with planktonic microbial cultures, achieving hydrocarbon removal efficiencies up to 90%. Molecular analyses confirmed the activation of key functional genes involved in hydrocarbon degradation pathways, highlighting the effectiveness of immobilized microbial systems as robust and sustainable tools for environmental remediation.

Additional experimental activities investigated the remediation of contaminated marine sediments using bioslurry technology. Bench-scale and pilot-scale bioslurry reactors were operated to evaluate the biodegradation of total petroleum hydrocarbons (TPH) in real sediments collected from industrial coastal areas. The results demonstrated that bioaugmentation and microbial stimulation can significantly enhance hydrocarbon degradation, achieving removal efficiencies exceeding 50% under optimized conditions and confirming the applicability of bioslurry processes for the treatment of dredged contaminated sediments.

Further research explored the enhancement of soil bioremediation through the application of indigenous microbial consortia combined with biosurfactant-producing strains. The addition of biosurfactants increased hydrocarbon bioavailability and degradation kinetics while reducing residual ecotoxicity, demonstrating the potential of integrated microbial–biosurfactant systems for the sustainable remediation of contaminated soils.

Finally, emerging bioelectrochemical approaches were investigated for the treatment of groundwater co-contaminated by chlorinated solvents and heavy metals. Laboratory-scale microbial electrolysis cells were tested to evaluate the simultaneous removal of trichloroethylene (TCE) and hexavalent chromium (Cr(VI)), demonstrating high removal efficiencies and strong resilience of electroactive microbial communities under different operating conditions. These results highlight the potential of bioelectrochemical systems as innovative tools capable of stimulating microbial reductive pathways and improving remediation performance in complex contamination scenarios.

Overall, the results obtained within Task 4.5.2 provide a comprehensive experimental basis for the development of operational guidelines for bioremediation implementation. The integration of microbial ecology, biodegradable carrier materials, innovative process configurations, and emerging technologies such as bioelectrochemical systems demonstrate the potential to significantly improve the efficiency, robustness, and scalability of sustainable remediation strategies for contaminated environments.

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## 4 Introduction

In 2018 the European Environmental Agency (EEA) has estimated that 2.8 million potentially contaminated sites exist in the European Union (EU) with 650,000 of them identified as requiring remediation. A large proportion of these are legacy sites (so-called brownfield sites), often with unknown ownership (Payá Pérez and Rodríguez Eugenio, 2018). This estimate is considered conservative and the number of potentially contaminated sites across the EU is likely to be underestimated (EEA, 2022) and the proportion of unregistered sites and sites that have not been risk assessed is more than 50%.

Significant progress has been made during the recent years in the remediation of contaminated sites: around 65 500 sites have already been remediated or are under aftercare measures (EEA, 2018); however, the levels of national action strongly differ across the EU.

Concerning the contaminants affecting soil and groundwater in Europe, mineral oils and heavy metals represent the main categories (Van Liedekerke et al., 2014) and the distribution is similar in the liquid and the solid matrices (Figure 1).

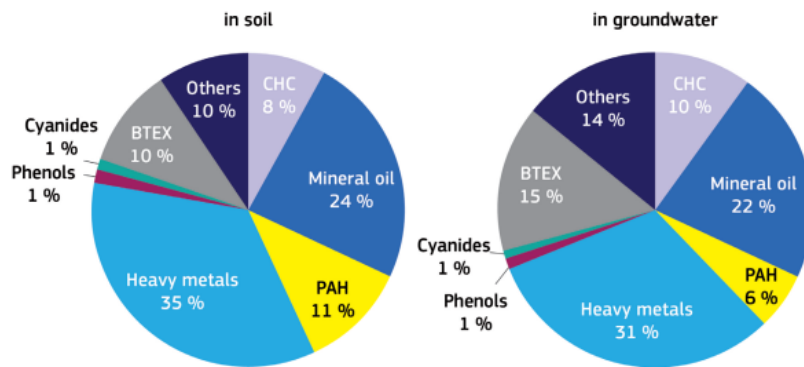


Figure 1 - Overview of contaminants affecting soil and groundwater in Europe (Van Liedekerke et al., 2014)

In Italy, the Italian Institute for Environmental Protection and Research (ISPRA) has recorded 42 Contaminated Sites of National Interest (Figure 2), defined by specific statutory provisions based on site characteristics, quantity and hazardousness of pollutants, extent of the environmental impact in terms of health and ecological risk, and 34.478 sites subjected to an administrative procedure of local contamination management according to Legislative Decree n. 152/06 (Figure 3). The main contaminants, similarly, to Europe, are mineral oils, heavy metals, CHC and BTEX.

Regards to remediation activities, “traditional” techniques still prevail for the treatment of contaminated soil, in particular soil excavation and disposal accounts for on average 30% of such activities. Furthermore, *in-situ* and *ex-situ* measures are applied about equally. With regard to the treatment of contaminated groundwater, *ex-situ* physical and/or chemical treatments are most commonly reported as being applied (37%) (Van Liedekerke et al., 2014). However, increasing regulatory control of landfill operations and associated rising costs, combined with the development of improved *ex-situ* and *in-situ* remediation techniques, is altering the pattern of remediation practices (Van Liedekerke et al., 2014) towards less impactful technologies such as

bioremediation. Bioremediation is an affordable and sustainable method to clean unsaturated soil, capillary fringe areas, and groundwater. It removes organic compounds, nitrobenzene, chlorobenzene, phenols, aromatic amines, some pesticides and plant protection chemicals, select metals, and other inorganics. This technique exploits the natural capacity of the micro-organisms present in the subsoil (or specifically introduced therein) to degrade organic contaminants, provided that the microbial colonies growth conditions are optimal (pH, temperature, redox potential, oxygen quantity, nutrients, etc.). They feed off polluting organic compounds and transform them into carbon dioxide, water and/or biomass.

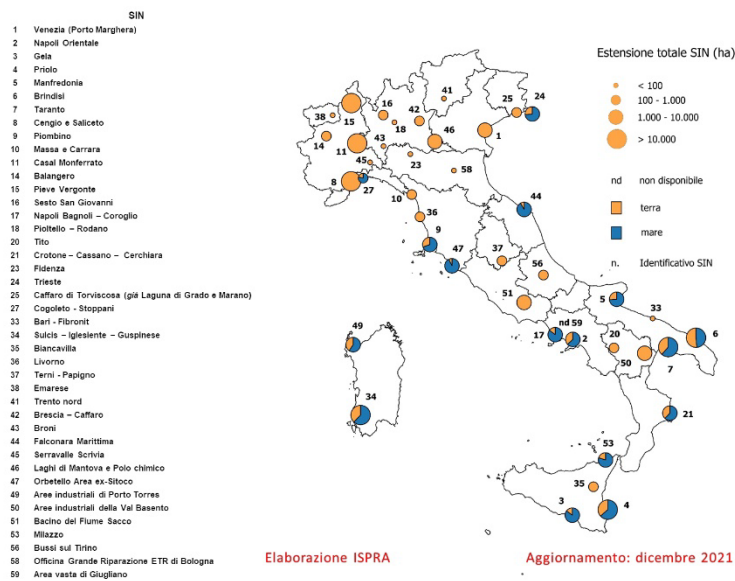


Figure 2 – Contaminated Sites of National Interest (ISPRA, 2021)

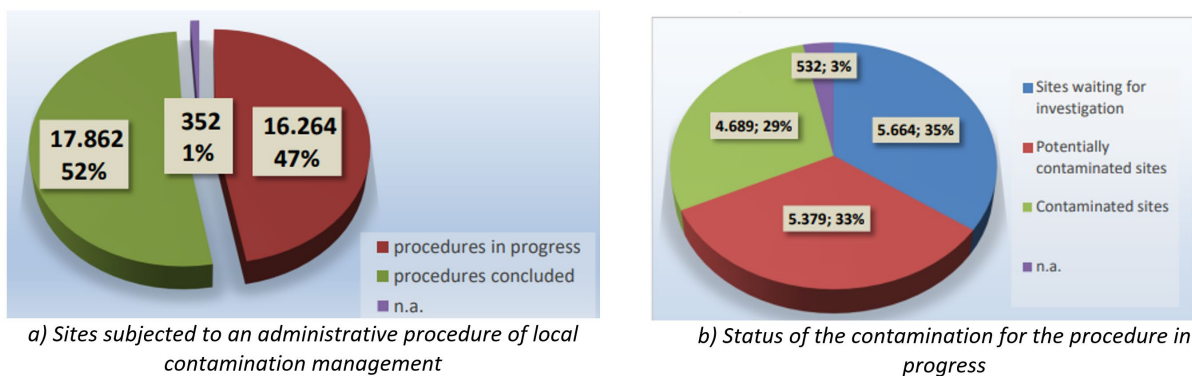


Figure 3 - Contaminated sites management at regional level. Data collected at 31.12.2019 (ISPRA, 2021)

To date, many degradative pathways of the main contaminants by higher organisms or microorganisms are known and defined. At the same time, the application of biomolecular technologies to bioremediation sector allowed a huge technological advance in the acquisition of site-specific data for process planning and

monitoring. Furthermore, the development of genetic databases together with the increase in experiences of biogenetic research applications, pilot tests in batches or micro-medium scale has further increased the basic knowledge relating to bio-removal processes and allowed to acquire high specific preliminary confirmations avoiding costly and unsuccessful field trials.

The next challenge is the scaling of processes from the laboratory to the field scale (both in pilot applications and on a large scale) together with the standardization of protocols to promote a large use of bioremediation even by non-ultra specialist operators and a general sense of reliability of bioremediation technologies for public authorities.

The achievement of Task 5.2, which involves the "development of innovative and eco-friendly bioremediation processes and technologies for contaminated soil, water, and groundwater using both aerobic and anaerobic pathways to remove organic and recalcitrant compounds", will require coordinated efforts from various operational units. These units possess multidisciplinary expertise, making them well-suited to develop bioremediation processes that can effectively address contamination issues in actual sites. To accomplish this task, a research plan has been devised that begins with laboratory investigations aimed at characterizing processes and bacterial communities that could be utilized in (bio)remediation. The plan will then move towards field applications, which may involve pilot-scale experimentation.

## 4.1 Microbiological characterization and bioremediation potential evaluation

The presence of microbial communities in contaminated environments provides an opportunity to naturally revive polluted sites by utilizing bacterial strains that can degrade and transform pollutants. Biodegrading bacteria have been identified in all matrices, including water, soil, and sediment, under different environmental conditions from the tropics to the poles. Hydrocarbon degrading bacteria, in particular, have been found to be ubiquitous in nature and can utilize a wide range of pollutant compounds as sources of carbon and energy for aerobic and anaerobic biodegradation. Although less common, degraders of chlorinated alkanes have also been detected in contaminated groundwater, with their presence and abundance dependent on hydrogeochemical characteristics. Over the years, promising and high-performing bacterial species and consortia have been characterized, isolated, and exploited for bioremediation projects. However, the use of environmental microbiota for bioremediation purposes requires deep knowledge regarding the variety of microorganisms, their catabolic capabilities, and how they function under different biotic and abiotic environmental constraints. The challenge in environmental research is activating the natural degradation potentials in environmental media to develop effective remediation methods. To do so, we must consider that while most degradation potentials are widely distributed among microorganisms, indigenous microbes are typically present in low numbers. Additionally, the degradative metabolism towards specific pollutants often needs to be induced, and the presence of heavy metals may inhibit biodegradation processes in co-contaminated matrices. Biostimulation involves changing physicochemical parameters such as pH, temperature, electron donors, or acceptors to activate the degradation process. Another approach to overcome these limitations is bioaugmentation, which involves inoculating competent microorganisms into the system to adjust the niche. A way to increase the solubility, mobility, bioavailability and subsequent biodegradation of hydrophobic or less soluble organic compounds is the use of biosurfactants, amphiphilic compounds which can reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids. Microorganisms have been shown to produce potent surface-active compounds that enhance the rate of degradation by emulsification or

solubilization of hydrophobic hydrocarbons. Biosurfactants are produced extracellularly or as part of the cell membrane by bacteria, yeasts, and fungi, from various substrates including sugars, alkanes, oils, and waste.

## 5 Bioremediation of contaminated marine sediments using bioslurry technology

### 5.1 Research Focus and Methodology

The core of the study investigated the applicability of the **bioslurry process**—a microbial-based technology using a bioreactor—to remove **Total Petroleum Hydrocarbons (TPHs)** from real marine sediment samples. The goal was to investigate how the presence of erythromycin, considering the co-occurrence of TPH degraders and antibiotic resistance genes (ARGs), could **modulate the microbial community (microbiome)**, thereby change its structure and impact the TPH removal efficacy.

### 5.2 Materials and Setup

#### 5.2.1 Sediments sample

Marine sediment samples were collected from the northern part of **Augusta Bay**, near petrochemical plants. The sediments were slightly acidic and severely contaminated, with an initial TPH level of about  $888.57 \text{ mg}_{\text{TPH}} \text{ kg}_{\text{DW}}^{-1}$ , which exceeds the Italian regulatory limit ( $888.57 \text{ mg}_{\text{TPH}} \text{ kg}_{\text{DW}}^{-1}$ ).

The detailed characterization of sediments was shown in the published paper: particularly, in order to complete the initial information, in **Table 2.1** also the activated sludge properties, used for initial inoculum, were reported.

**Table 1** - Properties of sludge and marine sediment of Augusta Bay.

Activated Sludge	
Total Carbon ( $\text{mg L}^{-1}$ )	9.84
Total Organic Carbon ( $\text{mg L}^{-1}$ )	1.18
EC ( $\text{mS cm}^{-1}$ )	1050
Total solids ( $\text{g L}^{-1}$ )	3.1
Volatile solids ( $\text{g L}^{-1}$ )	0.35
Marine sediment	
Physical properties	Value
Density ( $\text{g cm}^{-3}$ )	2.57
pH	6.6
EC ( $\text{mS cm}^{-1}$ )	5
Moisture content (%)	48.6
Organic matter (%)	4.7
Particle size distribution	Fraction (%)
Sand	42.36
Silt	35.38
Clay	22.27
TPH content	Concentration ( $\text{mg}_{\text{TPH}} \text{ kg}_{\text{DW}}^{-1}$ )
$\Sigma$ TPH fractions ( $\text{C}_{12}\text{-C}_{40}$ )	888.57

## 5.2.2 Experimental Design

Activated sludge collected from a full-scale WWTP (Enna, Sicily) was used for initial bioaugmentation.

The campaign was divided into two main stages:

1. **Preliminary Microcosm Tests (Small & Large Volume):** Conducted to identify the optimal erythromycin dosage. Five concentrations were tested ( e.g. 0.05, 0.1, 0.5, 1, 5  $\mu\text{g mL}^{-1}$ , respectively), with 0.5  $\mu\text{g mL}^{-1}$  being selected for further study based on its effect on Total Organic Carbon (TOC) and TPH biodegradation activity.
2. **Pilot Plant Study (75 days):** Two bioslurry reactors (R1 and R2) were set up ( working volume, sediment to water ratio) and operated in three phases:
  - **PHASE 1 (Day 1-30):** Both reactors worked under identical operating conditions (Control phase).
  - **PHASE 2 (Day 31-60):** Erythromycin (0.5  $\mu\text{g mL}^{-1}$ ) was added to the R2 reactor to study its effect as a potential bio-stimulant.
  - **PHASE 3 (Day 61-75):** The liquid phase in both reactors was replaced with saline water to investigate the "**rebound effect**," a phenomenon typical of slurry phase systems related to biological desorption.

Pilot plant scheme is reported in [Figure 1](#).

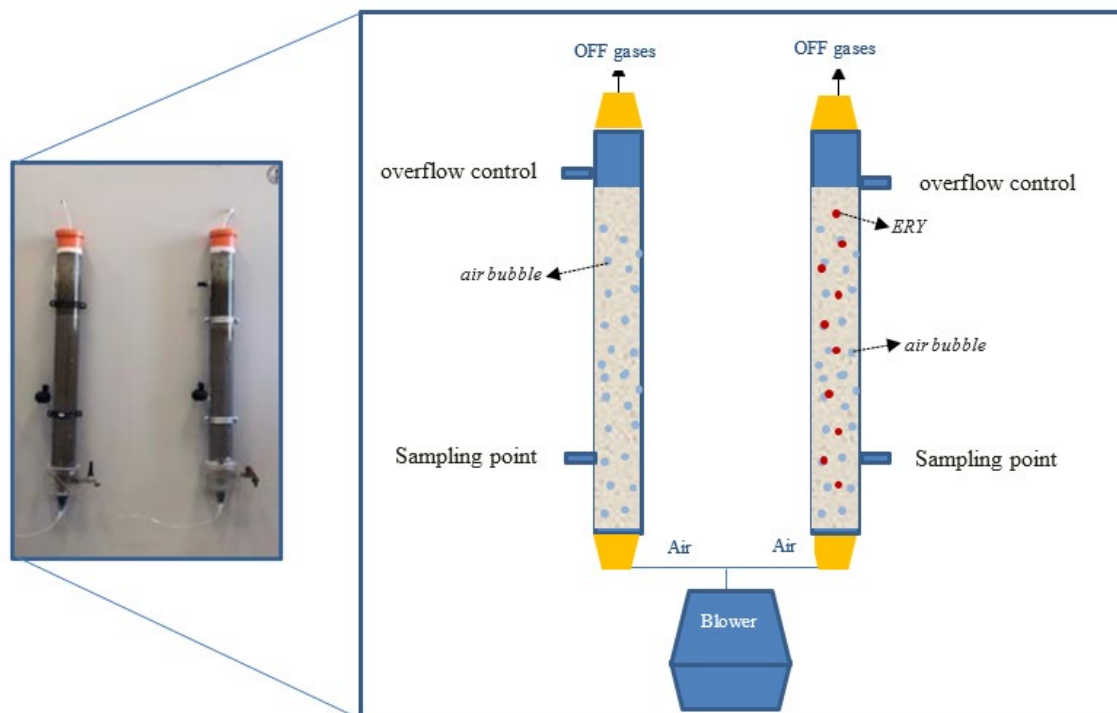


Figure 1 - BIOSLURRY Pilot plant scheme.

## 5.3 Key Findings

### 5.3.1 Microcosm Tests

- In small volume tests, the variation of TC and TOC concentrations were evaluated with reference to different incubation times (i.e. 0.5, 1, 24, and 96 h) and different erythromycin concentrations (i.e. 0.05, 0.1, 0.5, and 5  $\mu\text{g mL}^{-1}$ ). In general, at the concentrations of 0.1, 0.5 and 1  $\mu\text{g mL}^{-1}$  the TC and TOC values are quite stable after 0.5 and 1 h of activity, and partially after 24 h. On the contrary, the variations of the antibiotic response at 96 h prove to be heterogeneous at all used antibiotic concentrations. Interestingly, the results of TOC concentrations in the respect of erythromycin concentration and exposure time in small volume microcosm tests suggested that the antibiotic addition affected microbial activity and growth.
- Microcosm testing continued by analyzing a further batch test, mixing water and sediments in large volume (1 L) assays adding the antibiotic based on the best dosage previously detected. This analysis showed that TOC concentration increased rapidly from 1.5 to 2.44  $\mu\text{g mL}^{-1}$  after the first 24 h, remaining stable at around 2.2–2.3  $\mu\text{g mL}^{-1}$  in the following three days. This observation paralleled the 12–15 % removal of TPHs from the microcosm sediments in just 4 days, going from 888  $\text{mg}_{\text{TPH}} \text{kg}_{\text{DW}}^{-1}$  to 777  $\text{mg}_{\text{TPH}} \text{kg}_{\text{DW}}^{-1}$ . Based on these encouraging results, it was decided to dose the antibiotic directly in the pilot plant column and study the potentially stimulating effect of erythromycin during the continuous treatment of sediments with bioslurry. The results obtained in large volume microcosm tests confirm and validate the data obtained in the small volume microcosm assays. In particular, it appears that by injecting 0.5  $\mu\text{g mL}^{-1}$  of erythromycin the microbial cells of the bioslurry system manage to survive and, based on the temporal evolution of the TOC and TPH values, it seems that the erythromycin selective pressure parallels metabolic adaptation to the presence of TPHs in the sediments whose concentration decrease of 15 % in 4 days.

### 5.3.2 Pilot Plant Results

- **PHASE 1 (Baseline):** TPH residual concentrations were similar: for R1 and for R2, indicating a Removal Efficiency (RE) of approximately similar.
- **PHASE 2 (Erythromycin added to R2):** The antibiotic addition in R2 significantly **increased the TPH removal rate**, demonstrating an effective bio-stimulating process.
  - R1 (Control): Final TPH was (RE 35%).
  - R2 (with Erythromycin): Final TPH was (RE 55%).
- **PHASE 3 (Rebound Effect):** Following the liquid phase replacement, TPH residual concentrations increased in both reactors due to biological desorption (release of sequestered TPHs). However, R2 still proved to be more efficient than R1, and residual values remained lower than the initial TPH contamination.

The accumulation/utilization behavior of TPHs in the liquid phase, and their rebound effects, are diametrically opposed to the increase and present of TPH concentrations in the solid matrix, as observed in [Figure 2](#).

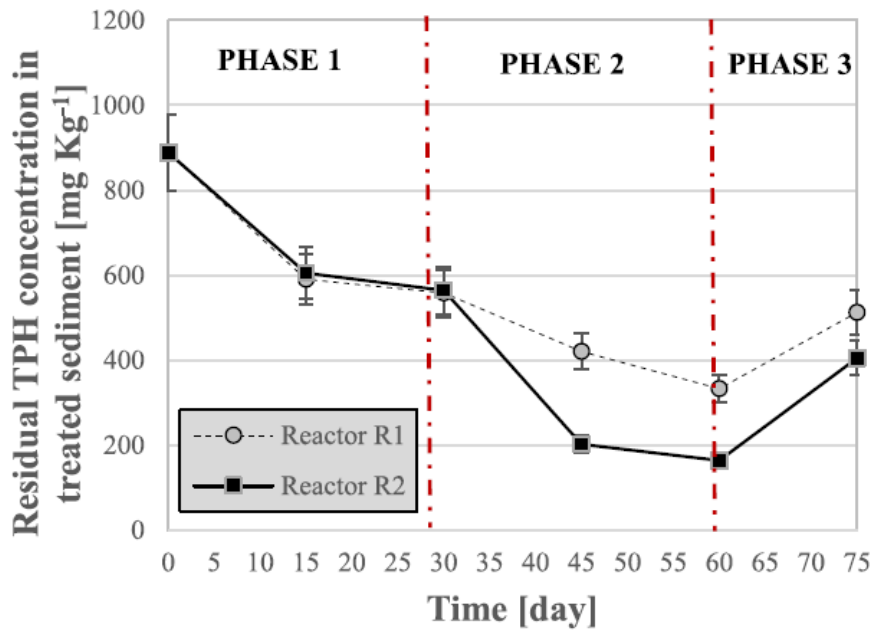


Figure 2 - TPH concentrations in different sediment phases

- Microbial Modulation:** The presence of erythromycin successfully affected the dynamic changes of the microbial community structure, selecting for microbiota members with an optimized metabolism for TPH removal. This confirms that the antibiotic, even at low concentrations, can promote further TPH reduction through **biostimulation**. The “Relative abundance” is shown in **Figure 3**.

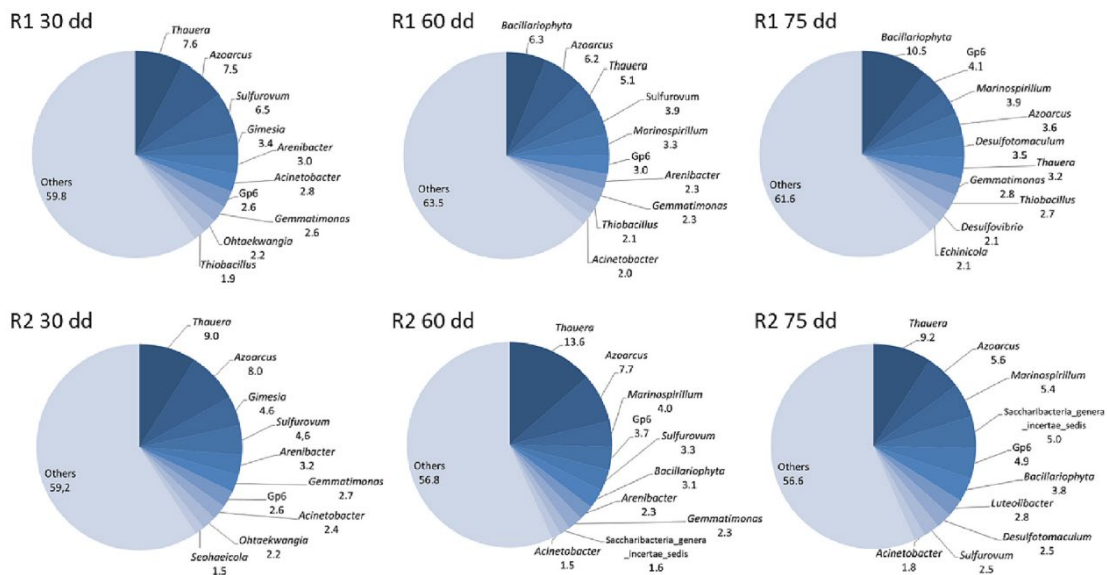


Figure 3 - Relative abundance of the ten most abundant bacterial genera identified in R1 and R2 at the indicated days by NGS analysis of the V3-V4 region of the 16S rRNA encoding gene

## 5.4 Ongoing research and future prospects for contaminated sediment management

A - The **biological "bioslurry"** treatment was conducted by means of autochthonous microorganisms, already present within the contaminated sediments, assisted by an inoculum of activated sludge adapted to the salinity. The experimental campaign involves four batches (R1, R2, R3, R4), each with a working volume of 4 liters, a sediment/water ratio of 10% by weight, and operating in parallel for a total period of 60 days. In order to evaluate possible "rebound" effects of the TPH present in the contaminated sediments, a seawater exchange was performed every 15 days in reactors R2, R3, and R4 according to the operating conditions reported below:

- **R1:** Inoculum of activated sludge + 400 g of contaminated sediment + 4000 g of seawater. The reactor is maintained in batch mode for the entire duration of the experiment.
- **R2:** Inoculum of activated sludge + 400 g of contaminated sediment + 4000 g of seawater. The reactor is maintained in batch mode for the entire duration of the experiment. However, every 15 days, a mass of fresh seawater equal to 25% by weight (%wt) of the slurry was exchanged.
- **R3:** Inoculum of activated sludge + 400 g of contaminated sediment + 4000 g of seawater. The reactor is maintained in batch mode for the entire duration of the experiment. However, every 15 days, a mass of fresh seawater equal to 50% by weight (%wt) of the slurry was exchanged.
- **R4:** Inoculum of activated sludge + 400 g of contaminated sediment + 4000 g of seawater. The reactor is maintained in batch mode for the entire duration of the experiment. However, every 15 days, a mass of fresh seawater equal to 75% by weight (%wt) of the slurry was exchanged.

At the beginning and end of the batch tests, liquid-liquid and solid-liquid extractions of TPH were performed to evaluate the hydrocarbons removal efficiency. This is expected to be predominantly biological TPH removal and, to a lesser extent, physical TPH removal (i.e. mainly associated with stripping phenomena). The potential "rebound" effect in reactors R2, R3, and R4 should be associated with the concentration gradient between the contaminated sediment and the liquid bulk. This concentration gradient is expected to increase as the mass percentage of the exchanged seawater increases.

## 5.5 Main Conclusion

The project's activities confirmed the important potential of *ex-situ* bioslurry treatment, demonstrating **satisfactory remediation performance** on real sediments (Augusta and Genoa) with limited financial investment. The primary **strengths** are the high removal rates, while the main **weaknesses** are the long and sometimes unpredictable treatment periods and the complexity of distinguishing between true bioaugmentation and biostimulation effects.

Crucially, this work is novel in showing that antibiotics like erythromycin can play a key role in **microbiome modulation strategies** for process improvement, promoting a further reduction of TPH concentrations by selecting for more suitable microorganisms. However, this finding underscores the necessity for **further investigations** into the antibiotic's impact on the spread of **ARGs** (Antibiotic Resistance Genes) and the development of advanced technological solutions to address this critical aspect.

## 6 Treatment of hydrocarbon – contaminated marine sediments by mycoremediation techniques

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### 6.1 Introduction

The contamination of marine sediments caused by anthropogenic activities represents a critical environmental issue, particularly in coastal areas subjected to prolonged industrial pressure. Sediments play a key role in aquatic ecosystems, acting both as sinks and secondary sources of contaminants through adsorption–desorption processes (Zhang et al., 2021). Petroleum hydrocarbons are among the most relevant pollutants due to their persistence, hydrophobic nature, and potential toxic, mutagenic, and carcinogenic effects (Xu et al., 2024; Alao et al., 2025). Conventional remediation technologies for contaminated sediments often rely on physical – chemical or thermal treatments, which may be costly and environmentally invasive. In contrast, biological approaches have gained increasing attention as sustainable alternatives. Bioremediation exploits the metabolic capabilities of microorganisms to transform or mineralize contaminants, offering advantages such as reduced environmental impact, lower operational costs, and applicability to a wide range of organic pollutants. Within this framework, mycoremediation and the use of fungi for contaminant degradation has shown particular promise. Filamentous fungi are capable of producing extracellular oxidative enzymes that can attack complex hydrocarbon structures, often under environmental conditions less favourable to bacterial activity. Moreover, synergistic interactions between fungal and bacterial communities may further enhance degradation efficiency (Harms et al., 2011). Among *ex situ* biological treatments, bioslurry reactors provide controlled conditions that enhance mass transfer and contact among contaminants, nutrients, and microorganisms. The present study aims to assess the effectiveness of mycoremediation in a bioslurry system treating hydrocarbon-contaminated marine sediments, comparing different operational strategies, including the use of biochar as a fungal support material. Experimental activities were carried out in a laboratory – scale bioslurry system.

### 6.2 Methodologies

#### 6.2.1 Experimental methods

The experimental campaign, with a total duration of 60 days and currently ongoing, was conducted using a laboratory-scale bioslurry apparatus consisting of four parallel reactor lines (Figure 1). Each line was equipped with a 5 L jacketed reactor fitted with a hermetically sealed lid, sampling ports, an aeration system with porous stone diffusers, and a mechanical mixing system. Airflow was continuously regulated using flowmeters. Experiments were performed in slurry mode by mixing sediments with sterilized and 0.45 µm-filtered seawater at a solid-to-liquid ratio of 1:10. The operating temperature was maintained at 24°C throughout the experimental period. The four reactor configurations were as follows: (A) abiotic control, with sodium azide added to inhibit microbial activity; (B) biotic control, containing native microbial communities; (C) mycoremediation system with fungal inoculation; and (D) enhanced mycoremediation system with fungal biomass immobilized on biochar.

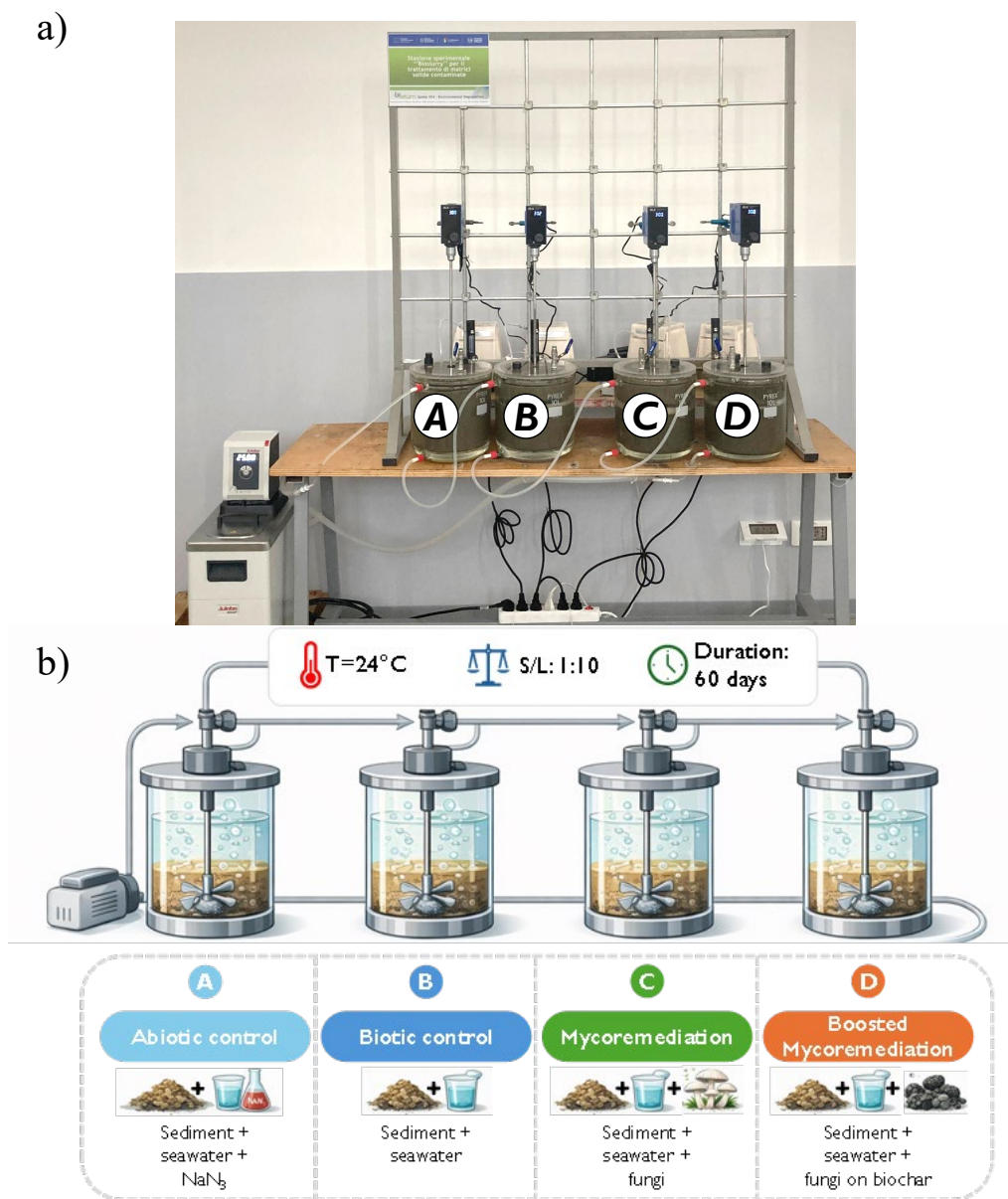


Figure 1. Panoramic overview (a) and layout (b) of the experimental apparatus

### 6.2.2 Materials

Marine sediments collected from the Port of Genoa were artificially contaminated with diesel fuel at 1% (w/w), resulting in an initial TPH concentration of approximately 5000 mg kg<sup>-1</sup> dry solids. Prior to experimentation, sediments were homogenized for 15 days to allow volatilization of lighter fractions, dried at 40°C for 72 h, and subsequently ground and sieved to <2 mm. The biochar used (B440) was produced from woody biomass derived from pruning residues at the University of Palermo. The biomass was dried, milled to <5 mm, and pyrolyzed in a muffle furnace at 440°C for 3 h. Autochthonous fungal strains were selected through functional screening assays and are preserved in the Cold Culture Collection (University of Genoa). Fungal inocula were

prepared by culturing biomass on specific media at 24°C for three weeks, followed by suspension in sterile seawater and standardization to conidial concentrations  $\geq 10^6$ .

### 6.2.3 Analytical methods

An integrated monitoring protocol was adopted to assess the microbiological, chemical, and environmental evolution of the system over time. Mycological analyses were performed biweekly in reactors C and D to assess fungal viability and abundance. Direct examinations, reinoculation of sediments on plates, and isolation of colonies grown after 7 days were used both to verify inoculum viability and to quantify fungal presence. In reactor D, where fungal colonies were immobilized on biochar, scanning electron microscopy (SEM) analyses were conducted to investigate biochar colonization, biological immobilization, and mycelial morphology, and to correlate these features with system performance. At the same time intervals and in all lines, TPH concentrations were measured in both liquid phase and solid phases using standardized analytical methods: “Oil Index”, UNI EN ISO 9377-2 and “Procedure for the analysis of hydrocarbons >C12 in contaminated soils – Manuals and Guidelines 75/11”, ISPRA, 2011, respectively. Moisture content, required for solid – phase TPH calculations, was determined according to Official Method no. II.2., Suppl. Org. G.U. no. 248 of 21/10/99. Additionally, key process parameters, including dissolved oxygen, pH, temperature, and electrical conductivity, were monitored twice weekly. Volatilized hydrocarbons (VOC) emissions were quantified using a portable flame ionization detector (FID) coupled with a hot-wire anemometer for the off-gas flow measurements. At the end of the experiment, residual phytotoxicity was assessed using a germination index test on *Lepidium sativum* (garden cress), following the APAT (2004) procedure.

## 6.3 Results and discussion

### 6.3.1 Fungal Colonization of Biochar: Temporal Dynamics Observed by SEM

SEM micrographs (Figure 2) reveal a significant temporal dynamic in fungal colonization of the biochar. Biochar not exposed to fungal colonies (Figure 2a) shows a well-defined porous structure typical of pyrolyzed lignocellulosic materials, characterized by elongated channels, open cellular cavities, and layered walls. After 48 hours of incubation, images (Figure 2b) show a network of branched filaments and numerous spheroidal particles associated with the biochar surface, consistent with hyphal and conidial development, demonstrating rapid substrate colonization. This behaviour aligns with literature reports indicating that biochar can promote microbial adhesion due to its porosity and capacity to provide protected microhabitats (Yin et al., 2023). After 21 days, SEM images (Figure 2c) no longer show a widespread filamentous network on the surface; instead, the biochar surface appears more exposed, with original pores and channels preserved. The reduced presence of hyphae and conidia may suggest several interpretations, including loss of organism viability due to stressful reactor conditions. Further investigations are ongoing.

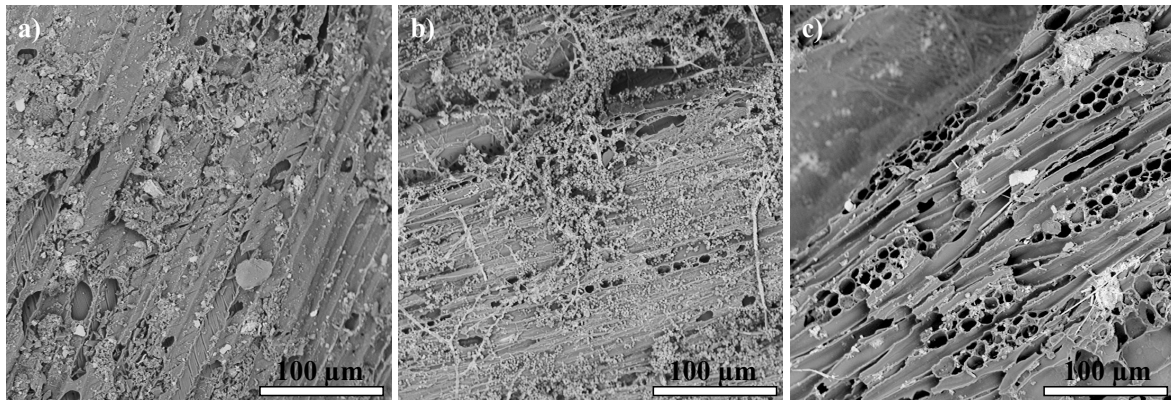


Figure 2. SEM images of biochar before contact with fungal colonies (A), 48 hours after contact (B), and after 21 days of experimentation (C)

### 6.3.2 TPHs removal efficiencies

Figure 3 shows removal efficiency and biodegradation rate over time. As it is possible to observe, after the first two weeks of experimentation, TPH concentration in the abiotic control (reactor A) decreased by 23% (residual concentration in sediment: 2855 mg/kg), likely due to desorption and volatilization phenomena. In the biotic control (reactor B), TPH reduction was significantly higher at 41% (2205 mg/kg), confirming the contribution of aerobic biodegradation by the autochthonous microbial community. In reactors C and D, where mycoremediation was applied, TPH removal reached approximately 55% (residual concentrations in both lines around 1675 mg/kg). The additional TPH reduction observed in reactor C suggests a synergistic effect between bacterial and fungal communities, highlighting the potential role of fungi in enhancing bioremediation processes in marine sediments. Conversely, the unchanged TPH removal in reactor D indicates that process efficiency is primarily governed by fungal biological activity, while biochar addition does not appear to provide further benefits in terms of removal efficiency. At the second sampling, carried out after another 14 days, the removal rates remained almost constant in all 4 reactors, indicating saturation effects or mass transfer limitations. Conversely, biodegradation rate resulted consistently higher in the second sampling day than in the first one, indicating improved biological activity over time. This increase in biodegradation percentage, despite the stabilization of overall TPH removal, suggests that the residual hydrocarbons became progressively more bioavailable or that microbial consortia underwent acclimation and enzymatic induction during the experimental period. The apparent decoupling between total removal efficiency and biodegradation rate in the later phase of the experiment may indicate that easily degradable hydrocarbon fractions were preferentially consumed during the first two weeks, while the remaining compounds were more recalcitrant and strongly sorbed to sediment particles.

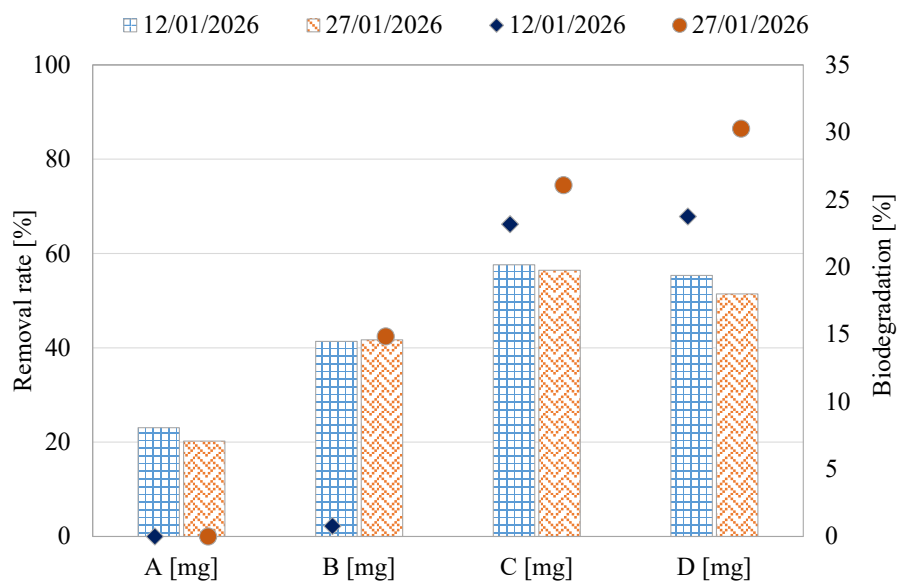


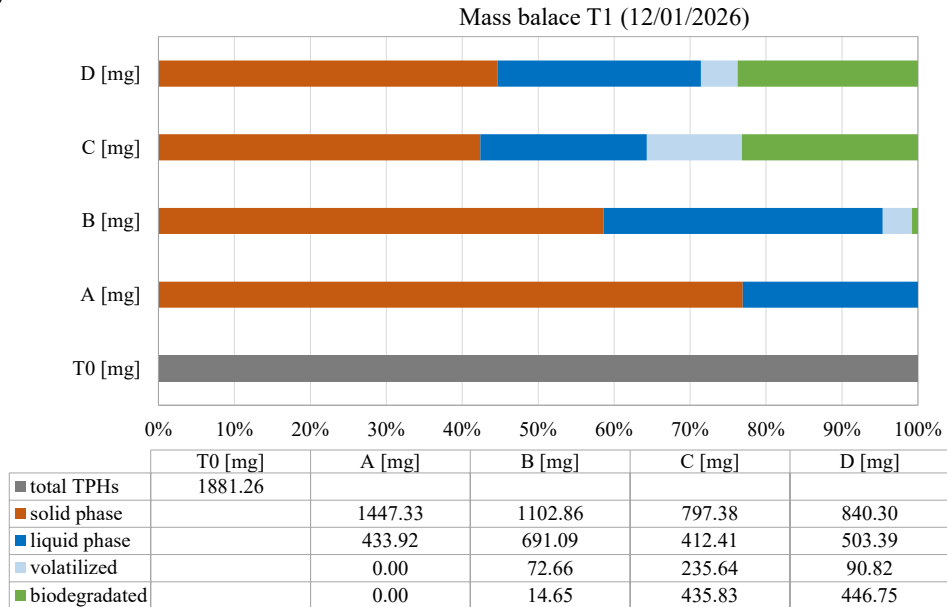
Figure 3. Removal efficiency and biodegradation rate over time

The mass balance analysis (Figure 4) at T1 (12/01/2026) and T2 (27/01/2026) provides further insight into the mechanisms governing TPH removal in the different reactors.

At T1 (Figure 4a), in reactor A (abiotic control), most of the residual TPHs remain associated with the solid phase (1447 mg), with a relevant fraction detected in the liquid phase (434 mg) and no biodegradation observed. This confirms that the ~23% reduction measured in this reactor is entirely attributable to physical processes, mainly desorption and phase transfer phenomena, without any biological contribution. In reactor B (biotic control), TPHs are distributed between the solid (1103 mg) and liquid phases (691 mg), with a small but detectable biodegraded fraction (14.65 mg) and a volatilized portion (72.66 mg). Although biodegradation at T1 is still limited in absolute terms, the higher overall removal compared to the abiotic control indicates the onset of aerobic microbial activity by the indigenous community. In reactors C and D (mycoremediation treatments), the biological contribution is clearly evident already at T1. The biodegraded fraction reaches 435.83 mg in reactor C and 446.75 mg in reactor D, representing the main removal pathway. Correspondingly, the residual TPH in the solid phase is markedly lower than in the control reactors, confirming the effectiveness of fungal inoculation in enhancing hydrocarbon degradation. The presence of a non-negligible volatilized fraction, particularly in reactor C, may also suggest interactions between biological activity and mass transfer processes.

At T2 (Figure 4b), the mass balances show a distinct evolution. In reactor A, the distribution remains substantially unchanged, with TPH predominantly in the solid phase and no evidence of biodegradation, confirming stabilization of purely physical processes. In reactor B, however, the biodegraded fraction increases significantly (279.33 mg), while the liquid and volatilized fractions decrease, indicating microbial acclimation and improved metabolic efficiency over time. In reactors C and D, a further increase in the biodegraded fraction is observed (490.39 mg in C and 569.31 mg in D), accompanied by a reduction in the liquid-phase fraction compared to T1. Nevertheless, total residual concentrations do not decrease proportionally, suggesting that degradation primarily affects the more bioavailable hydrocarbon fractions, while a portion of TPH remains strongly sorbed to the sediment matrix or consists of more recalcitrant compounds.

a)



b)

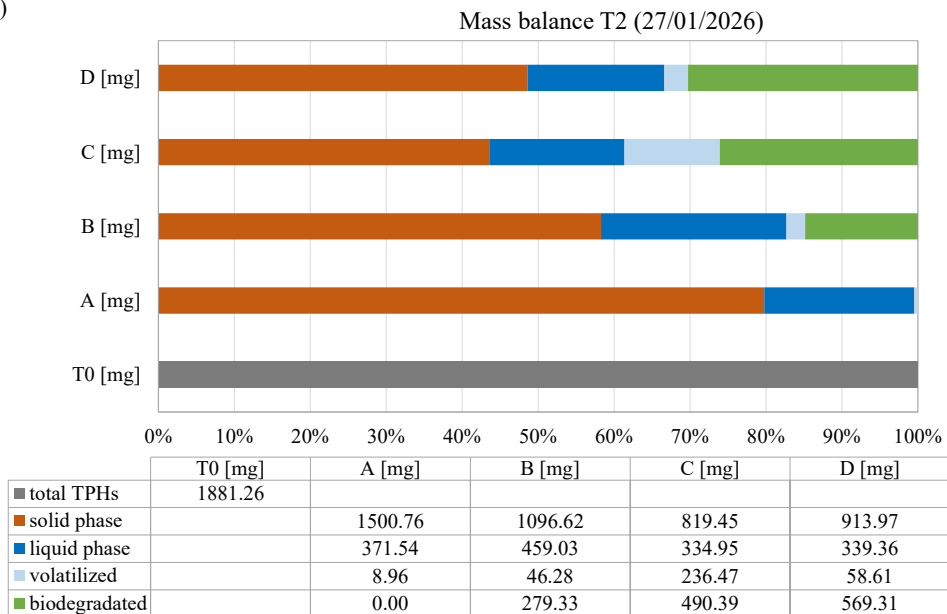


Figure 4. Mass balance over time in the 4 reactors

### 6.3.3 Mycological analysis

Figure 5 shows fungal colonization at T1 in reactors C and D. From the observation of Figure 5, for both reactors the Petri dish show complete fungal colonization across the entire surface, indicating that the fungal strain successfully adapted to the sediment matrix and was able to grow extensively under the experimental conditions. Alongside the fungal development, a bacterial component originating from the native sediment microbiota is also present. From a morphological perspective, the two microbial components can be clearly distinguished. The bacterial colonies appear gelatinous and display a coloration similar to that of the sediment,

suggesting their direct origin from the native microbial community. In contrast, the fungal mycelium is characterized by a green coloration and a more spongy, filamentous structure, typical of active hyphal growth. Importantly, no evident competitive exclusion between bacteria and fungi was observed. Instead, their coexistence suggests a synergistic interaction. The fungi likely contribute through the secretion of extracellular enzymes capable of degrading TPHs, while bacteria may further metabolize intermediate degradation products, enhancing overall process efficiency. The enzymatic activity released by the fungi plays a key role in this mechanism. These extracellular enzymes are active in the liquid phase, meaning they preferentially attack TPH fractions that have desorbed from the sediment matrix. As a consequence, degradation is not limited strictly to the physical presence of viable fungal biomass. Even after fungal cells lose viability, the enzymes already released into the surrounding medium may continue to catalyze hydrocarbon breakdown, sustaining the remediation process beyond the active growth phase.

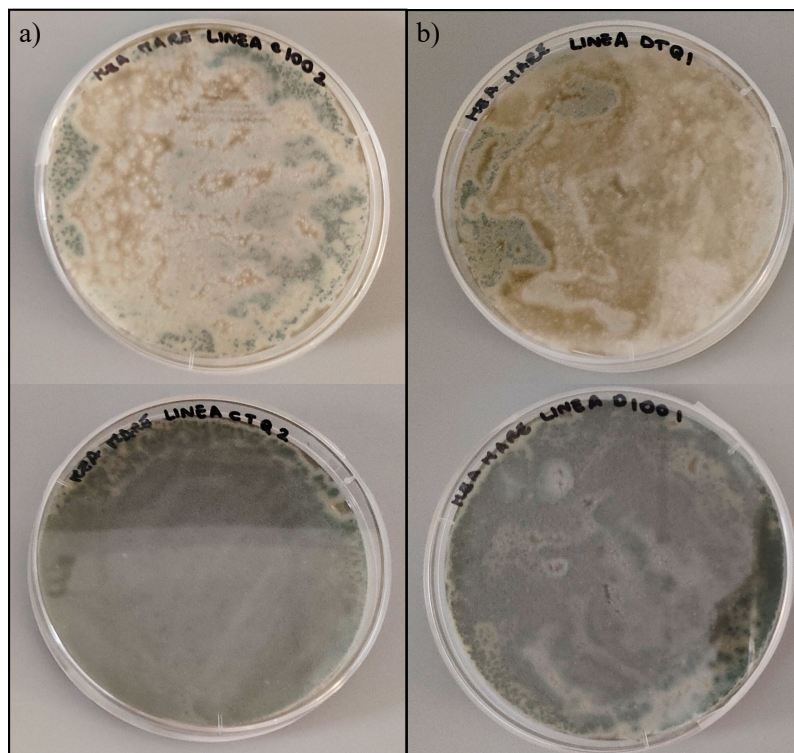


Figure 5. Petri dish and fungal colonization in reactor C (a) and D (b)

## 6.4 Conclusions and perspectives

Preliminary results indicate that mycoremediation in bioslurry systems is a promising strategy for the treatment of hydrocarbon-contaminated marine sediments, achieving higher removal efficiencies than both abiotic and conventional biotic controls. Fungal inoculation promoted synergistic interactions with the native bacterial community, enhancing biodegradation of the more bioavailable hydrocarbon fractions. Immobilization of fungal biomass on biochar enabled rapid initial colonization but did not produce significant additional improvements in overall removal efficiency. After an initial phase of rapid degradation, the process appeared to be limited by mass transfer constraints and the presence of more recalcitrant compounds. The persistence of extracellular fungal enzymatic activity beyond active biomass growth represents a key mechanism sustaining

hydrocarbon degradation. Future research should focus on optimizing reactor operating conditions, increasing contaminant bioavailability, and assessing the long-term stability of microbial consortia. Integration with physicochemical pretreatments or alternative support materials may further improve the removal of resistant fractions. These findings support the development of scalable and sustainable technologies for the remediation of contaminated port sediments.

## 7 Biological 1,2-DCA removal under anaerobic and aerobic conditions

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### 7.1 Introduction

Chlorinated organic compounds (COCs) represent a broad class of synthetic chemicals derived from aliphatic or cyclic hydrocarbons in which one or more hydrogen atoms are replaced by chlorine. Owing to their molecular structure, these compounds are typically composed of carbon, hydrogen, and chlorine atoms and are often referred to as “chlorinated solvents” due to their notable solvent capacity. Their physicochemical properties—such as low aqueous solubility, poor biodegradability, high toxicity, and, in many cases, carcinogenicity—render them hazardous environmental pollutants. Among the most widely encountered chlorinated solvents are 1,2-dichloroethane (1,2-DCA), 1,1,1-trichloroethane (TCA), carbon tetrachloride, methylene chloride, chloroform, tetrachloroethylene (PCE), and trichloroethylene (TCE).

These compounds have been extensively used in industrial applications, including degreasing of mechanical and electronic components, dry cleaning of metals and textiles, and as solvents or intermediates in the chemical and pharmaceutical industries. Historically inadequate management and disposal practices have led to pervasive contamination of soils and groundwater systems across numerous industrial sites. Due to their resistance to natural attenuation and their persistence in subsurface environments, contamination by COCs has become a significant environmental challenge.

Chlorinated solvents found in groundwater typically include chlorinated methanes, ethanes, and ethenes. Many of these compounds exhibit characteristic physicochemical traits that govern their subsurface behavior, such as high vapor pressure, density higher than water, and limited water solubility. These features favor the formation of dense non-aqueous phase liquids (DNAPLs) when released in sufficient quantities. DNAPLs migrate vertically through the saturated zone until reaching low-permeability layers, thus creating persistent source zones that continuously release dissolved contaminants into groundwater. These conditions make conventional remediation strategies, such as Pump & Treat (P&T) systems, largely ineffective. P&T often fails to achieve contaminant reductions sufficient for reinjection, ultimately requiring surface discharge of treated water. These limitations have prompted increasing interest in innovative in-situ remediation technologies, particularly permeable reactive barriers (PRBs).

Traditional PRBs employing zero-valent iron (ZVI) have proven highly effective in promoting reductive dechlorination of numerous COCs. However, ZVI has shown limited efficacy toward certain contaminants, particularly 1,2-DCA. This has driven the development of alternative remediation strategies, including biologically-based permeable reactive barriers (biological PRBs or PRBBs). These systems have emerged as promising solutions for sites characterized by high concentrations of 1,2-DCA.

In this context, the Environmental Sanitary Engineering research group at the University of Palermo, in collaboration with microbiologists from the same institution and researchers from the Kore University of Enna, conducted an experimental study aimed at assessing the technical feasibility of PRBBs under both anaerobic and aerobic conditions. The investigation was carried out using real groundwater sourced from a National Interest Site (SIN) in Southern Italy contaminated predominantly by 1,2-DCA. Under anaerobic conditions, the PRBBs were amended with polyhydroxybutyrates (PHBs), a class of biodegradable biopolymers applied as slow-release electron donors capable of sustaining reductive dechlorination. Under aerobic conditions, the

barriers were supplemented with an oxygen-release compound (ORC), designed to provide a sustained oxygen supply as an electron acceptor to promote aerobic oxidation of chlorinated solvents.

The experimental campaign was structured into two main phases (referring to both anaerobic and aerobic process).

**Phase 1** involved batch microcosm tests aimed at evaluating the intrinsic biodegradation potential of the native groundwater microbiota and the effectiveness of biostimulation and bioaugmentation strategies. Multiple batch reactor configurations were used to explore the influence of nutrient addition and inoculation with external microbial cultures. Throughout the incubation period, the degradation of 1,2-DCA was monitored using headspace gas chromatography coupled with mass spectrometry (HS-GC/MS), allowing precise quantification of contaminant removal.

**Phase 2** involved the operation of a continuous-flow laboratory-scale column system designed to simulate in-situ treatment conditions. Preliminary work focused on optimizing the system configuration to maximize biodegradation performance under both anaerobic and aerobic conditions. Throughout the experimental period, key physico-chemical parameters—dissolved oxygen (DO), oxidation–reduction potential (ORP), electrical conductivity, and pH—were routinely monitored to characterize the evolving geochemical environment. Groundwater samples were periodically collected for 1,2-DCA quantification using the same HS-GC/MS analytical protocol. In the anaerobic system, the concentration of acetate, a key fermentation product supporting long-term microbial activity and reductive dechlorination, was measured using ion chromatography (IC) to assess PHB fermentation efficiency.

A central innovation of this study lies in the use of PHB-based biodegradable biopolymeric carriers. These engineered scaffolds act simultaneously as physical supports for microbial colonization and as slow-release electron donors, enhancing long-term biological activity while maintaining environmental sustainability and low operational costs. The aerobic phase employed an ORC material chosen for its economic advantages over conventional aeration systems and its suitability for long-term in-situ applications. The ORC served as a stable oxygen source, allowing evaluation of the feasibility of aerobic biodegradation pathways for 1,2-DCA in contaminated aquifers.

## 7.2 Materials And Methods

### 7.2.1 Anaerobic Period

#### 7.2.1.1 Experimental Design Overview

The experimental activity lasted a total of 171 days and was divided into two 90-day phases:

1. **Phase I (Microcosm Study):** Assessment of microbial growth and activity under anaerobic conditions, with the aim of evaluating the biodegradation potential of native microbial consortia and the effectiveness of PHB as an electron donor.
2. **Phase II (Laboratory-Scale Continuous System):** Construction and operation of a PRB-simulating apparatus fed with groundwater collected from a contaminated aquifer, based on optimal conditions identified in Phase I.

### 7.2.1.2 Contaminated Groundwater Characterization

Groundwater was collected from a chlorinated-solvent-impacted aquifer and stored at 4°C in airtight, headspace-free containers to prevent oxygen exposure and contaminant volatilization. The groundwater exhibited:

- Presence of multiple chlorinated solvents (1,2-DCA = 4 ppm as primary contaminant)
- Negative redox potential
- Neutral pH
- High electrical conductivity and total dissolved solids

### 7.2.1.3 Microcosm Biodegradation Tests (Phase I)

Microcosms were prepared using contaminated groundwater to identify optimal conditions for 1,2-DCA biodegradation. Three experimental conditions were tested:

1. **Electron donor: sodium lactate**
2. **Electron donor: PHB**
3. **Abiotic control:** no electron donor, for assessing non-biological losses

Microcosms were incubated anaerobically for 30 days. 1,2-DCA degradation was quantified by HS-GC/MS. Results indicated:

- 90% removal in microcosms amended with PHB and inoculated with an anaerobically enriched microbial consortium
- 30% removal in abiotic control microcosms

These findings supported the selection of PHB as the electron donor for Phase II.

### 7.2.1.4 Laboratory-Scale Apparatus (Phase II)

The continuous-flow system (Figure 1) consisted of:

- A 20-L self-collapsing feed bag
- A five-way splitter
- A MasterFlex multichannel peristaltic pump
- Four parallel lines (A, B, C, D), each composed of:
  - One glass column (PRB zone)
  - One plexiglass column (downstream aquifer simulation)

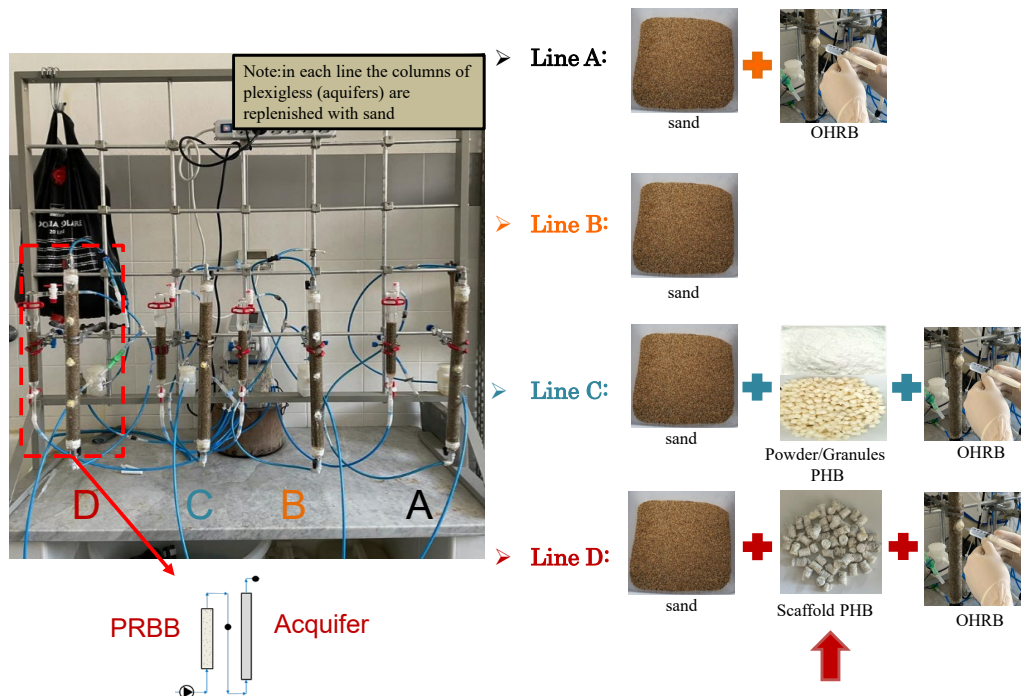
All glass columns were packed with sterilized silica sand (80 g). The lines were configured as follows:

- **Line A (biotic control):** inoculated microbial consortium
- **Line B (abiotic control):** no biological inoculum
- **Line C (PHB granular/powder):** inoculum + 5 wt% PHB
- **Line D (PHB scaffold):** inoculum + 5 wt% engineered PHB scaffolds

PHB scaffolds provided both structural support and slow-release electron donation.

Operational conditions:

- Flow rate: 6 mL/h
- Hydraulic retention time: 25 h
- Biomass inoculated 10 days before start-up
- Columns flushed with N<sub>2</sub> (0.5 L/min) prior to operation



**Figure 1.** Panoramic view of the experimental apparatus for the continuous anaerobic test

### 7.2.1.5 Analytical Methods

The following parameters were monitored:

- **Dissolved oxygen (DO), pH, ORP, conductivity:** using a WTW 3430 multimeter
- **1,2-DCA concentration:** HS-GC/MS
- **Acetate and chloride concentrations:** Ion Chromatography (DIONEX ICS-1100), APAT IRSA CNR 4020 method

## 7.2.2 Aerobic Period

### 7.2.2.1 Test for the assessment of oxygen release

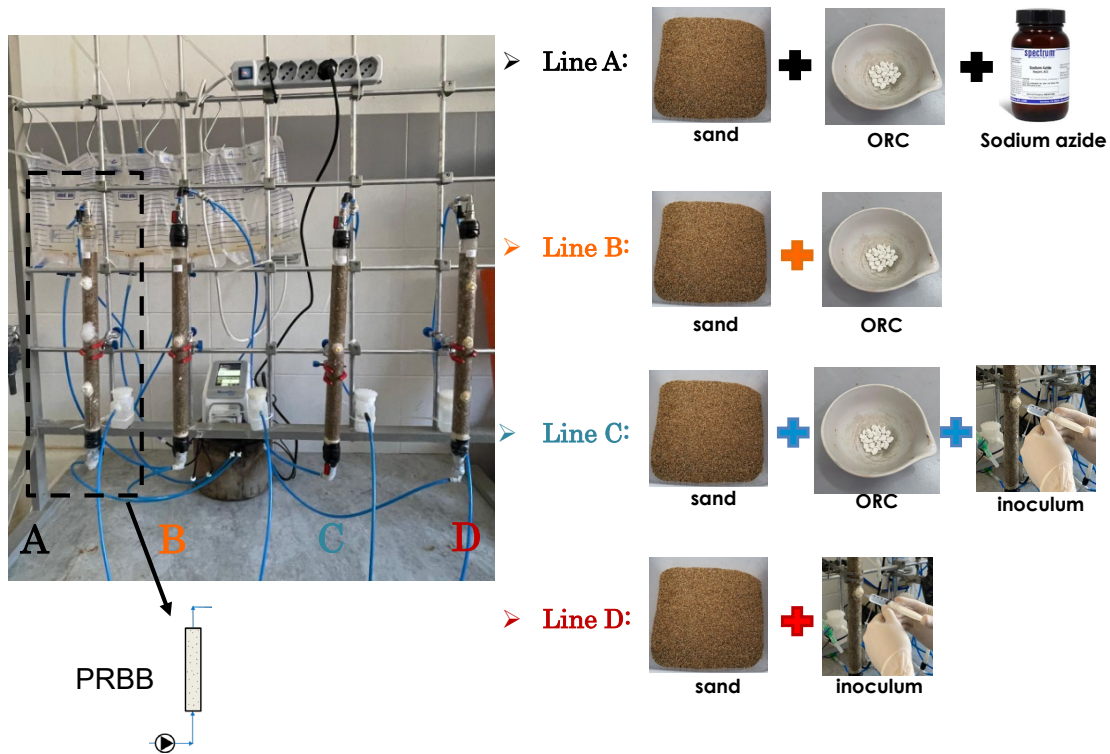
The column test was conducted using a feed bag containing deoxygenated tap water (Capodici et al., 2019). The bag was connected via a five-way manifold to a precision MasterFlex multichannel peristaltic pump, which supplied a system consisting of four parallel sand-filled columns, designated CORC2, CORC4, CORC6, and CORC8, containing 2 g, 4 g, 6 g, and 8 g of ORC, respectively. Aeration within the columns was not promoted by atmospheric air sparging; consequently, since the oxygen supply could not be varied over time, a system with increasing amounts of ORC was implemented to monitor the dissolved oxygen (DO) concentration within the columns. Moreover, the study aimed to investigate whether a correlation exists between the ORC mass and the dissolved oxygen concentration measured at the column outlet. In any case, the metabolism of aerobic bacteria implies a consumption of free oxygen in proportion to the bacterial concentration and, consequently, to the amount of biodegradable organic material present.

### 7.2.2.2 Description of the Experimental Setup

The experimental setup (Figure 2), assembled in the Laboratory of Environmental and Sanitary Engineering at the Department of Engineering, University of Palermo, consisted of four feed bags with a maximum capacity of 2 liters, containing water from the contaminated aquifer. These bags were connected via a five-way splitter to the same MasterFlex multichannel peristaltic pump used in the oxygen release tests, which supplied a system composed of four parallel lines, labeled A, B, C, and D, identical in geometric configuration.

Each line consisted of a plexiglass column, 44 cm in length and 2.8 cm in diameter, filled with 360 g of silica sand, aimed at simulating a portion of the aquifer. The differences between the lines were as follows:

- **Feed water:** Line A was supplied with sterilized groundwater, whereas lines B, C, and D were supplied with contaminated groundwater.
- **Filling material:** In line D, no ORC was present, while in lines A, B, and C, the ORC was placed at the bottom of the columns without mixing with the rest of the sand.
- **Bacterial inoculum:** This was added only in lines C and D.



**Figure 2.** Panoramic view of the experimental apparatus for the continuous aerobic test

In summary, line A was designed to evaluate potential physicochemical phenomena (abiotic control), since groundwater sterilization with sodium azide inhibited the possibility of natural attenuation by indigenous microorganisms. Line B (biotic control) was intended to simulate natural attenuation processes in the aquifer if oxygen were available. Finally, lines C and D represented configurations specifically designed for enhanced biodegradation via bioaugmentation. Line C, containing both bacterial inoculum and ORC, allowed the simultaneous study of aerobic biodegradation and chemical oxidation phenomena, whereas line D, containing only the bacterial inoculum, was used to study enhanced biodegradation alone.

The sand used to fill the columns was previously sterilized in an oven at 105°C for 24 hours.

Each line was equipped with sampling ports located at the inlet, midpoint, and outlet of the aquifer. These sampling ports were constructed by inserting a cap fitted with a perforable membrane. The details of the sampling/monitoring system were analogous to those shown in Figures 4.13a and 4.13b.

All four lines were supplied with the same groundwater containing 1,2-DCA at a concentration of approximately 10 ppm.

### 7.2.2.3 Analytical methods

During the entire experimental period, the concentrations of dissolved oxygen (DO) and  $K_s$ , as well as the pH and oxidation-reduction potential (ORP) values, were monitored; the 1,2-DCA concentrations were monitored periodically through HS-GC/MS. The analysis of the bacterial community structure was carried out at the end

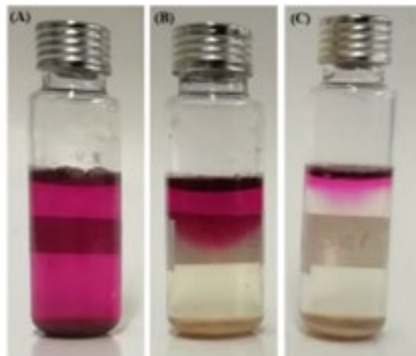
of experiments (the report of this activity is provided by the microbiologic research team of Palermo University).

## 7.3 Results and Discussion

### 7.3.1 ANAEROBIC PERIOD

#### 7.3.1.1 *Microcosms test results*

The series of anaerobic microcosms established using samples of contaminated groundwater under the lac, PHB, and K conditions, had the primary objective of optimizing the laborious setup under anaerobic conditions. Additionally, preliminary data were obtained regarding the biodegradation of 1,2-DCA and the structure and composition of the microbial communities enriched within the microcosms. To evaluate the establishment of anaerobic conditions during setup and the maintenance of these conditions throughout the incubation period, resazurin was added to the BTZ culture medium as a redox potential indicator. Figure 3 shows, as an example for all anaerobic microcosms, one replicate of the microcosm under lac conditions immediately after setup (T0) (A), after 7 days (B), and after 10 days of incubation (T10) (C).

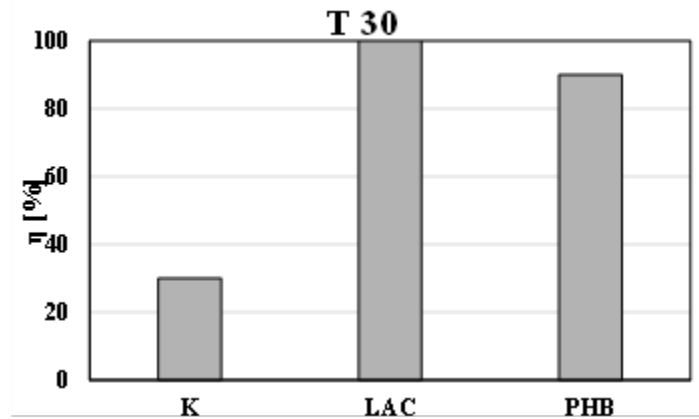


**Figure 3.** Monitoring of anaerobic conditions in lac microcosms using the redox potential indicator resazurin. (A) Microcosm at T0, (B) at T7, and (C) at T10.

The pink coloration observed at T0 indicates the reduction of resazurin (blue/purple) to resorufin (pink), demonstrating the establishment of anoxic conditions during microcosm setup inside the glove box. During incubation, a progressive decrease in redox potential was observed, evidenced by the increasingly transparent appearance of the microcosm, resulting from the further reduction of resorufin to dihydroresorufin. The state shown in Figure 3C persisted throughout the entire incubation period (45 days), demonstrating that anaerobiosis was not only established from the beginning but also maintained over time within the vials where the microcosms were prepared.

Time-course monitoring of 1,2-DCA concentrations in the microcosms, performed by HS-GC/MS, provided conclusive evidence of contaminant degradation. Unlike the observations reported by Cruciata (2023), where excessive variability rendered comparisons between results over different incubation periods and among conditions and controls unreliable, a clear trend of 1,2-DCA removal was derived for microcosms biostimulated with lactate and PHB compared to the abiotic control (K).

Specifically, microcosms biostimulated with either lactate or PHB exhibited 1,2-DCA degradation, with complete removal in lac microcosms and approximately 90% removal in PHB microcosms after 30 days of incubation (Figure 4). Therefore, both LAC and PHB microcosms were used as inoculum for the pilot-scale column test. In particular, a mixed culture derived from these microcosms was added at 10% of the total groundwater volume (present in the Plexiglas column simulating the aquifer).



**Figure 4.** Percentage removal of 1,2-DCA in lac and PHB microcosms and in abiotic controls (K-) after 30 days of incubation.

These results are consistent with literature; in particular, several studies (Aulenta et al., 2005; Munro et al., 2017) indicate that the abundance of the *dhlA* gene, found in hydrolytic DCA degraders, is positively correlated with DCA concentration and is unexpectedly higher under low-oxygen conditions.

Specifically, referring to the current results, the greatest decrease in DCA was observed in the lac and PHB microcosms, consistent with previous findings (Jugder et al., 2016; Türkowsky et al., 2018; Hug et al., 2013) that anaerobic DCA enrichments can dechlorinate a wide range of aliphatic and aromatic substrates, although no clear correlation has been found between phylogenetic affiliation and substrate specificity.

Furthermore, the results confirm previous studies by Aulenta et al. (2005) and Munro et al. (2017), which highlighted that chlorinated hydrocarbons (CHCs) may also act as electron acceptors, and that the abundance of the *dhlA* gene in hydrolytic DCA degraders is positively correlated with DCA concentration.

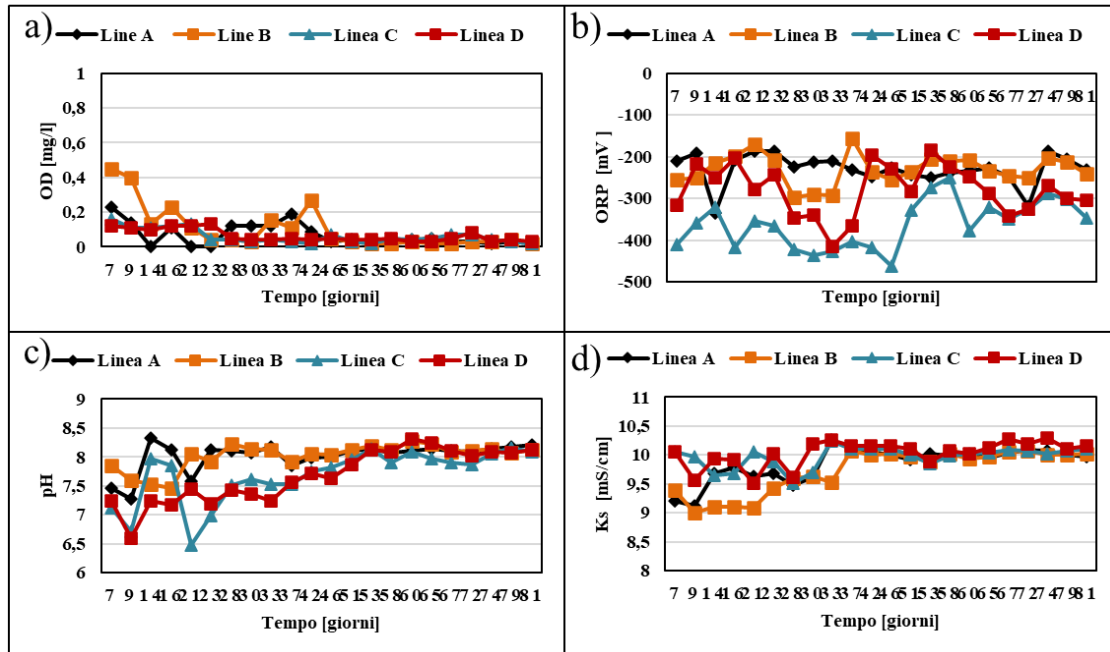
### 7.3.1.2 Results of continuous column study

#### Physico-Chemical Parameters

Figure 5 summarizes the trends of DO, ORP, pH, and conductivity across all four lines:

- **DO:** consistently near zero, confirming stable anaerobic conditions
- **ORP:** negative values throughout (up to -462 mV), supporting reducing conditions
- **pH:** increasing in lines C and D, likely due to:
  - PHB hydrolysis → acetate production (pH decrease)

- Microbial acetate consumption → alkalinity increase; the net effect suggests faster acetate consumption than production
- **Conductivity:** stable between 9–10 mS/cm in all lines



**Figure 5.** trend profile of Dissolved Oxygen (a), ORP (b), pH (c) and electric conductivity in the four lines

### PHB Fermentation and Acetate Production

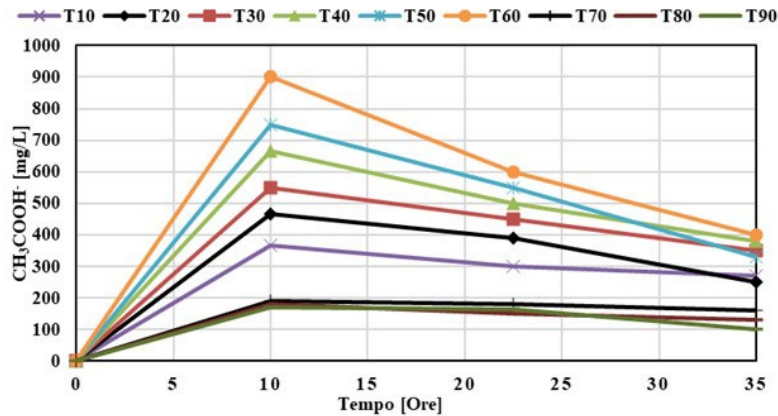
During the entire experimental period, nine sampling events were conducted to monitor acetate concentrations.

Specifically, the measured acetate concentrations showed that, in lines C and D, the two forms of PHB used exhibited different behaviors in terms of fermentative capacity. These differences can likely be attributed to the distinct particle sizes of the materials (scaffolds versus powder/granules), which result in different specific surface areas available for microbial fermentation of the substrate and, consequently, different kinetic patterns.

In line C (Figure 6), it can be observed that during the first 30 days the overall rate of acetate production was higher than the consumption rate, indicating a greater fermentative activity compared to the microbial consumption of acetate. On day 30 of the experiment (T30), acetate production reached approximately 400 mg/L.

Subsequently, from T30 onward, acetate production continued to increase, reaching its peak at day 60 (T60) with a concentration of 647 mg/L, accompanied by a corresponding increase in the consumption rate. This demonstrates that the microbial populations exhibited higher acetate uptake. From day 60 (T60) until day 90 (T90), a significant decrease in acetate production was observed, with the lowest concentration (70 mg/L) recorded at T90. This reduction was accompanied by a decrease in fermentation kinetics, which also led to stabilization of pH values. Indeed, based on the results discussed above, a strong interconnection between pH, acetate consumption, and microbial activity clearly emerges.

Thus, in line C, an initial increase in acetate concentration was followed by a decline, suggesting a high microbial activity rate within the aquifer. Notably, De Marines et al. (2023), in a study aimed at evaluating 1,2-DCA removal under anaerobic conditions using powdered PHB, observed a significant increase in acetate production by day 90, reaching a maximum concentration close to 267 mg/L. This value is considerably lower than the maximum obtained in the present study, likely due to the greater heterogeneity in PHB particle size, which resulted in a larger available specific surface area.

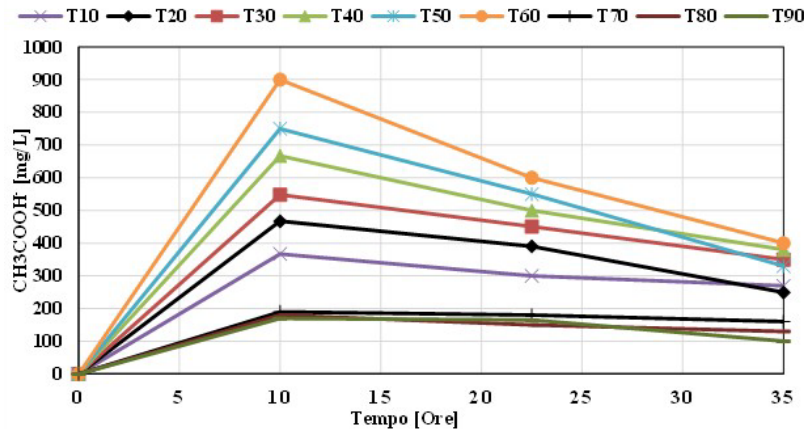


**Figure 6. Acetate concentration trend in line C**

In line D (Figure 7), the acetate production rate was consistently higher than that observed in line C throughout the experiment. This behavior is likely related to two main factors:

- the different PHB granulometries in the two lines, with scaffolds exhibiting a higher specific surface area than powder/granules;
- the composition of the scaffolds, which consist of 10% PHB and 90% PLA and PCL—two highly biodegradable and therefore easily fermentable polymers.

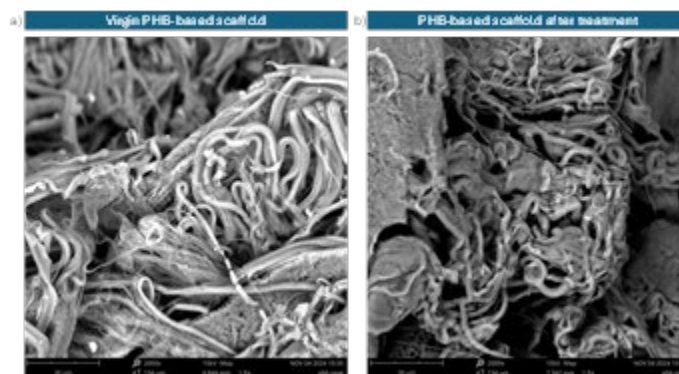
Similarly to line C, in line D the acetate production rate exceeded the consumption rate during the first 30 days. From T30 to T60, acetate production increased continuously, reaching its maximum peak at T60 (900 mg/L), again accompanied by an increase in the microbial acetate consumption rate. From T60 to T90, a marked decrease in acetate production occurred, with the lowest concentration (169 mg/L) recorded at T90, as fermentation kinetics declined and pH values stabilized.



**Figure 7. Acetate concentration trend in line D**

The results obtained are in good agreement with recent studies, which demonstrated that the use of an electron donor significantly enhances the dechlorination process compared to biotic and/or abiotic controls (Dell'Armi et al., 2021; Rossi et al., 2021; Zeppilli et al., 2021; Amanat et al., 2022). As shown by Baric et al. (2012), the high specific surface area of PHB may explain its rapid degradation; in their study, the authors observed a high initial production of VFAs followed by a marked decrease after two months of experimentation. Similarly, as reported by Amanat et al. (2022), Valentino et al. (2019), and Abbruzzese et al. (2024), even PHA produced by MMC ensured continuous acid production over extended periods, representing an efficient and sustainable electron-donor source for bioremediation processes.

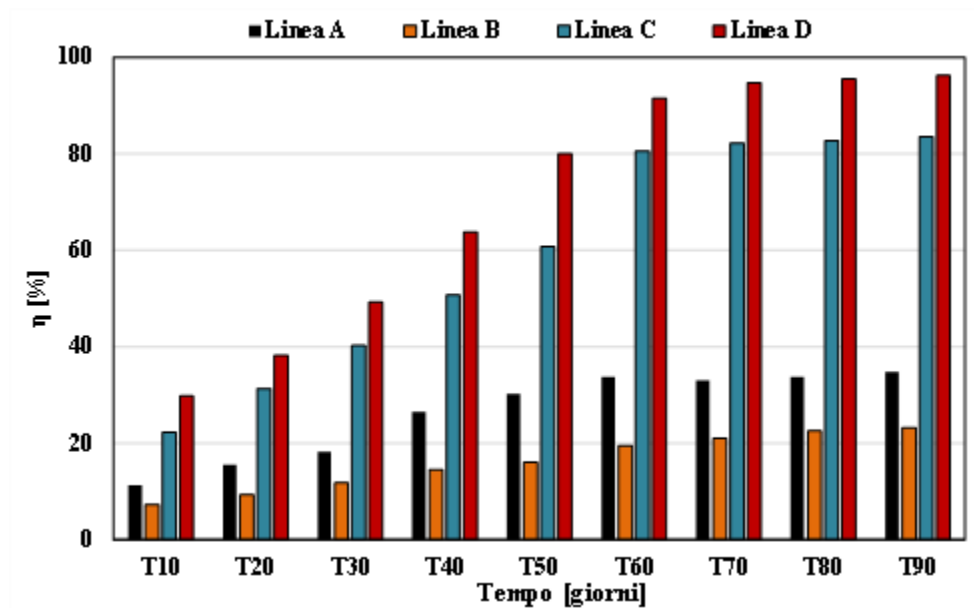
This study is innovative and original, as it is the first to evaluate the potential use of PHB embedded within scaffolds (line D) as a slow-release electron donor for in situ bioremediation. From a morphological perspective, a noticeable swelling of the fibers and partial cohesion among them was observed at the end of the treatment (Figure 8). These changes are likely linked to fermentative processes that led to progressive fiber degradation.



**Figure 8. Morphology of scaffolds before (a) and after (b) the experiment**

### 1,2-DCA Removal Efficiency

In Figure 9, the DCA removal efficiencies in the columns during the experimental period are presented:



**Figure 9.** 1,2-DCA removal efficiencies in the different lines recorded throughout the experimental period

It is important to note that the graph shows comparisons among the four removal efficiencies of the four lines over the entire experimental period. The x-axis represents the days on which sampling was conducted. As shown in Figure 9, at the end of the experiment (T90), DCA removal efficiencies of 33%, 22%, 83%, and 96% were observed in lines A, B, C, and D, respectively. These results indicate that in the presence of PHB fermentation products, the microbial consortium was able to significantly degrade 1,2-DCA.

The natural attenuation phenomena in line B were consistent with expectations, as the removal efficiencies were both low and comparable to each other relative to the other configurations. Regarding line A, by the end of the experiment, a removal efficiency 10% higher than that obtained under natural attenuation conditions was achieved, demonstrating that the combination of biostimulation and bioaugmentation was suitable for groundwater contaminated with chlorinated solvents.

Lines C and D achieved, at the end of the experiment, removal efficiencies that were approximately three and four times higher, respectively, than those observed under simulated natural attenuation conditions. From Figure 9, it is evident that in lines C and D, from T60 onward, DCA removal efficiencies reached approximately 80% and 90%, respectively; these values increased slightly from T60 to T90, ultimately reaching the final removal efficiencies of 83% for line C and 96% for line D.

Specifically, the observations from T60 to T90 differ markedly from those between T10 and T60. During the first two months of the experiment, substantial DCA removal was recorded. These results are closely related to the simultaneous acetate production and consumption occurring in the two lines. In line C, the maximum acetate production peak occurred at T60. From T60 to T90, a significant reduction in fermentation kinetics, decreased acetate production, and consequently lower microbial activity were observed.

Similarly, in line D, acetate consumption kinetics increased up to the maximum acetate production peak at T60. From T60 until the end of the experiment, acetate production decreased significantly, along with a

reduction in fermentation kinetics. This effect may be attributed to the biodegradation of PHB in the form of scaffolds within the column.

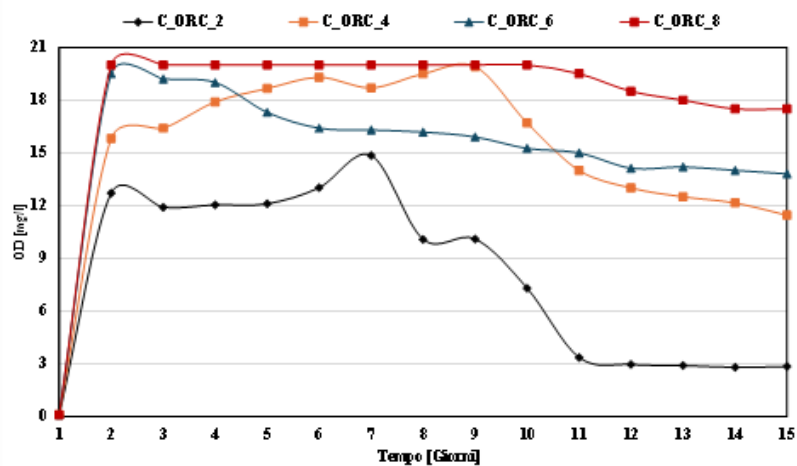
During the experiment, as reported in the literature by Amanat et al. (2022), when the VFA concentration decreased, dechlorination ceased. This likely occurred from T60 onward. Unlike the study by Amanat et al. (2022), the slow-release electron donor source remained constant; thus, no alternative acetate production was provided, and consequently, the concentration of dechlorination products did not decrease rapidly when PHB fermentation drastically slowed. These results indicate that both PHB in mixed form and within scaffolds provided sustained high acetate production over an extended period, representing an effective and sustainable electron donor source for the biological remediation process.

Although, as discussed previously, VFA concentrations decreased from T60 onward, their production remained sufficient to stably sustain the BRD process.

## 7.3.2 AEROBIC PERIOD

### 7.3.2.1 Assessment of PermeOx® Ultra Effectiveness in Terms of Oxygen Release

As previously discussed, during the preliminary column test, both dissolved oxygen (DO) concentration and pH were monitored. Figure 10 shows the temporal trend of DO concentration for the four lines CORC2, CORC4, CORC6, and CORC8.



**Figure 10. Temporal trend of DO concentration in the lines during the preliminary test.**

From the graph reported in Figure 10, it can be observed that lines CORC2, CORC4, CORC6, and CORC8 exhibited an immediate oxygen release due to the presence of ORC, reaching initial maximum effluent concentrations of 12.7 mg/L, 15.8 mg/L, 19.5 mg/L, and 20 mg/L, respectively. At the end of the 15-day test period, measured concentrations in lines CORC2, CORC4, CORC6, and CORC8 were 2.85 mg/L, 11.45 mg/L, 13.8 mg/L, and 17.5 mg/L, respectively. Thus, DO increased significantly in all four lines. Furthermore, the results confirmed the expected correlation between the mass of ORC and the oxygen concentration in the system.

Since the objective of this experimental phase was to evaluate the biodegradation capacity of the inoculated aerobic bacteria, the availability of dissolved oxygen represented a necessary condition for metabolism, while simultaneously preventing the growth of strictly anaerobic bacterial species.

In conclusion, based on the results obtained, in order to ensure sufficient oxygen availability to avoid oxygen-limiting conditions, while at the same time minimizing the risk of excessive pH increases and maintaining DO concentrations above 2 mg/L, a dose of 2 g of ORC was selected (CORC2).

### 7.3.2.2 1,2-DCA Removal Efficiency in continuous column test

The graph shown in Figure 11 presents the comparisons among the four removal efficiencies observed in the four lines over the entire experimental period.

In lines A, B, C, and D, analysis of the results at the end of the experiment (T60) revealed 1,2-DCA removal efficiencies of 52.9%, 64.3%, 99.9%, and 91.5%, respectively. The physicochemical and natural attenuation phenomena observed in lines A and B were consistent with expectations, as the removal efficiencies were lower compared to the other setups. For lines C and D, the results obtained at the end of the experiment indicated that the inoculated aerobic microbial consortium was capable of significantly degrading 1,2-DCA.

Temporal analysis of the 1,2-DCA removal efficiencies showed that, as early as T10, high contaminant removal rates had already been achieved in lines A, B, C, and D, with values of 41.8%, 56.7%, 88.1%, and 84.9%, respectively. Specifically, in lines A, B, and C, most of the contaminant was removed at the beginning of the experiment; from T20 to T60, the overall removal efficiency increased by 11%, 7.6%, and 11.8%, respectively. These results confirm that an immediate oxygen release occurred in these three lines due to the presence of ORC, and that calcium peroxide oxidized the 1,2-DCA present in the groundwater.

In line D, a similar trend was observed; from T20 to T60, the removal efficiency increased by approximately 6.6%. However, in this case, only enhanced aerobic biodegradation phenomena occurred, as no ORC was present in the column.

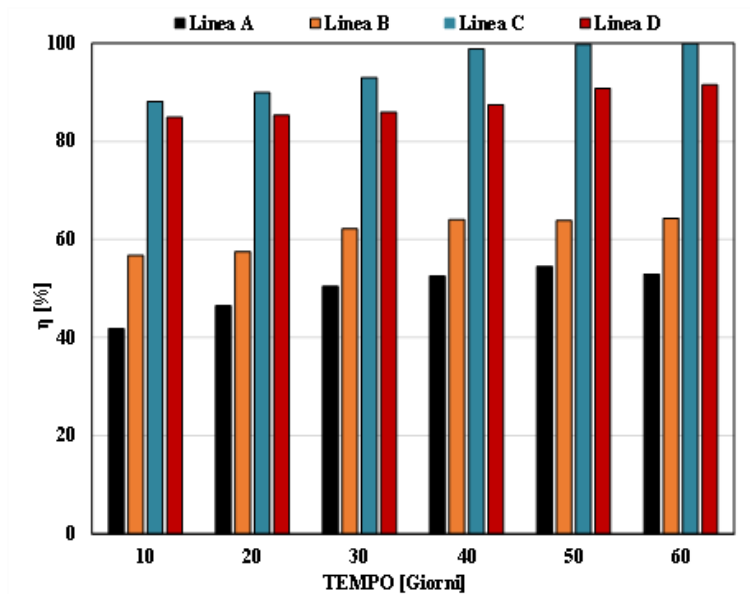


Figure 11. 1,2-DCA removal efficiencies in lines A, B, C, and D during the experimental period.

## 7.4 Conclusions

The experimental results indicate that anaerobic biodegradation supported by PHB is an effective and sustainable approach for removing 1,2-DCA from contaminated groundwater. The use of biodegradable PHB scaffolds aligns with circular economy principles and offers operational advantages for long-term in-situ remediation. The findings support further development and potential field application of PHB-based biological permeable reactive barriers for the treatment of chlorinated-solvent plumes.

Referring to aerobic conditions, at the end of experiments the results indicated significant degradation of 1,2-DCA across all columns. Notably, column C exhibited the highest process performance and removal efficiency, owing to the combined presence of the active bacterial inoculum and the ORC compound. These results suggest that a synergistic effect of enhanced aerobic biodegradation and chemical oxidation contributed to a 1,2-DCA removal efficiency of 99.9%. Such exceptionally high removal rates ensured compliance with the concentration limit established by Legislative Decree 152/06 (CSC = 3 µg/L), thereby confirming the effectiveness of the treatment.

Overall, the study highlights the potential of biological PRBs—supplemented with PHB under anaerobic conditions and ORC under aerobic conditions—as viable, sustainable, and cost-effective technologies for the remediation of groundwater contaminated with 1,2-DCA, particularly at complex contaminated sites where DNAPL sources hinder the performance of conventional treatments.

## 8 Bioelectrochemical systems (BES) for the remediation of groundwater co-contaminated by trichloroethylene (TCE) and hexavalent chromium (Cr VI)

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### 8.1 Introduction

The research was divided into two main lines. The first concerned the application of bioelectrochemical systems (BES) for the remediation of groundwater co-contaminated by trichloroethylene (TCE) and hexavalent chromium (Cr VI). The second explored the integration of BES with more conventional remediation technologies, in particular adsorption on carbonaceous materials, in order to increase contact time, contaminant removal efficiency and overall robustness of the system. Column reactors were employed to investigate removal capacities, transport processes, electron transfer and microbial activities under controlled electrochemical conditions. The energy requirements, efficiency and scalability of the systems were also assessed.

### 8.2 Context and state of the art

Groundwater contamination by chlorinated aliphatic hydrocarbons (CAHs) and heavy metals, such as Cr(VI), still represents a critical environmental issue due to their persistence, toxicity and frequent co-presence. Traditional remediation methodologies, such as pump & treat, adsorption and chemical reduction, show limitations in terms of efficiency, selectivity and sustainability in the long term. Bioelectrochemical systems (BES) have established themselves in recent years as a promising approach, capable of stimulating reductive microbial pathways through targeted control of electron supply. Recent studies have highlighted their application potential for the simultaneous removal of CAHs and heavy metals, while the challenges related to integration with established technologies and their applicability on a real scale remain open. Instead, the combination of BES with adsorbent materials, such as biochar or activated carbon, has attracted increasing interest for their ability to improve mass transfer, smooth out fluctuations, and broaden applicability to complex mixtures of contaminants.

### 8.3 Objectives

The project aimed to develop and optimise bioelectrochemical systems (BES) for complex groundwater remediation scenarios. The specific objectives were:

1. To study the simultaneous removal of TCE and Cr(VI), with particular attention to the effects of chromium on the dechlorinating activity of microorganisms and to any inhibitory or competitive interactions.
2. To evaluate the synergistic combination of BES with carbon-based adsorbent materials to improve the removal capacity, stability and durability of the system.
3. Characterize the electrochemical and microbiological removal processes in column reactors operated under different conditions.
4. Analyze energy consumption and efficiency, robustness to contaminant fluctuations, and system scalability.

## 8.4 Results

For the research on co-contaminations, a tubular microbial electrolysis cell (MEC) without membrane was studied, consisting of a granular graphite working electrode and an internal counterelectrode also made of graphite granules. The MEC was fed continuously, for a total of 250 days, with a solution contaminated by TCE and Cr (VI) and kept in galvanostatic polarization with the cathode as the working electrode. The system was evaluated under different operating conditions, varying the applied current (in electroplating mode) and the hydraulic residence times (HRT).

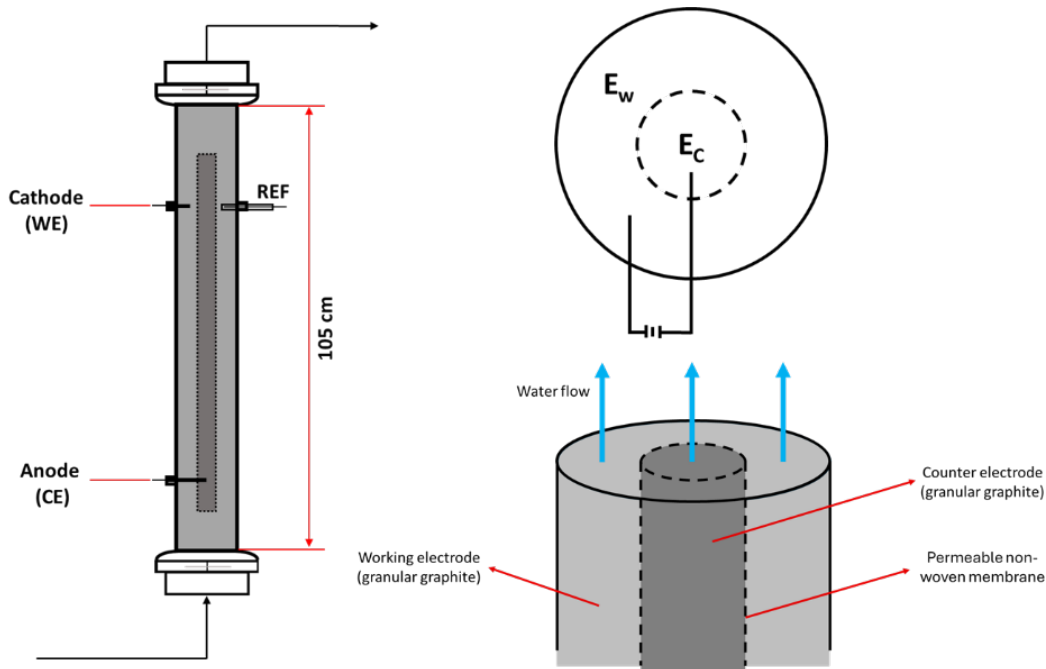


Figura 1 Schema del reattore utilizzato

The system demonstrated high resistance to the presence of Cr(VI), consistently achieving near-complete (>99%) TCE removal and total (100%) Cr(VI) reduction under all explored conditions. It has been observed that the distribution of dechlorination by-products varies with varying operating conditions (Figure 2), highlighting the dynamic response of the microbial community to process parameters and to the introduction of Cr(VI) into the feed. Despite an initial inhibitory effect of Cr(VI) on dechlorinating bacteria, the system showed a strong adaptability, with a recovery of dechlorination efficiency over time. These results highlight the potential of bioelectrochemical technologies as a resilient and adaptable solution for the simultaneous bioremediation of chlorinated solvents and heavy metals in contaminated groundwater.

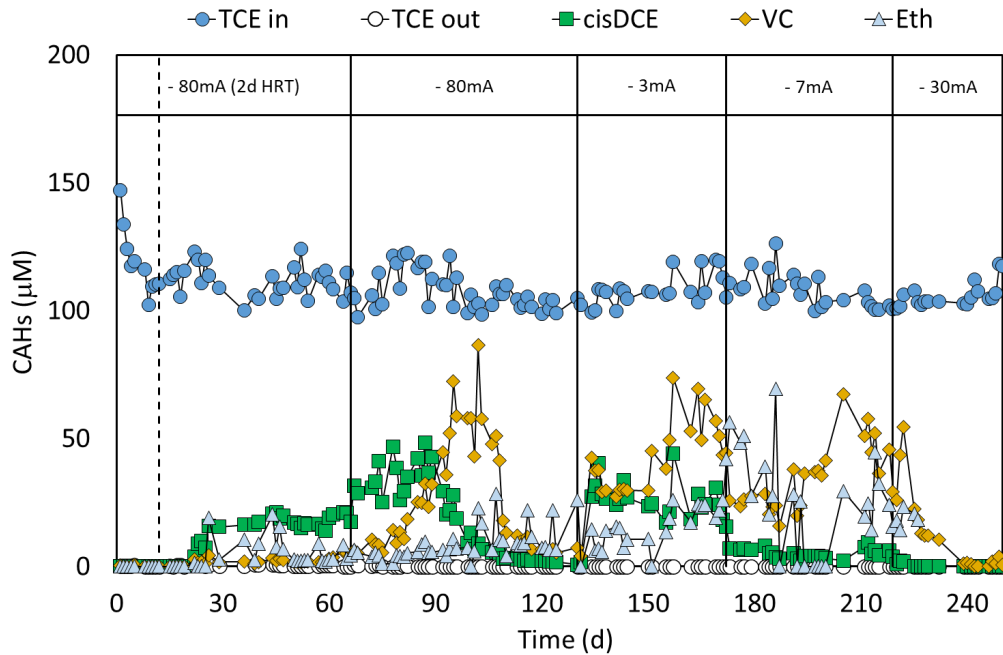


Figura 2 Andamento delle concentrazioni del TCE e dei sottoprodotti

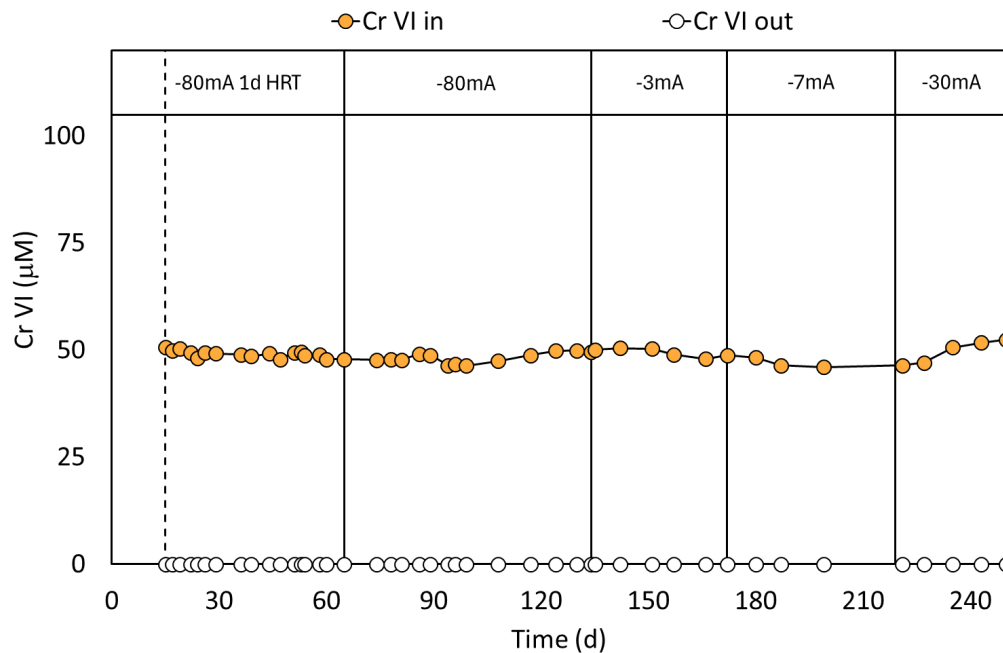


Figura 3 Andamento della rimozione del Cr VI

In parallel, three column reactors were tested to evaluate the combination of BES and carbonaceous adsorbent materials. The experimental apparatus included three glass columns of 380 mL each (Figure 4): a control BES filled only with sand (0.4–0.8 mm), a second control consisting of sand and biochar (4% w/w, < 0.5mm) and finally the combined column filled with sand and biochar in a polarized BES. Biochar was produced from pine wood by pyrolysis at about 1040 °C.

The BES adopted provides a concentric configuration with three electrodes, with a central anode in graphite bar wrapped in graphite felt and enclosed in a filter tube to avoid short circuits with the working electrode, and an external cathode also in graphite felt (Figure 5). The system did not involve the use of ion exchange membranes. All columns were inoculated with a dechlorinating microbial culture and subsequently characterized from a hydraulic point of view.

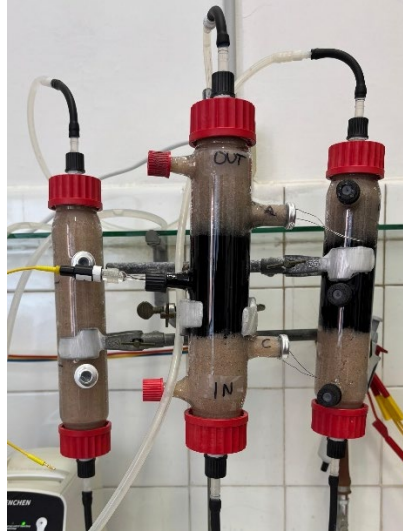


Figura 4 Foto delle tre colonne sviluppate

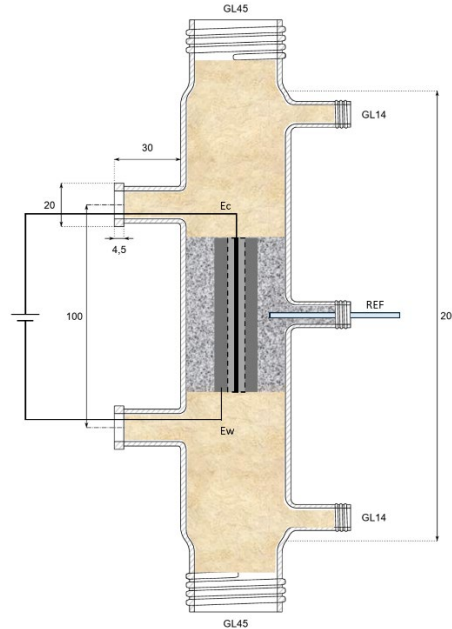
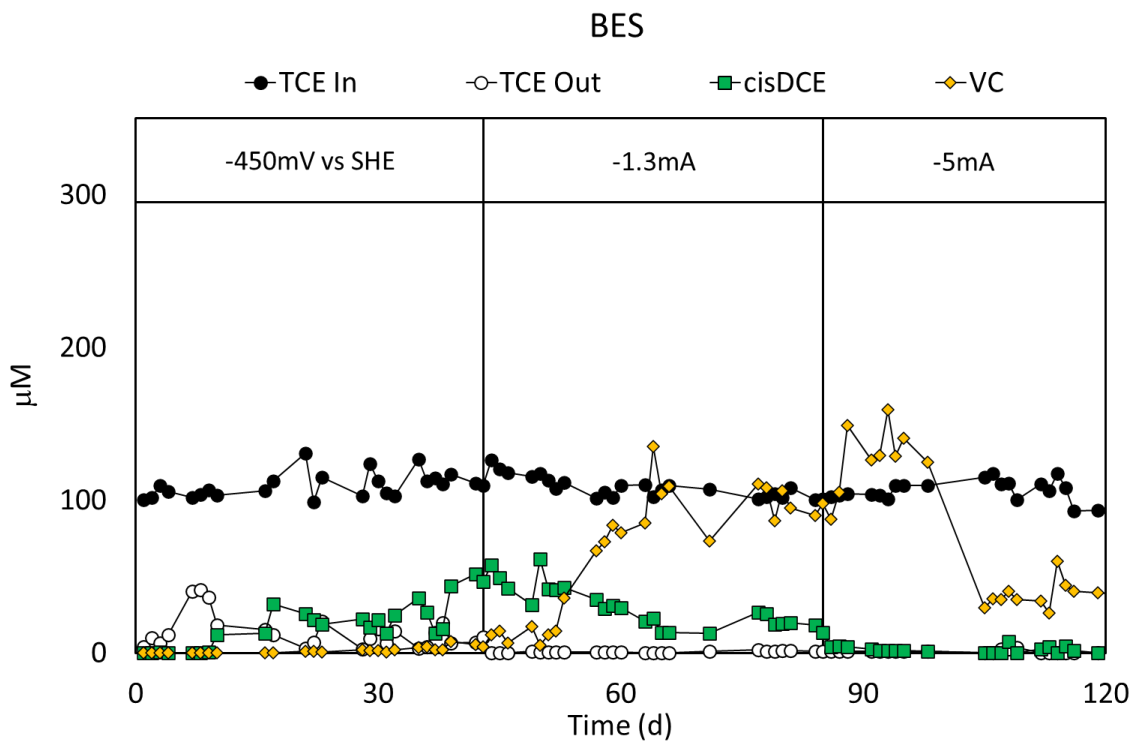
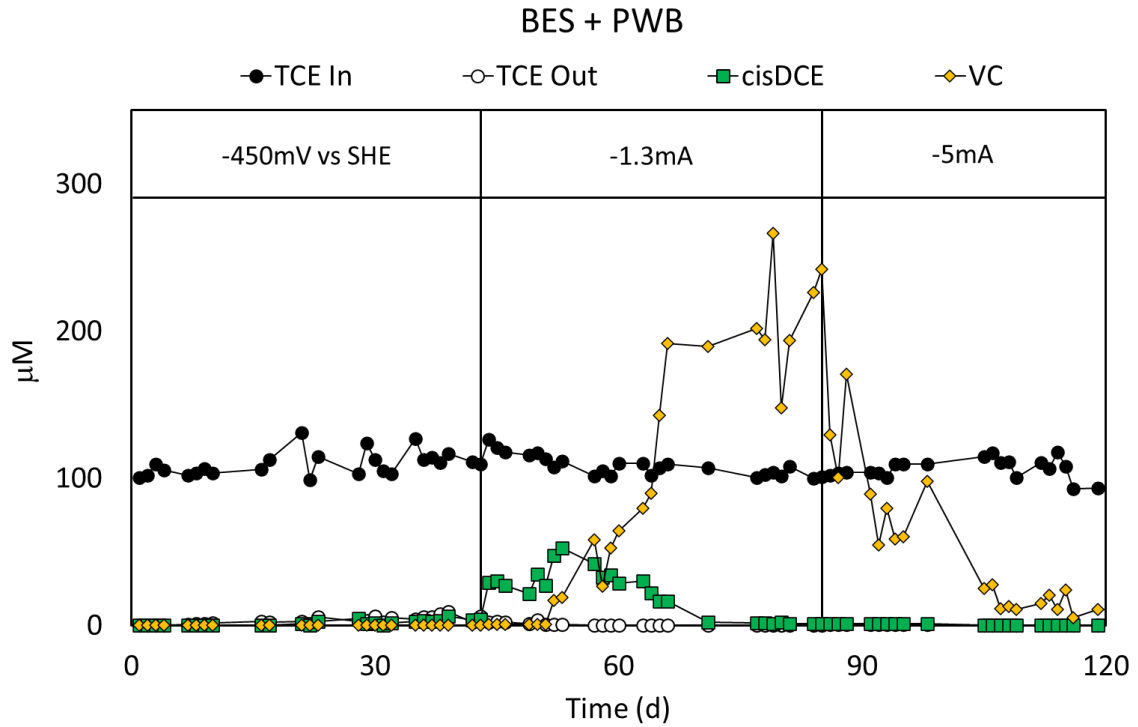


Figura 5 Schema della colonna combinata

The comparison between the three systems, one containing only BES, one containing only biochar and one combined BES-biochar, showed that the combined system achieved superior performance compared to the individual technologies, confirming a clear synergistic effect. The bioelectrochemical activity allowed the degradation of contaminants adsorbed on the biochar, regenerating the active adsorption sites and prolonging the efficiency of the material. At the same time, the presence of biochar increased the contact time between microorganisms and contaminants, resulting in lower output concentrations. These results confirmed the

feasibility and effectiveness of the integration between BES and conventional technologies, paving the way for new design criteria for remediation strategies applicable to complex groundwater contamination scenarios.



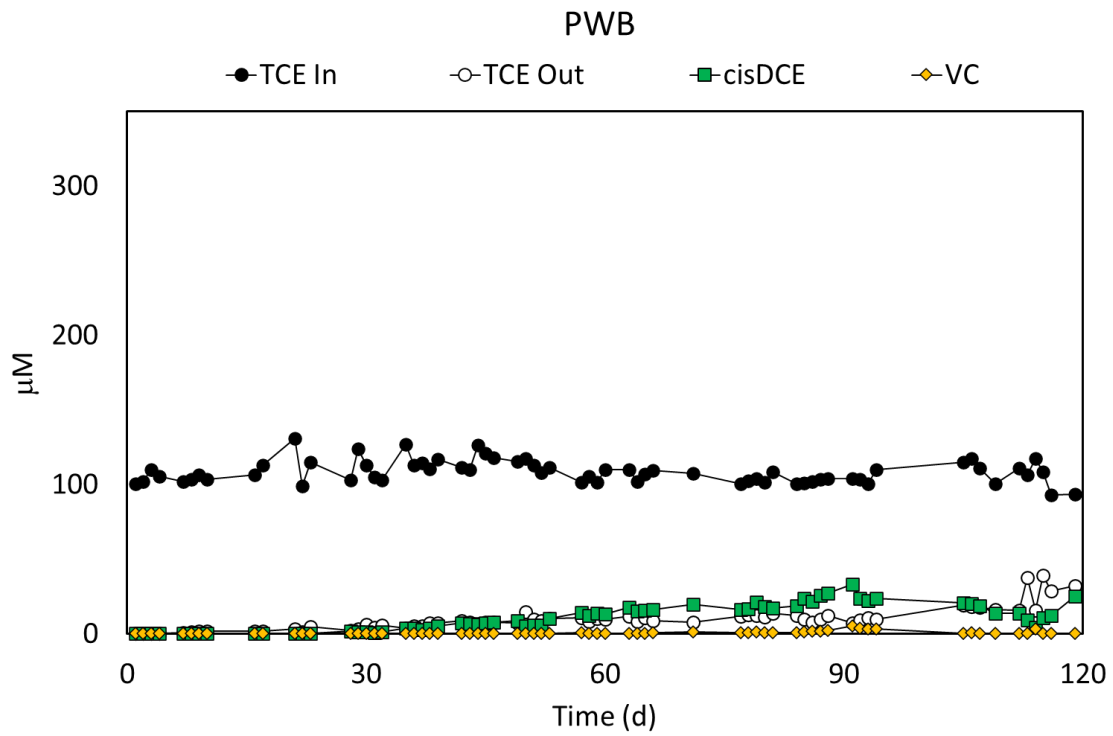


Figura 6 Andamento delle concentrazioni del TCE e dei sottoprodotti nelle tre colonne

## 9 Multidisciplinary Assessment of Soil Bioremediation by Indigenous Hydrocarbon-Degrading Bacteria: Chemical, Microbial, and Ecotoxicological insights

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The main objective of the activity carried out by **ENEA group in Task 4.5.2** was to **boost the natural bioremediation potential** in a hydrocarbon-contaminated soil by applying a **native microbial consortium**, validated in earlier bioaugmentation experiments, through the addition of a **biosurfactant** designed to enhance hydrocarbon bioavailability and microbial activity and to perform an ecotoxicological evaluation to assess the environmental safety and effectiveness of the bioremediation approach.

### 9.1 Activities

#### 9.1.1 Microbial Consortium: Screening of Biosurfactant Production and Hydrocarbon-Degrading Abilities

- **Revitalization** of eight bacterial strains composing the consortium, previously isolated from the same contaminated soil and stored at  $-80\text{ °C}$  in the EMCC (ENEA Microbial Culture Collection).
- **Evaluation of biosurfactant production potential** of the eight strains using E24, drop collapse and CTAB-blue assays.
- **Assessment of hydrocarbon-degrading ability** of the eight strains by growth and degradation tests on minimal agar medium containing petroleum as the sole carbon source.

#### 9.1.2 Biosurfactant Production and Characterization

- **Selection** of the best-performing EMCC strain showing the highest biosurfactant production capacity and hydrocarbon degradation potential (based on the aforementioned tests), followed by the **optimization of the experimental conditions** for biosurfactant biosynthesis to be used in the subsequent trials.
- **Compatibility assessment** between bacterial consortium strains and the selected biosurfactant-producing strain to ensure synergistic activity.
- **Optimization of production parameters and evaluation of the biosurfactant's physicochemical properties**, including surface tension reduction and emulsifying activity, assessed through oil-spreading, drop-collapse, and emulsification index tests.

#### 9.1.3 Hydrocarbon Degradation trials

- **In-Batch degradation trials** on hydrocarbon-contaminated soil were conducted with periodic **microbiological, chemical, and ecotoxicological analyses** performed at 15, 45, 80, and 120 days from the start of the experiment, with the following experimental conditions:
  1. **Abiotic control:** sterilized soil ( $\text{HgCl}_2$ ).

2. **Biotic control:** soil without bioaugmentation.
3. **Biosurfactant control:** soil without bioaugmentation, with biosurfactant addition.
4. **Treatment:** soil with bioaugmentation combined with biosurfactant.

#### 9.1.4 Ecotoxicological evaluation

- **Ecotoxicological tests** were carried out to evaluate the potential reduction of soil toxicity during the bioremediation process and to verify the environmental safety of the treatments. Analyses were performed at 15, 45, 80, and 120 days in parallel with chemical and microbiological monitoring.

The following bioassays were conducted on soil eluates:

- ✓ **Bacteria:** *Vibrio fischeri* (Microtox® test) for acute toxicity evaluation through inhibition of bioluminescence.
- ✓ **Crustaceans:** *Daphnia magna* immobilization test to assess acute effects on aquatic invertebrates.
- ✓ **Algae:** *Raphidocelis subcapitata* growth inhibition test to determine effects on primary producers.

## 9.2 RESULTS

### 9.2.1 MICROBIAL CONSORTIUM: Screening of biosurfactant-producing and hydrocarbon-degrading bacteria used for bioaugmentation

#### 9.2.1.1 Biosurfactant Production

The eight strains of the **microbial consortium** were identified as biosurfactant-producing bacteria using the emulsification activity assay (E24) and CTAB agar plate method (Table 1 and Fig.1).

*Brevibacterium aurantiacum* RET4 and *Paenibacillus mobilis* R1 showed the highest biosurfactant activities based in both tests.

Name	Strain Id	E24%	CTAB Agar Assay
R1	<i>Paenibacillus mobilis</i>	38,5%	+++
R3	<i>Pseudoxanthomonas beigongshangi</i>	19,2%	+
R4	<i>Cellulosimicrobium funkei</i>	23,1%	+
R13	<i>Sphingopyxis macrogoltabida</i>	11,5%	+
R24	<i>Lysobacter soli</i>	12%	++
R25	<i>Niallia circulans</i>	16,7%	++
RET2	<i>Micrococcus aloeverae</i>	15,3	-
RET4	<i>Brevibacterium aurantiacum</i>	46,7%	+++

Table 1. Results of biosurfactant production assays by indigenous isolates

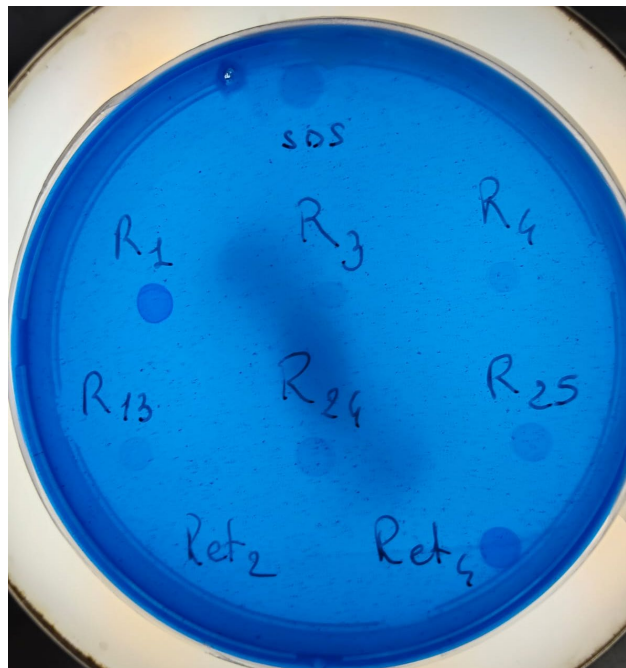


Fig. 1 Blue dark color indicating anionic surfactant secretion by the 8 strains

### 9.2.1.2 Hydrocarbon-degrading ability

All eight strains of the consortium grew on minimal agar medium containing oil; some showed intracellular accumulation zones of hydrocarbons, while others formed dissolution halos around the cells (Fig. 2)

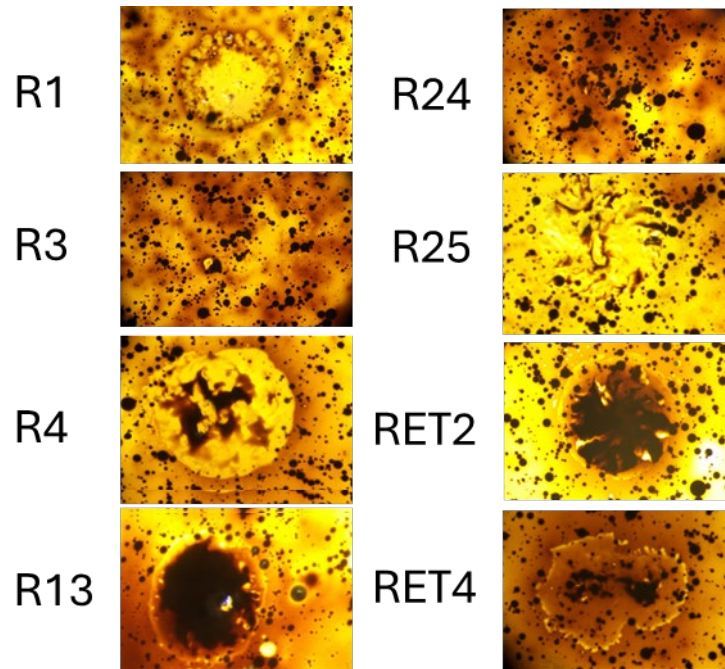


Fig. 2. Colonies of the eight indigenous strains grown on solid medium plate supplemented with oil.

## 9.2.2 *Rhodococcus qingshengii* OSS19b: BIOSURFACTANT PRODUCTION

### 9.2.2.1 Selection of the best biosurfactant producer from EMCC

The EMCC strain OSS19b, identified as *Rhodococcus qingshengii*, exhibited the highest biosurfactant production (E24 values around 60%) and stability, and the best hydrocarbon degradation activity, even compared to the native bacteria used for the bioaugmentation consortium (Fig. 3)

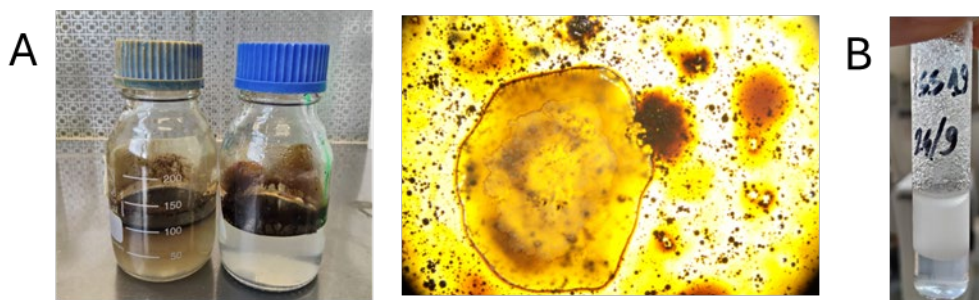


Fig. 3. A) Growth and hydrocarbons-degradation on solid and liquid media with oil. B) Emulsification index test of OSS19b culture

### 9.2.2.2 Cross-streak test between co-inoculated strains

Cross-streak test on agar plate was performed to assess the compatibility between the biosurfactant-producer strain OSS19b and the strains composing the bioaugmentation consortium. No inhibition zones were observed (Fig.4). The co-existence of strains was essential for formulating bioinoculant used in the batch experiment setup.

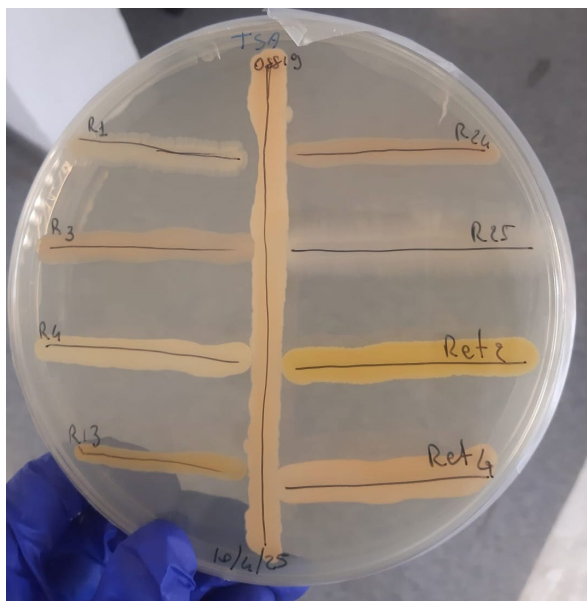


Fig. 4. Cross-streak assay showing no inhibition zones among strains.

### 9.2.2.3 Optimization of production parameters and evaluation of the biosurfactant's physicochemical properties

*Rhodococcus qingshengii* OSS19b optimal growth conditions were achieved in a minimal medium containing 0.2% sodium gluconate as the sole carbon source, at 28°C under incubation in a rotating shaker (110 rpm). A scale-up process was carried out to obtain the amount of biosurfactant required for the following experimental phase. The whole culture broth was used directly, without any extraction or purification steps. When production was scaled up to larger volumes (1 L) maximum biosurfactant production was reached on the fifth day of cultivation, showing a decrease in the E24 value compared to that recorded in the initial small-scale experiments (Table 2). Since the emulsifying activity of *Rhodococcus* is mainly associated with the cellular fraction (Zheng et al., 2009), centrifugation markedly decreased the emulsifying activity, making the resulting cell-free supernatant unsuitable for use (29.6% E24). The culture broth was then sonicated to induce a partial cell lysis yielding an increase in E24 value (44.4%); a further step of centrifugation was performed to reduce the number of bacterial cells ( $2 \cdot 10^7$  CFU/ml).

Production scale-up: biosurfactant treatments	E24 %
Crude biosurfactant (day 5 of cultivation)	<b>41.4%</b>
After first centrifugation	29.6%
After sonication (no centrifugation)	<b>44.4%</b>
After sonication + centrifugation	33.3%

Table 2. E24 value of the biosurfactant in a 1 L volume after the different treatments

### 9.2.3 Hydrocarbon Degradation Trials

The monitoring of the microbial load during the 120-day trial shows values ranging from  $10^{12}$  to  $10^8$  CFU/ml in the different treatments, indicating the viability of the native and bioaugmented bacteria. Although the chemical analyses are still underway, initial data indicate promising outcomes regarding the use of biosurfactants in enhancing the biodegradation of petroleum hydrocarbons in contaminated soils. Specifically, the addition of biosurfactant appears to significantly accelerate the breakdown of Total Petroleum Hydrocarbons (TPHs), with a notable impact on the heaviest and most recalcitrant fractions, such as hopanes. These compounds, typically resistant to microbial degradation due to their complex structure and low solubility, were removed from the soil by up to 90%, suggesting a substantial improvement in remediation efficiency.

### 9.2.4 Ecotoxicological evaluation

The ecotoxicological assessments conducted on the same samples show a reduction in toxicity towards *Chlorella*, a sensitive microalgal species commonly used as a bioindicator. This decrease in toxicity further supports the hypothesis that although biosurfactants enhance TPH solubility, they can contribute to the detoxification of the soil. Conversely, the *Daphnia magna* assays revealed relatively high toxicity levels in some treatments, particularly during the early stages of incubation, likely due to the transient increase in hydrocarbon bioavailability and intermediate degradation products. However, a progressive reduction in toxicity was observed over time, indicating the effectiveness of the bioaugmentation–biosurfactant strategy in mitigating long-term ecotoxic effects.

## 9.3 Conclusion

The experimental results confirmed the synergistic effectiveness of bioaugmentation, performed using an autochthonous microbial consortium in combination with a biosurfactant-producing strain, in enhancing hydrocarbon degradation in contaminated soils. The observed effects are likely attributable to the biosurfactant's ability to increase the bioavailability of hydrophobic contaminants, making them more accessible to microbial communities capable of degrading them. By reducing surface tension and emulsifying hydrocarbons, biosurfactants facilitate microbial uptake and boost the metabolic activity, thereby accelerating the overall biodegradation process.

The established methodological framework—including strain selection, compatibility assessment, biosurfactant optimization, and multiparametric testing—provides a solid foundation for the development of a standardized protocol for assessing the biological treatability of contaminated sites.

Such a protocol would enable the systematic evaluation of microbial performance, surfactant contribution, and treatment sustainability, thereby supporting decision-making in site-specific bioremediation strategies and feasibility studies.

## 10 General Conclusions and Recommendations for Bioremediation Implementation

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The remediation of contaminated soils, sediments, and groundwater remains a major environmental challenge in Europe. According to the European Environmental Agency, approximately 2.8 million potentially contaminated sites exist within the European Union, of which about 650,000 require remediation. In Italy alone, national inventories report dozens of contaminated Sites of National Interest (SIN) and tens of thousands of sites managed at the regional level under Legislative Decree 152/06. Despite the progress achieved in recent decades, remediation activities are still largely dominated by conventional approaches such as excavation and disposal for soils and pump-and-treat for groundwater.

However, increasing regulatory pressure, rising disposal costs, and the need for more sustainable remediation strategies are progressively shifting the focus toward in-situ and nature-based technologies, including bioremediation. Bioremediation offers significant advantages in terms of environmental sustainability, operational costs, and reduced disturbance of contaminated matrices. Nevertheless, its broader application requires improved knowledge of microbial processes, optimization of operational parameters, and the development of standardized approaches capable of supporting reliable field implementation.

Within the framework of the RETURN project, Task 4.5.2 aimed to contribute to this objective by investigating innovative and eco-friendly bioremediation strategies applicable to different contaminated matrices. The activities carried out by the participating research units addressed complementary aspects of microbial remediation processes, including groundwater remediation through biological permeable reactive barriers, sediment treatment through bioslurry technology, soil bioremediation enhanced by biosurfactants, and the application of emerging bioelectrochemical systems.

The experimental results obtained across these different research lines confirm the strong potential of biologically based technologies for the remediation of complex contaminated environments. In groundwater systems contaminated by chlorinated solvents, the use of biodegradable polymers such as polyhydroxybutyrate (PHB) proved particularly effective as a slow-release electron donor for sustaining reductive dechlorination processes. Laboratory-scale column experiments demonstrated that PHB-based materials can maintain long-term microbial activity, enabling high removal efficiencies for 1,2-DCA under anaerobic conditions. Under aerobic conditions, the addition of oxygen-release compounds further enhanced contaminant removal, highlighting the importance of controlling redox conditions in order to activate specific microbial degradation pathways.

For the treatment of contaminated marine sediments, bioslurry technology demonstrated promising performance in enhancing hydrocarbon degradation under controlled bioreactor conditions. The results showed that the modulation of microbial communities and the optimization of operational parameters can significantly improve the removal efficiency of petroleum hydrocarbons, although treatment duration and the management of desorption or rebound phenomena remain critical aspects to be addressed during process design.

In contaminated soils, the combined use of indigenous microbial consortia and biosurfactant-producing strains proved effective in increasing hydrocarbon bioavailability and accelerating degradation processes. The experimental results confirmed that biosurfactant-mediated bioaugmentation can significantly enhance biodegradation kinetics, particularly for recalcitrant hydrocarbon fractions, while also contributing to the progressive reduction of soil ecotoxicity.

Finally, the investigation of bioelectrochemical systems demonstrated the potential of electro-stimulated microbial processes for the simultaneous removal of chlorinated solvents and heavy metals from groundwater. The results highlighted the resilience and adaptability of electroactive microbial communities and showed that the integration of bioelectrochemical systems with adsorbent materials such as biochar can further improve contaminant removal efficiency and process stability.

Based on the experimental results obtained within Task 4.5.2 and on the current scientific and technological state of the art, several practical recommendations can be formulated to support the optimization and implementation of bioremediation processes for contaminated soils, sediments, and groundwater.

- **Perform a comprehensive site characterization before selecting the bioremediation strategy**

A detailed assessment of hydrogeochemical conditions, contaminant distribution, and microbial community structure is essential to determine whether intrinsic biodegradation processes are already active and to identify potential limitations. Advanced molecular techniques can support the identification of functional microbial populations and guide the design of appropriate remediation strategies.

- **Optimize redox conditions to promote specific biodegradation pathways**

The efficiency of microbial degradation processes strongly depends on the redox conditions of the system. The supply of suitable electron donors (e.g., biodegradable polymers such as PHB) or electron acceptors (e.g., oxygen-release compounds) should be carefully calibrated to stimulate the targeted microbial metabolic pathways, such as reductive dechlorination or aerobic oxidation.

- **Ensure long-term availability of substrates through slow-release materials**

The use of slow-release substrates represents a key factor for sustaining microbial activity over extended time periods. Materials such as biodegradable polymers, oxygen-release compounds, or engineered carriers can help maintain stable process conditions and reduce the need for repeated substrate injections.

- **Improve contaminant bioavailability when mass-transfer limitations occur**

In many contaminated soils and sediments, the bioavailability of hydrophobic pollutants represents the main limiting factor for biodegradation. The use of biosurfactants or other mobilizing agents can significantly enhance contaminant desorption and solubilization, thereby improving microbial access to the pollutants and accelerating degradation processes.

- **Consider integrated or hybrid remediation approaches for complex contamination scenarios**

In cases of mixed contamination or highly heterogeneous subsurface conditions, combining biological processes with complementary technologies may significantly improve remediation performance. Examples include the integration of bioelectrochemical systems with adsorption materials or the coupling of biological barriers with physical or chemical treatment methods.

- **Promote pilot-scale testing to support field-scale implementation**

Although laboratory-scale experiments provide valuable insights into microbial processes, pilot-scale studies are essential to evaluate process performance under realistic environmental

conditions. Pilot tests allow the assessment of system robustness, operational stability, and scalability prior to full-scale implementation.

- **Monitor microbial activity and environmental parameters during remediation**

Continuous monitoring of key parameters—such as redox potential, dissolved oxygen, pH, microbial community dynamics, and contaminant concentrations—is necessary to verify process performance and to adapt operational conditions when required.