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Research paper

Multi-evidence approach for the optimisation and management of bioremediation in an ex-situ plant

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ABSTRACT

Soil contamination from petroleum hydrocarbons is a widespread environmental problem requiring effective and sustainable remediation strategies. Biopile bioremediation is a widely used technology thanks to its operational adaptability and reduced environmental impact. However, conventional monitoring that is based solely on the quantification of hydrocarbons is often inadequate for reliably describing the evolution of the process due to the high heterogeneity of soils and the analytical limitations associated with measuring a composite parameter. This study applied a multi-evidence approach to evaluate the effectiveness of bioremediation treatments at a pilot scale plant, integrating chemical, respirometric, biomolecular and modelling evidence.

The results demonstrate that hydrocarbon analysis alone can lead to misleading interpretations, particularly in the early stages, whereas compositional indicators, such as the isomeric ratios of linear alkylbenzenes, enable more accurate assessment of biodegradation processes. Respirometric measurements provided direct information on microbial activity, enabling the efficiency of treatments to be assessed, and operational thresholds for oxygenation management to be defined. Meanwhile, biomolecular analyses highlighted the role of selected microbial communities and their functional consistency with pollutant degradation trends. Kinetic modelling of oxygen concentrations and consumption supported a quantitative interpretation of the observed processes, enabling an objective comparison of different treatment solutions. Overall, the multi-evidence approach proved to be an effective tool for overcoming the uncertainties associated with traditional monitoring and improving the ability to interpret processes.

1. Introduction

Soil contamination by petroleum hydrocarbons remains a major environmental concern globally, particularly in areas affected by industrial activities, accidental spills, and the mismanagement of oil-derived products. Petroleum hydrocarbons, including alkanes and aromatics, pose risks to ecosystems and human health due to their toxicity, persistence, and potential for bioaccumulation [1,2]. Recent studies have highlighted that petroleum hydrocarbon contamination may impair soil ecological quality even when contaminant concentrations remain below regulatory thresholds. Consequently, the assessment of remediation success should not rely exclusively on chemical parameters, but also consider biological indicators capable of reflecting the recovery of soil functionality and ecosystem services [3]. Addressing such contamination in a sustainable and effective manner has become a priority in environmental management and policy [4]. Among the available remediation technologies, bioremediation has gained wide acceptance due to its environmental sustainability, cost-effectiveness, and ability to mineralize organic pollutants [5]. Effective and sustainable remediation of hydrocarbon-contaminated soils also contributes to broader environmental policy objectives, including those related to the transition to climate neutrality and the reduction of pollution as a key priority in sustainable development frameworks [6]

Bioremediation relies on the metabolic activity of indigenous or

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introduced microorganisms capable of degrading petroleum compounds into less harmful or inert products such as carbon dioxide, water, and biomass [7]. In particular, ex situ biopile systems are commonly employed for the treatment of excavated hydrocarbon-contaminated soils [8]. These engineered systems allow for the optimization of environmental conditions such as oxygenation, nutrient supply, temperature, and moisture content to enhance microbial degradation [9]. Although ex situ biopiles offer operational control and scalability, achieving optimal performance remains challenging due to site-specific heterogeneity, dynamic microbial responses, and the complexity of pollutant mixtures [9,5].

A large body of research on hydrocarbon biodegradation has been conducted at bench scale under idealized conditions, whereas pilot scale studies and field trials remain comparatively scarce [10]. Nevertheless, recent investigations have highlighted the potential of biological treatments, including bioaugmentation and organic amendments, to enhance hydrocarbon degradation under field-relevant conditions [11]. As a result, there is limited understanding of how laboratory findings translate to industrial-scale operations. Upscaling to pilot and industrial scale is crucial for the circular economy frameworks, as bioremediation enables contaminated soil to be recovered and reintegrated into the ecosystems, mitigating the environmental impacts of petroleum hydrocarbons [12,13].

Each contaminated site presents unique soil properties, microbial communities, and pollutant profiles, which strongly affect bioremediation outcomes and highlight the need for more pilot-scale investigations to evaluate bioremediation performance under realistic, heterogeneous conditions [14,15]. In most practical applications, the progress and effectiveness of biopile treatment are monitored through periodic measurements of residual hydrocarbon concentrations, typically expressed as total petroleum hydrocarbons (TPH) [16]. While this metric is required by regulatory frameworks to demonstrate achievement of cleanup targets, its usefulness for process optimization and monitoring is limited [17].

One major challenge lies in the collection of representative soil samples from large and spatially heterogeneous piles [18]. Variability in contaminant distribution, even within a single pile, introduces significant uncertainty [19]. Additionally, traditional TPH analyses often suffer from limited reproducibility and low compound-specific resolution [20]. Interferences from co-extracted organic matter, analytical artifacts, and inconsistencies in sample handling further reduce the reliability of TPH data [21]. These limitations make it difficult to draw robust conclusions about biodegradation progress and hinder informed decision-making.

To overcome these shortcomings, this study proposes a multi-evidence monitoring approach for the optimisation and management of ex situ bioremediation processes. This approach integrates complementary methodologies that together provide a robust and mechanistic understanding of biodegradation dynamics. Microbial respirometry is used to quantify oxygen consumption and carbon dioxide production, offering a direct, real-time measure of microbial metabolic activity and an indirect indicator of hydrocarbon biodegradation. Respirometric data allow operators to detect active microbial degradation phases and identify stagnation points in real time.

Compositional fingerprinting, based on advanced gas chromatography–mass spectrometry (GC–MS), enables detailed characterization of hydrocarbon mixtures [22]. This technique provides insight into the relative abundance and degradation of specific compounds, highlighting which fractions are more readily degraded and which remain persistent [23,24]. When interpreted alongside diagnostic ratios-such as n-C17/pristane and n-C18/phytane for alkanes - these fingerprints can indicate the occurrence and extent of biodegradation [25]. In the present study, attention is focused on linear alkylbenzenes (LAB), prevalent in the contaminant profile of the soil under treatment [26]. LABs are alkylated aromatic hydrocarbons characterized by relatively high carbon numbers and physicochemical properties associated with the less volatile and less mobile fractions of petroleum-derived contaminants. Heavy hydrocarbons comprise a heterogeneous mixture of high-carbon-number compounds, including alkylated cyclic hydrocarbons, resins and asphaltenes [27]. For the purposes of this study, the heavy hydrocarbon fraction was operationally defined as the $C > 12$ fraction determined by GC analysis, in accordance with the classification commonly adopted in the Italian regulatory framework for contaminated sites (*Italian Legislative Decree 152/2006, Part IV, Title V, Annex 5*). Although LAB do not represent the entire heavy hydrocarbon fraction, their environmental behaviour is representative of the more persistent petroleum-derived contaminants present in the investigated soil.

Biomolecular tools, including high-throughput sequencing, provide insights into the structure and functional potential of microbial communities involved in the treatment [28]. The presence and abundance of hydrocarbon-degrading taxa, as well as specific catabolic genes, can be monitored throughout the process to evaluate the biological drivers of contaminant removal [29,30]. These biomolecular data support the interpretation of chemical trends and reveal shifts in microbial community dynamics that may influence treatment outcomes. Kinetic modelling further supports quantitative interpretation by fitting concentration–time data to mathematical models to estimate degradation rates and predict treatment durations [31].

This paper presents a case study applying the multi-evidence approach to a pilot-scale biopile treating soil contaminated by linear alkylbenzenes (LAB). The experimental design included the use of low-cost organic amendments and commercial bioremediation products in various combinations, with the aim of identifying the most effective treatment formulations and operational conditions under realistic field constraints. Traditional monitoring based solely on TPH analysis yielded inconclusive and sometimes conflicting results, limiting the ability to identify the most effective treatment conditions. In contrast, the integrated approach provided a comprehensive understanding of bioremediation dynamics, enabling the identification of optimal strategies based on microbial activity, compound-specific degradation, community structure, and toxicity reduction. The results demonstrate that multi-evidence monitoring enhances process understanding, supports real-time optimisation, and enables more informed and reliable decision-making. This approach is particularly valuable in complex contamination scenarios, where single-metric monitoring may fail to capture the full picture. By providing a holistic view of bioremediation performance, the multi-evidence framework supports the development of more efficient, tailored, and ecologically sound treatment strategies for hydrocarbon-contaminated soils.

2. Material and methods

2.1. Pilot scale biopile test

Two pilot-scale test campaigns using biopiles were performed, aimed at evaluating different bioremediation treatments under controlled conditions. The first experimental set consisted of eight biopiles (BP1 – BP8), whereas the second experimental set included six additional biopiles (BP9 – BP14). All biopiles were constructed with unsaturated soil from the same source characterized by heavy hydrocarbon (TPH: C10-C40) concentrations ranging from 2000 to 2500 mg/kg (dry weight).

The first experimental campaign began in October 2023 and lasted 5 months while the second one was launched in May 2024 year and monitored for 9 months. This second campaign was designed to further investigate treatment combinations that had shown promising results in the first phase and to test additional formulations.

Each biopile was treated with a distinct combination of amendments, including compost, inorganic fertilizers, and commercial bioremediation products. The names of the commercial products are not disclosed; a description of their main characteristics and the mode of action is provided in the Supplementary Materials (Table S1). Detailed treatment conditions and amendment dosages for both experimental sets are

summarized in Table 1.

Prior to pile construction, soil moisture was adjusted to 14% (w/w), corresponding to approximately 55% of the water holding capacity, for all treatments except the BP1 which served as dry control. Humidification was performed using sprayed tap water, applied in 30 cm layers and followed by mechanical turning. Water-soluble amendments (NPK fertilizer, Commercial product 1, Commercial product 2, Commercial product 3, Commercial product 4) were pre-dissolved in the humidification water and applied during this step. Mixed compost (BP3, BP4, BP10) and Green compost (BP12, and BP14) was added after moistening and homogenized with the soil using mechanical equipment. All compost materials were supplied by a local certified provider. Detailed information on the origin, chemical properties, and microbial community composition of the green and mixed composts used in this study is provided in Table S2 of the Supplementary Material.

Each biopile consisted of approximately 30 m^3 of soil (around 37,5 tons) and was constructed by layering the amended soil to a final height of approximately 1.50 m. Perforated plastic pipes were installed horizontally at mid-height and connected to the site's forced aeration system, operated in extraction mode. Each biopile received approximately 10 Nm^3/h of airflow to maintain aerobic conditions. A slotted piezometric tube (30 cm perforated section) and a guide tube for internal temperature measurements were installed centrally in each pile to monitor interstitial gas composition and thermal conditions throughout the treatment period.

2.2. Respirometric tests

Respirometric monitoring was carried out in parallel with chemical sampling to assess the rates of oxygen consumption, carbon dioxide production, and the accumulation of gases indicative of anaerobic respiration. The measurements were performed as a single test at each sampling time directly within the interstitial air of each biopile by temporarily interrupting the forced aeration system. The gas sampling point was located as far as possible from the biopile surface in order to reduce the effect of the atmospheric air.

Gas concentrations were monitored at fixed intervals (0, 2, 4, 6, 24, 28, and 30 h) following the cessation of air extraction. Prior to each measurement, the sampling tube located within the piezometer was purged to ensure representative gas sampling. The following gases were analyzed: O_2, CO_2 CH_4, and H_2S. Additionally, internal and external temperatures of the biopiles were recorded during each respirometric session.

Table 1
Summary of treatment conditions and amendment dosages for both experimental sets.

Biopile ID	Treatment Description	Amendment Dosage	Initial Moisture (%)
BP1	Unamended, not moistened (control)	-	5
BP2	Unamended, moistened (control)	-	14
BP3	Mixed compost	10% v/v compost	14
BP4	Mixed compost + NPK fertilizer	10% v/v compost + NPK (C:N = 5:1)	14
BP5	NPK fertilizer	NPK (C:N = 5:1)	14
BP6	Commercial product 1 (CP-1)	According to the producer's instructions	14
BP7	Commercial product 2 (CP-2)	According to the producer's instructions	14
BP8	Commercial product 3 (CP-3)	According to the producer's instructions	14
BP9	Unamended, moistened (control)	-	14
BP10	Mixed compost	5% v/v	14
BP11	Green compost	10% v/v	14
BP12	Green compost + NPK	Compost: 10% v/v NPK: C:N = 5:1	14
BP13	Oxidant + Green compost	Oxidant: 10% of the dose suggested by the providers for In situ chemical oxidation Compost: 10% v/v	14
BP14	Oxidant + Green compost + NPK fertilizer	Oxidant: 10% of the dose suggested by the providers for In situ chemical oxidation Compost: 10% v/v NPK: C:N = 5:1	14

2.3. Sampling

Sampling for chemical and biomolecular analyses was carried out at predefined intervals using representative composite samples from each biopile (BS), in accordance with UNI EN ISO 10,802:2023 and UNI TR 11,682:2017. All sampling equipment was properly cleaned and reconditioned according to the analytical parameters to be investigated.

Each biopile was conceptually divided into six sections. From each section, three subsamples were collected using a 1-meter manual auger: the first by vertically inserting the auger from the top of the pile, the second through an oblique insertion from the right side, and the third from the left side. This procedure yielded a total of 18 increments per biopile.

The increments were homogenized to form a bulk representative sample, which was then subjected to quartering to obtain an average sample of approximately 2 kg. From this composite sample, 3 sample replicates were formed for chemical analyses and one replicate for the biomolecular ones. Composite sampling was adopted to obtain a representative microbial profile of each biopile treatment while minimizing the influence of local spatial heterogeneity, an approach commonly employed in soil microbiology studies investigating treatment-level community responses [32].

2.4. Quantitative and qualitative analysis for determination of hydrocarbon mixture

The quantitative determinations of Total Petroleum Hydrocarbons were carried out according to UNI EN 14,039:20.

For qualitative analysis the soil samples from the set 1 were analyzed by gas chromatography coupled with mass spectrometry (GC–MS) to qualitatively characterize the composition of extractable hydrocarbon fractions. Analyses were performed using the same instrumentation and chromatographic conditions employed for the quantification of TPH (C10–C40 hydrocarbons), without fractionation of the organic extracts. Compound identification was based on mass spectral interpretation and comparison with commercial spectral libraries (e.g., NIST and Wiley). The recognition of specific hydrocarbon structures was achieved through the analysis of diagnostic fragmentation patterns and confirmed, where available, by matching with authentic reference spectra. Additionally, selected isomer ratios were evaluated as diagnostic indicators of biodegradation, an approach commonly used to assess hydrocarbon weathering and microbial degradation in contaminated environments [33]. This approach enabled the detection of qualitative changes in the hydrocarbon mixture over time and among treatments, providing supporting information on the nature and transformation of petroleum-derived compounds during bioremediation.

All analyses were performed in ISO/IEC 17,025 accredited laboratories. Appropriate quality assurance and quality control protocols were followed, including the use of calibration standards, procedural blanks, and duplicate samples to ensure data reliability and traceability.

To estimate the significance of the difference in biodegradation rate among the treatments a one-way ANOVA was performed with treatment as the fixed factor considering biodegradation fraction at 120 days. Pairwise differences between treatments were assessed using Tukey's significant difference (HSD) test.

2.5. Modelling of hydrocarbon degradation and oxygen consumption

2.5.1. Biodegradation kinetics modelling

Residual hydrocarbon concentration data were interpreted through kinetic modelling to describe the time-dependent dynamics observed during biopile treatment. Two distinct processes were identified and modelled accordingly. In some biopiles an initial increase in extractable hydrocarbon concentrations was observed during the early stages of treatment. This phenomenon is attributed to the physical mobilization of hydrocarbons from micropores or from sorption sites on soil organic matter, following mechanical manipulation, humidification, and biological activation of the matrix [34]. This release phase was described using a first-order asymptotic model (Eq. (1)) [35]:

$$C_{\{released\}(t)} = C_0 + R \cdot \left(1 - e^{-k_r t}\right) \quad (1)$$

where C_0 is the initial measurable concentration (mg/kg), R is the maximum releasable hydrocarbon fraction (mg/kg), k_r is the release rate constant (1/day), and t is the time in days.

The subsequent decrease in concentrations due to microbial degradation was modelled using a first-order decay equation (Eq. (2)) [36]:

$$C(t) = C_{\{released\}(t)} \cdot e^{-k_d t} \quad (2)$$

where k_d is the first-order biodegradation rate constant (1/day).

The overall model describing both processes concurrently is expressed by Eq. (3):

$$C(t) = \left(C_0 + R \cdot \left(1 - e^{-k_r t}\right)\right) \cdot e^{-k_d t} \quad (3)$$

The time zero concentration for each biopile was estimated as the mean of all replicate values.

To avoid overestimation of the release component, a model constraint was applied such that $R \leq 0.5\ C0$. The selection of the model was based on the shape of the observed concentration trends. Model parameters were estimated by nonlinear least squares (Levenberg–Marquardt algorithm). For each pile, selection of the modele between the complete relese-decay model (eq. (3)) and only-decay model (Eq. (2)) was carried out using the Akaike information criterion corrected for small sample sizes (AICc). The model with the lower AICc was retained; a difference $\Delta AICc > 2$ was taken as substantial support for the more complex release-and-decay model, while $|\Delta AICc| \leq 2$ was treated as statistical equivalence, in which case the more parsimonious pure-decay model was preferred.

The adequacy of each model was evaluated through a residual analysis Goodness of fit was quantified by the coefficient of determination (R^2) and the root-mean-square error (RMSE). The normality of the residuals was tested with the Shapiro–Wilk test, with $p < 0.05$ indicating departure from normality. All computations were carried out in Python 3.11 with SciPy.

2.5.2. Oxygen consumption modelling

To evaluate the influence of oxygen concentration on the biodegradation rate during treatment, a modelling approach was developed based on the analysis of oxygen consumption curves recorded in the centre of the biopiles during static respirometric tests (post-aeration) [37].

Initial attempts to fit the experimental data using classic models from the literature—such as first-order or Monod-type kinetics—did not adequately describe the observed O_2 depletion trends. These traditional models assume either exponential or hyperbolic relationships between oxygen concentration and consumption rate [38–40]. However, these models failed to capture the specific curvature and slowdown of O_2 consumption observed experimentally.

On the basis of the experimental data an empirical model was therefore proposed, starting from a simple linear relationship (Eq. (4)) between oxygen consumption rate and its concentration:

$$dO_2/dt = m \cdot O_2 + b \quad (4)$$

Where m is the slope for the equation, consequently (-b/m) is the value of oxygen concentration where the oxygen consumption rate is zero or null.

To introduce a more meaningful and normalized interpretation, this equation was reformulated by making explicit the minimum threshold concentration C_{O2}^{min}, below which aerobic microbial activity ceases, and the maximum oxygen consumption rate observed at atmospheric O_2 levels (in %/h).

The final model was (Eq. (5)):

$$dO_2/dt = k \cdot \left(O_2 - {C_{o2}}^{min}\right)/\left(20.9 - {C_{o2}}^{min}\right) \quad (5)$$

where:

- O_2 is the oxygen concentration (in % v/v) in soil gas at time t,
- C_{O2}^{min} is the threshold oxygen level (in % v/v) below which respiration is negligible,
- O_2_atm is atmospheric oxygen concentration (set to 20.9% v/v),
- k is the maximum oxygen consumption rate observed at atmospheric O_2 levels (in % v/v/h).

The ratio (O_2 - $C_{O_2}^{min}$) / (20.9 - C_{O2}^{min}) represents the relative biodegradation efficiency, which decreases as oxygen availability diminishes. This model allows the calculation of the effective O_2 concentration above which a desired biodegradation efficiency is maintained. Consequently, the oxygen level corresponding to 80% efficiency ($O_2^{80\%}$) is calculated as (Eq. (6)):

$${O_2}^{80\%} = {C_{o2}}^{min} + 0.8 \cdot \left(20.9 - {C_{o2}}^{min}\right) = 0.2 \cdot {C_{o2}}^{min} + 16.72 \quad (6)$$

2.6. Biomolecular analyses

To assess microbial community structure and activity within the biopiles, a targeted biomolecular analysis set was employed. This included both qualitative and quantitative approaches aimed at characterizing total bacterial abundance and functional potential for hydrocarbon degradation.

Bacterial community structure was investigated through high-throughput sequencing of the 16S rRNA gene. DNA was extracted with FastDNA™ Spin Kit for Soil (MP Biomedicals, Solon, OH, United States) from soil samples collected at different timepoints and subjected to amplification of the bacterial V5–V6 hypervariable region of the 16S rRNA gene by using 783F 1027R primers [41], followed by paired-end sequencing using the Illumina MiSeq platform. The resulting sequences were quality-filtered, clustered into OTU and taxonomically annotated using RDP reference databases [42]. This approach provided qualitative insights into shifts in microbial composition and the enrichment of specific taxonomic groups potentially involved in biodegradation. Estimation of the relative abundance of bacterial populations with hydrocarbon- degrading capabilities has been carried out as previously reported [43]. Briefly, the original relative abundance of each genus was multiplied by a coefficient ranging from 0 to 1, representing the estimated fraction of a certain genus that harbors the marker genes relevant for aerobic and anaerobic degradation processes of aliphatic and aromatic hydrocarbons. In particular, the marker genes *alk*B (associated with the degradation of short- to medium-chain alkanes) and *lad*A (involved in the initial oxidation of long-chain alkanes) were investigated [44,45]. To obtain these coefficients, the Hidden Markov Models of the main marker genes for hydrocarbon degradation were searched (hmmseach command) against the GTDB genome database [45,46].

3. Results and discussion

3.1. Assessing biodegradation by TPH analysis

Total Petroleum Hydrocarbon (TPH, C > 12) concentrations were monitored throughout both pilot test campaigns to evaluate treatment performance under varying amendment conditions. Despite all biopiles being prepared from the same homogenized batch of contaminated soil, initial concentrations at T0 varied widely but with similar range in both sets. In the first set, values ranged from 1480 mg kg^{-1} to 3660 mg kg^{-1}, while in the second set they spanned from approximately 1600 mg kg^{-1} to 3100 mg kg^{-1}. This high variability - confirmed by the large standard deviations shown in Figs. 1 and 2- reflects both sampling heterogeneity and the limited analytical reproducibility of conventional TPH quantification methods.

During the early stages of treatment, apparent concentration increases were observed in most biopiles in both sets. These were particularly pronounced in BP2 and BP6 in the first set, and in BP9 and BP13 in the second set, all exceeding 3000 mg kg^{-1} at 15 days (T15). These early spikes are not uncommon and are attributed to the physical mobilization of hydrocarbons from micropores and sorption sites triggered by humidification and microbial activation. These processes increase the extractability of hydrocarbons by organic solvents, artificially elevating their apparent concentrations [34,35].

Over time, TPH concentrations generally decreased in all treatments, although with markedly different patterns. In the first set, the unamended controls (BP1 and BP2) and BP6 (treated with a commercial biostimulant, CP-1) showed minimal decline, remaining above 1700 mg kg^{-1} throughout the monitoring period. In contrast, amended piles such as BP7 (CP-2), BP8 (CP-3 + NPK), BP3 (compost 10%), and BP4 (compost 10% + NPK) showed substantial reductions, with BP7 achieving the lowest final concentration (574 mg kg^{-1}). BP5 (NPK) initially reached the target level of 750 mg kg^{-1} at day 120 but exhibited an anomalous value of 1790 mg kg^{-1} at day 150, further illustrating the susceptibility of TPH data to random fluctuations.

In the second set, similar trends were observed. The unamended control (BP9) again showed low removal efficiency, while amended treatments such as BP13 (oxidant + compost 10%) and BP14 (oxidant + NPK+ compost 10%) achieved final TPH concentrations close to or below the cleanup target (750 mg kg^{-1}).

At 120 days, the one-way ANOVA indicated a significant overall effect of treatment on biodegradation percentage ($F(13,28)=5.76$, $p < 0.001$). However, the subsequent pairwise Tukey comparisons resolved only a small number of significant differences among the individual treatments (Table S3). In particular, despite the prolonged treatment period, most of the amended microcosms were not statistically distinguishable from the humified controls BP2 and BP9. This limited resolution is largely attributable to the high variability of the replicate measurements, which inflates the within-group error and widens the minimum difference required for significance. It should also be noted that this pairwise approach relies solely on the t = 0 and t = 120 day data and does not exploit the information contained in the intermediate sampling points; differences in degradation dynamics between treatments are therefore not captured, and are better addressed through the time-series (regression) analysis.

These results highlight the significant limitations of relying solely on TPH monitoring to assess bioremediation performance. High baseline uncertainty, sampling artifacts, and erratic concentration spikes undermine its reliability as a quantitative indicator for estimating degradation rates, tracking treatment dynamics, or selecting the most effective amendment strategy.

Several studies have documented how solvent-based TPH measurements are sensitive to extraction efficiency, soil heterogeneity, and operational variability. Even at baseline (T0), TPH values often vary by >50% across replicates due to localized variability and inconsistent extraction, as shown by [47], who reported large inter-laboratory and inter-method discrepancies when comparing infrared and GC-FID techniques. Moreover, disturbances such as aeration and humidification frequently mobilize previously sorbed hydrocarbons, leading to transient increases in extractable concentrations unrelated to actual contaminant input or degradation reversal [48,49]. These “spikes” are particularly problematic in early-stage monitoring, giving a false impression of contamination persistence.

In addition to these analytical uncertainties, TPH represents a non-specific sum of an heterogeneous range of compounds. As a result, reductions in TPH may not reflect the degradation of the most hazardous fractions, while persistence in TPH values can mask the removal of specific target hydrocarbons. It has been emphasized that TPH measurements fail to distinguish between biodegradation and redistribution processes and cannot resolve which hydrocarbon groups are actually being metabolized [50,51]. This non-specificity limits the capacity of TPH to assess the relative performance of different treatment formulations or to help real-time decisions during biopile management.

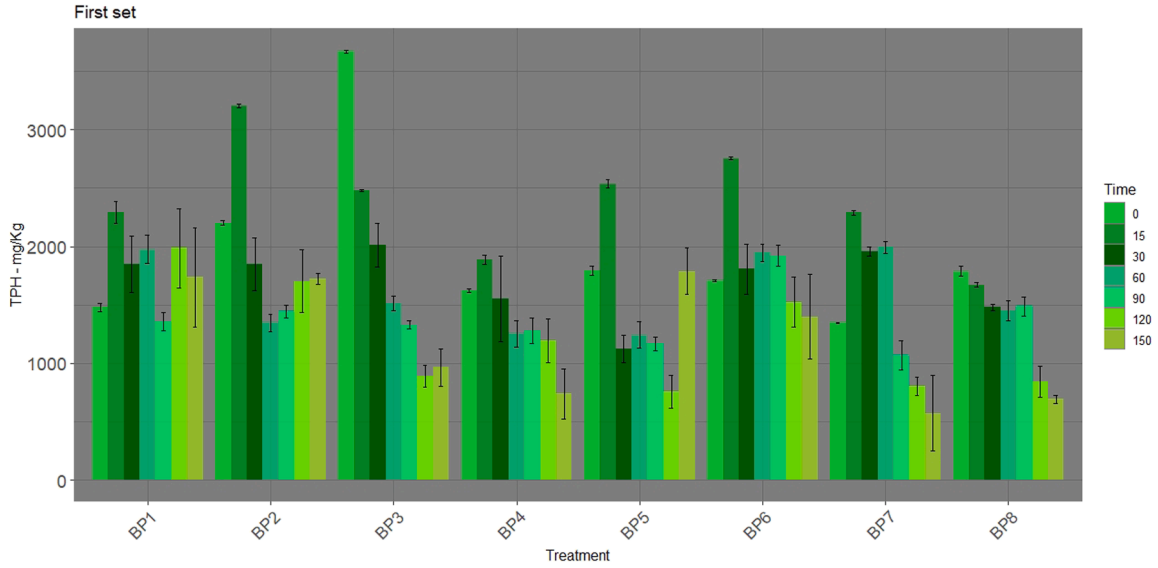

Fig. 1. TPH concentration trend in the first pilot scale biopile experiment.

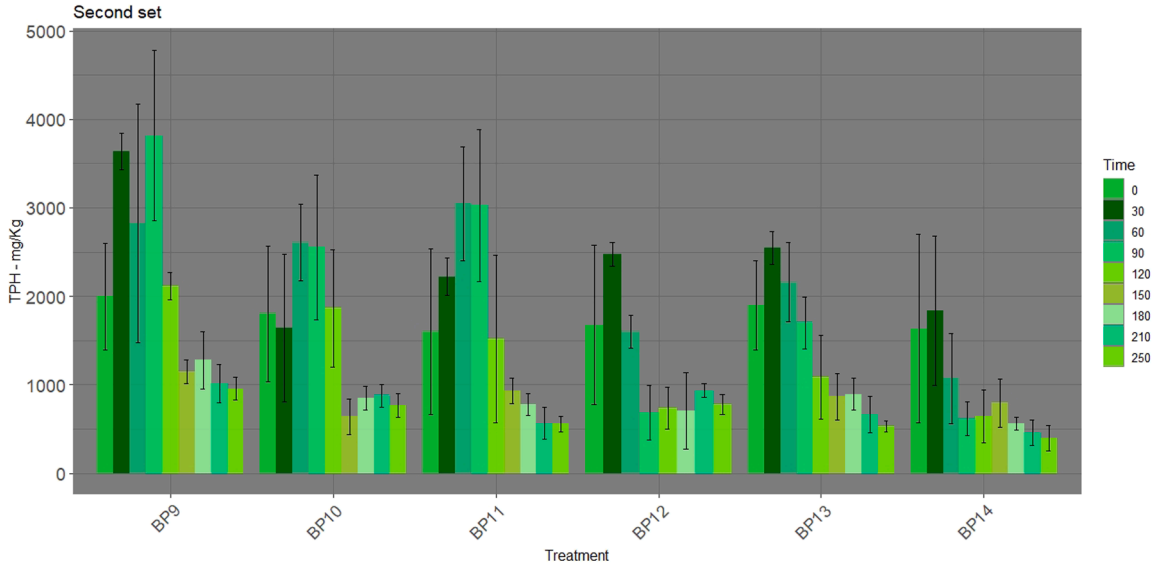

Fig. 2. TPH concentration trend in the second pilot scale biopile experiment.

3.2. Qualitative analysis and biodegradation diagnostic ratios

Qualitative GC–MS analysis of selected soil samples from the original soil batch allowed the identification of 18 major analytes (peaks), corresponding to structurally related organic molecules. This analysis supported the hypothesis of a homogeneous contamination profile across the tested matrix, although minor differences in peak intensities reflect the inherent heterogeneity of field-collected soil samples and confirmed the presence of aromatic compounds belonging to the class of linear alkylbenzenes (LABs), characterized by benzene rings substituted with straight or branched alkyl chains of variable length (with a total number of alkyl-chains carbon atoms ranging from 10 to 14). In environmental matrices, their isomeric composition provides valuable insights into biodegradation processes. Scientific literature shows that LAB isomers with identical alkyl chain length but differing in the position of the benzene substitution exhibit markedly different biodegradation behaviours. Specifically, when the benzene ring is bonded to an external carbon in the alkyl chain (E-isomers), the molecule is more susceptible to microbial degradation compared to when the bond is located closer to the internal carbons (I-isomers) [52,53]. As a consequence, the I/E concentration ratio for a given homologous group increases over time and can be used as a semi-quantitative biomarker for the extent of aerobic biodegradation.

For example, in the C12 LAB group, 1-methylundecylbenzene (also referred to as 2-C12 LAB), where the benzene ring is attached to the second carbon, is classified as an E-isomer and degrades more rapidly. In contrast, 1-pentylheptylbenzene (6-C12 LAB), where the ring is bonded to the sixth carbon, is an I-isomer and persists longer in the environment. This principle underlies the calculation of I/E ratios as diagnostic indicators.

In the GC–MS analyses on the original soil batch, 18 LAB compounds were identified and assigned structural isomers based on the position of the aromatic substitution and the length of the two alkyl branches. A subset of these, particularly from the C11 and C12 homologous groups, was used to compute isomer ratios, selecting representative compounds where standard isomer classification (I vs. E) was unambiguous. Table 2 summarizes, for each identified LAB, the carbon length of each alkyl chain, and their classification as I- or E-isomers.

The temporal evolution of I/E ratios for LAB homologues C11 and C12 across the different biopiles of the first set provides useful insights into the biodegradation dynamics occurring during the treatment (Fig. 3). As expected, treatments that showed higher efficacy in reducing TPH concentrations also exhibited a marked increase in the I/E ratio, consistent with preferential degradation of the more labile E-isomers. This trend is particularly evident in BP4 (compost + NPK) and BP3 (compost), where both C11 and C12 I/E ratios increased steadily over time, reaching values above 2.0 and 1.6, respectively, at T150. These values suggest an advanced stage of microbial transformation and are coherent with the observed reduction in TPH concentrations in these piles.

Conversely, the control treatments (BP1 and BP2) and BP8 (CP-3 + NPK) showed stable or only slightly increased I/E ratios throughout the monitoring period, indicating limited biodegradation activity. BP7 (CP-2) also exhibited only moderate increases in isomer ratios, despite showing relatively low final TPH concentrations, suggesting that mechanisms other than selective degradation of LAB isomers (e.g., physical mobilization or sorption) may contribute to hydrocarbon removal in that case.

Notably, the evolution of the I/E ratios over time displayed greater consistency and interpretability than the bulk TPH measurements,

Table 2
Summary of the characteristics of the identified linear alkylbenzenes.

Compound	Alkyl chain C1	Alkyl chain C2	Total alkyl C	Benzene bond position	I/E isomer
1-methyldecylbenzene	1	10	11	2	E
1-propyloctylbenzene	3	8	11	4	E
1-butyloctylbenzene	4	7	11	5	I
1-pentylhexylbenzene	5	6	11	6	I
1-methylundecylbenzene	1	11	12	2	E
1-propylnonylbenzene	3	9	12	4	E
1-butyloctylbenzene	4	8	12	5	I
1-pentylheptylbenzene	5	7	12	6	I

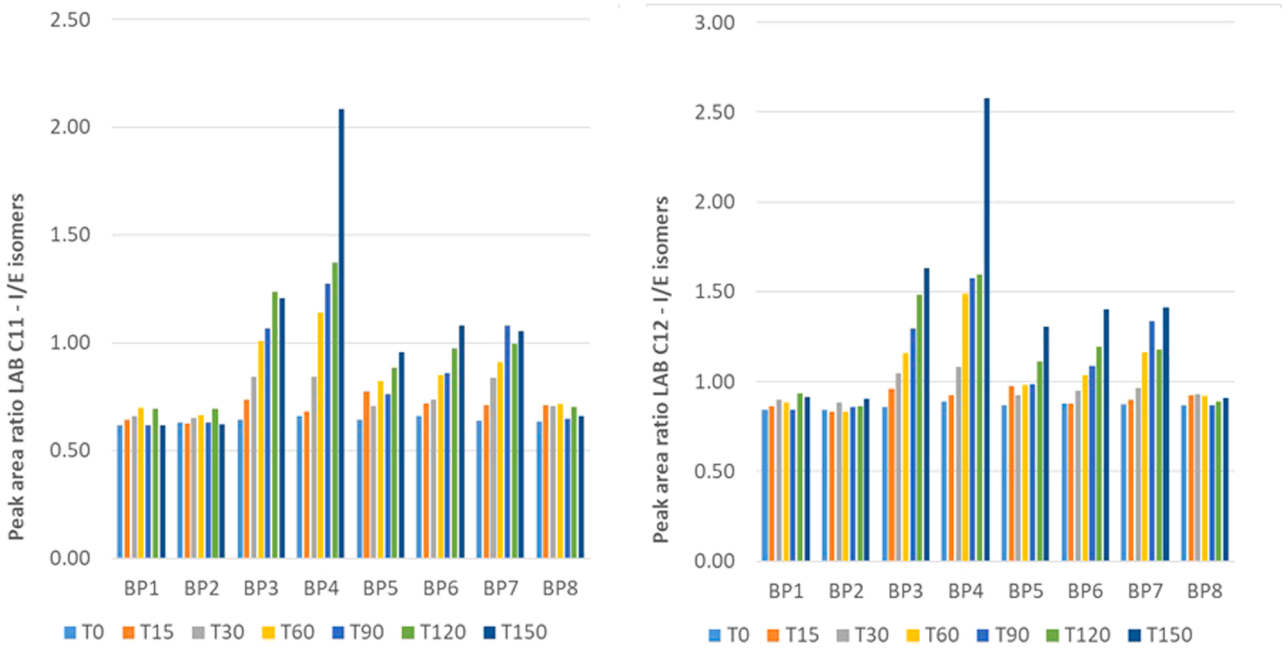

Fig. 3. Evolution of the LAB I/E isomer diagnostic ratios.

which were affected by substantial analytical variability and concentration rebounds. These isomeric indicators thus offer a more reliable proxy for tracking the actual biodegradation of hydrocarbon constituents and align more closely with expected treatment performance, supporting their use as complementary diagnostic tools in bioremediation monitoring.

3.3. Hydrocarbon kinetic modelling

Applying the first-order kinetic model with an asymptotic release term (Eqs. (1)–(3)) allowed us to integrate the fluctuating TPH measurements and analytically compensate for early-stage release artifacts. For all the biopiles of the same set, the initial concentration C0 was set as the mean of all T0 replicates, and the anomalous values from BP5 at day 150 was excluded to improve model fidelity. Indeed, the TPH measured at T150 values were significantly higher than the results obtained from the previous sampling times, thus showing inconsistency with the expected trend.

The release phase was observed in most piles; only treatments such as BP8 - where no early increase occurred - were modelled solely with Eq. (2). The estimated biodegradation rate constants are reported in Table 3, whereas the complete information about model selection, parameter estimationa and residual analysis is reported in Table S4. BP3 (Mixed compost) achieved the highest k_d (0.011 ± 0.001 d^{-1}), followed closely by BP7 (CP-2, 0.008 ± 0.001 d^{-1}) . In contrast, the dry control BP1 showed the slowest degradation (0.0004 ± 0.0001 d^{-1}). Additionally, this modelling suggested a potential inhibitory effect of NPK addition to the immature compost (BP4), which showed a lower k_d (0.0045 ± 0.0008 d^{-1}) since this estimated k_d is lower than the one obtained for compost only (BP3). Excessive nutrient amendment can adversely affect hydrocarbon biodegradation. In particular, Chaîneau et al. [66] observed that high levels of nitrogen and phosphorus fertilizers led to a permanent inhibition of hydrocarbon assimilation in soil. The authors suggested that excessive fertilization can disturb the balance of microbial populations and metabolic pathways, resulting in a reduced degradation efficiency for certain hydrocarbon classes, especially aliphatic compounds. In the second campaign, the highest biodegradation rates were seen in BP13 (green compost + oxidant, 0.0071 ± 0.0008 d^{-1}) BP14 (green compost + NPK + oxidant, 0.0065 ± 0.001 d^{-1}) and BP11 (green compost, 0.006 ± 0.003)B. Compost treatments at reduced dose (BP10,) and the humidified control (BP9) showed lower degradation rates ranging from 0.004 to 0.005 d^{-1}.

The kinetic modelling approach effectively mitigates the uncertainties associated with direct TPH data by providing a single, comparative biodegradation parameter for each treatment. This enables reliable assessment of amendment efficacy and process dynamics—even when raw concentration data are affected by noise and outlier points.

Table 3
Summary of the estimated biodegradation rate constants of each treatment.

Treatment (BP)	k_d ± standard error ($days^{-1}$)
BP1	0.0004 ± 0.0009
BP2	0.004 ± 0.001
BP3	0.011 ± 0.001
BP4	0.0045 ± 0.0008
BP5	0.0035 ± 0.002
BP6	0.0041 ± 0.0008
BP7	0.008 ± 0.001
BP8	0.0051 ± 0.0006
BP9	0.006 ± 0.001
BP10	0.004 ± 0.001
BP11	0.006 ± 0.003
BP12	0.005 ± 0.001
BP13	0.0071 ± 0.0008
BP14	0.006 ± 0.001

3.4. Oxygen consumption modelling

The application of the empirical oxygen consumption model allowed the estimation of key parameters for each biopile treatment, specifically the oxygen consumption rate constant (k) and the minimum interstitial oxygen concentration (C_{O2}^{min}) during respirometric tests.

In Table 4 the estimated values of k and C_{O2}^{min} as well as the calculated $O_2^{80\%}$ for the T30 time point in the second set of biopiles.

These values were then used to calculate the biodegradation process efficiency ($C_{O2}{}^{80\%}$), defined as the oxygen concentration corresponding to 80% of the maximum consumption rate under optimal conditions (i.e., air-saturated soil gas). As shown in Table 4, two treatments supplemented with compost (BP12 and BP14) exhibited higher k values,

Table 4
Parameters estimated through the empirical oxygen consumption model.

Biopile	Sampling time	k	C_{O2} min	O_2 80%
BP9	T30	0.092	12.52	19.2
BP10	T30	1.071	9.405	18.6
BP11	T30	0.655	11.25	18.7
BP12	T30	5.33	1.612	17.0
BP13	T30	1.09	8.527	18.4
BP14	T30	6.391	6.022	17.9

indicating accelerated oxygen consumption likely due to increased microbial activity supported by the additional organic carbon. Recent studies have highlighted the central role of oxygen availability in regulating microbial activity and the overall efficiency of aerobic TPH biodegradation in contaminated soils [54].

$C_{O2}^{80\%}$ values varied between 17% and 19.2% which might represent an optimal range of oxygen concentration in the biopiles. Indeed, maintaining atmospheric-level O_2 concentrations in the soil gas would require higher air fluxes, increasing the energy demand of the system.

Estimating the biodegradation efficiency at varying oxygen levels becomes a valuable tool to define the optimal trade-off between maximizing biodegradation rates and minimizing oxygenation costs. In particular, this approach enables the identification of oxygen thresholds below which the biodegradation process becomes significantly less efficient, allowing for informed decisions on the design and operation of aeration systems. By quantifying the relationship between interstitial oxygen concentration and biodegradation performance, it is possible to optimize airflow regimes that sustain high microbial activity while avoiding unnecessary energy expenditure. This is especially relevant in systems treated with organic amendments such as compost, where increased microbial respiration can rapidly deplete oxygen levels [55,56]. The ability to quantify and predict these dynamics supports a rational management of biopile systems aimed at enhancing remediation efficacy and cost-efficiency.

3.5. Biomolecular microbiological analyses

Biomolecular monitoring was conducted across both pilot-scale biopile sets to assess the effects of treatments on bacterial community structure and the selection of hydrocarbon-degrading microorganisms. High-throughput sequencing (NGS) of the 16S rRNA gene was used to characterize the taxonomic and functional composition of the microbial communities within the biopiles during the treatment times. Further details regarding the microbial community composition is provided in Table S5 and S6 of the Supplementary Material.

The biomolecular analyses were performed on composite samples designed to provide a representative characterization of each treatment. Consequently, the observed community shifts should be interpreted as treatment-level responses rather than indicators of microbial heterogeneity at the intra-pile scale. Similar composite sampling strategies are commonly adopted in studies aimed at evaluating the overall effects of remediation treatments on soil microbial communities [32].

Sequencing results revealed that the contaminated soil already hosted a microbial community with strong hydrocarbon-degrading potential, likely due to the selective pressure exerted by long-term contamination. Bacterial genera known for their hydrocarbon degradation capabilities were detected in all biopiles at both the start (T0) and at an advanced treatment stage (T120) of the treatment period, confirming the persistence of a functionally specialized community. In particular, *Pseudoxanthomonas and Pseudomonas* were among the most abundant genera [57–60]. Other microorganisms with documented hydrocarbon-degrading capabilities detected in the biopiles included *Rhodococcus, Solimonas, Bordetella, Mycobacterium* [61–65].

The use of compost (both mixed and green) as an amendment significantly impacted community structure. Mixed compost, derived from urban organic waste and green waste, introduced greater microbial biodiversity compared to green compost, which consists solely of plant residues. In both cases, the microbial input from compost was evident at T0, and the increased diversity was generally maintained throughout the treatment. In pre-oxidized treatments (BS13 and BS14), no substantial differences were observed between samples collected before and after chemical oxidation. However, following compost addition, a clear restructuring of the community was observed, with an enrichment of hydrocarbon-degrading taxa. This suggests that oxidation with sodium persulfate may enhance the bioavailability of certain hydrocarbon fractions, facilitating the growth of specialized microbial populations.

Principal Component Analyses (Figs. 4 and 5) illustrate the temporal evolution of bacterial communities and treatment effects. Among the samples at T0, it's possible to observe that the addition of compost considerably influences the structure of the microbial community, particularly in the biopiles BP3 and BP4. Furthermore, pre-treatment with the oxidant, and subsequent compost amendments in BP13 and BP14, shift the microbial communities closer to those of green and mixed compost. Compost-microbial communities show an enrichment in bacteria that can degrade long-chain alkanes (*lad*A). Over time, samples tend to shift along the principal components, indicating a transition from more generalist communities to ones dominated by aerobic and short-chain hydrocarbon-degrading bacteria (*alk*B) [44,45]. This shift is visually represented by blue arrows, particularly pointing toward *alk*B and aerobic bacteria. Samples from BP3, BP4, BP12, BP13, and BP14 show the strongest alignment in the direction of *alk*B, confirming the effectiveness of compost-amended treatments in selecting functionally relevant microbial taxa for bioremediation. In treatments receiving both NPK and compost (BP4, BP12, and BP14), this enrichment toward aerobic hydrocarbon-degrading communities is particularly pronounced, highlighting the synergistic effect of combining fertilization with compost amendment.

Overall, the biomolecular results confirm that compost amendments promote the selection of bacterial populations with high degradation capabilities, and that the addition of NPK and/or chemical oxidants can further enhance this selection, improving treatment efficacy from a microbiological perspective.

4. Conclusion

This study demonstrated the advantages of a multi-evidence

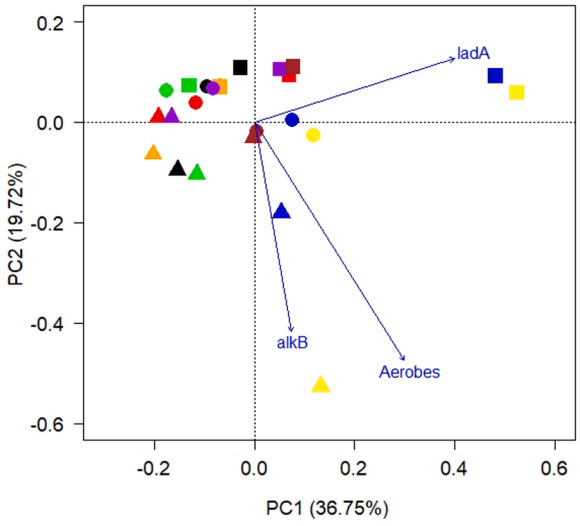

Fig. 4. PCA plot with fitting of functional prediction variables (First set). BP1 "black", BP2 "green", BP3:"blue",'BP4: "yellow", 'BP5:"red"; 'BP6:"orange"; BP7:"purple"; BP8:"brown"; Time 0: square; Time 30: "circle"; Time 120: "triangle".

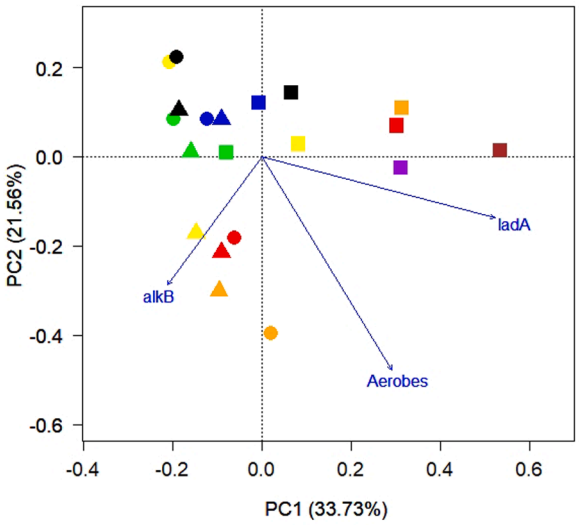

Fig. 5. PCA plot with fitting of functional prediction variables (Second set). BP9 "black", BP10 "green", BP11:"blue", BP12: "yellow", 'BP13:"red"; 'BP14:"orange"; Mixed compost:"purple"; Green compost:"brown"; Time 0: square; Time 30: "circle"; Time 120: "triangle".

monitoring framework for optimizing and managing ex situ bioremediation processes at pilot scale. By integrating chemical, biomolecular-microbiological, respirometric, and modelling data, it is possible to overcome the limitations of traditional monitoring approaches based solely on Total Petroleum Hydrocarbon (TPH) analysis.

The application of multiple, complementary lines of evidence enabled a more accurate and robust interpretation of bioremediation dynamics. Respirometric assays provided real-time insights into microbial activity and oxygen consumption kinetics, allowing the definition of optimal oxygenation thresholds and the identification of treatments that sustained high biodegradation rates. Modeling of O_2 consumption showed that treatments involving compost, particularly those supplemented with NPK (e.g., BP12 and BP14), reached higher microbial respiration rates, but also experienced rapid oxygen depletion—highlighting the need for tailored aeration regimes to avoid inefficient energy expenditure.

Chemical fingerprinting and diagnostic isomer ratios offered reliable indicators of hydrocarbon transformation, reducing uncertainties due to sampling variability and analytical artifacts. In particular, the evolution of LAB I/E ratios closely mirrored biodegradation trends and showed greater consistency than bulk TPH data. Kinetic modeling further refined the estimation of degradation rates, accounting for early-phase hydrocarbon release and enabling quantitative comparisons across treatments.

Biomolecular analyses confirmed the positive impact of compost amendments on microbial community structure and functional potential. High-throughput sequencing revealed a sustained enrichment of hydrocarbon-degrading taxa and highlighted the effectiveness of mixed compost + NPK treatments (e.g., BP4 and BP12) in promoting bacteria involved in the aerobic degradation of short-chain hydrocarbons. The addition of chemical oxidants further enhanced this selective enrichment by increasing hydrocarbon bioavailability. The experimental approach identified the treatments amended with mixed compost (BP3: only compost and BP4 with NPK fertilizer) as the most effective ones, as they consistently ranked among the best treatments across thelines of evidence. Indeed, sole compost amendments ranked first in kinetic constant estimation and second in diagnostic ratio. Conversely, compost and NPK ranked, by far, first in diagnostic ratio approach. Both of them gave strong evidence of selecting the bactetial communities towrads the enrichment of hydrocarbon-degrading taxa. Altogether, the multi-evidence approach enabled us to (i) more confidently identify the most effective treatment combinations (e.g., the synergistic effect of different treatment solutions from a microbiological standpoint); (ii) mitigate the uncertainty associated with analytical variability and soil heterogeneity; and (iii) support the real-time optimization of aeration and amendment strategies. By providing a mechanistic and multi-dimensional understanding of bioremediation processes, this framework supports more informed decision-making and the development of efficient, site-specific remediation protocols for hydrocarbon-contaminated soils. The multi-evidence monitoring framework proposed in this study inevitably entails higher analytical costs compared to conventional TPH-only monitoring, due to the additional labour, instrumentation, and expertise required for respirometric measurements, GC–MS fingerprinting, biomolecular analyses, and kinetic modelling. In routine operational settings where treatment conditions are well-established and contamination profiles are homogeneous, this additional investment may not be justified by a proportional gain in operational efficiency. However, the cost–benefit balance shifts substantially when the framework is applied in contexts where standard approaches are most likely to fail such as soils with atypical or heterogeneous contamination profiles, complex pollutant mixtures, or when the primary objective is the comparative evaluation and optimisation of alternative treatment strategies. In these scenarios, as demonstrated by the present study, the richer information content generated by the multi-evidence approach supports more accurate identification of effective amendment combinations and more informed management of aeration regimes. Early identification of the most performant treatment formulation, and avoidance of ineffective or sub-optimal amendments, can result in substantial savings in material costs, treatment duration, and energy expenditure associated with forced aeration. The oxygen consumption modelling presented here, for instance, directly enables the definition of aeration thresholds below which energy input can be safely reduced without compromising biodegradation efficiency. We therefore propose that the multi-evidence approach is not as a replacement for routine TPH monitoring, but as a decision-support tool during the optimisation phase of bioremediation projects.

Declaration of generative AI

During the preparation of this manuscript, the authors used ChatGPT to check and improve the English language and style of the text. After using this tool, the authors reviewed and edited the content as necessary, taking full responsibility for the content of the published article.

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CRediT authorship contribution statement

Alessandra Suagher: Investigation, Writing – original draft. **Davide Rossi:** Investigation, Writing – original draft. **Asia Rosatelli:** Investigation, Writing – review & editing. **Luca Serbolisca:** Conceptualization, Investigation, Writing – review & editing. **Alessandro Conte:** Investigation, Writing – review & editing. **Ilaria Pietrini:** Investigation, Writing – review & editing. **Fiora Bagnato:** Conceptualization, Writing – review & editing, Project administration. **Salvatore Crobu:** Project administration, Investigation, Writing – review & editing. **Massimiliano Baric:** Investigation, Methodology, Writing – review & editing. **Valentina Rivelli:** Investigation, Writing – review & editing. **Tatiana Stella:** Investigation, Writing – review & editing. **Andrea Franzetti:** Conceptualization, Investigation, Writing – original draft, Methodology, Formal analysis.

Declaration of competing interest

The authors declare the following financial interests/personal relationships that may be considered potential competing interests: Alessandro Conte, Ilaria Pietrini, and Luca Serbolisca are employees of Eni S.p.A.; Fiora Bagnato and Salvatore Crobu are employees of Eni Rewind S.p.A. Eni Rewind S.p.A. is the owner and operator of the facility where the experimental activities were conducted and is a subsidiary of Eni S.p.A.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rineng.2026.111882.

Data availability

Data will be made available on request.

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