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Assessing the ecological quality and reuse potential of bioremediated hydrocarbon-contaminated soils

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Bioremediation is increasingly recognized as a sustainable strategy for improving the environmental quality and supporting the beneficial reuse of hydrocarbon-contaminated soils. By preserving soil agronomic properties, this technique transforms bioremediated soils otherwise classified as waste into potential resources within circular-economy frameworks. However, current compliance criteria are primarily based on chemical thresholds, which do not account for ecological functionality or reuse potential in green infrastructure. In this study, we propose and preliminarily evaluate a multidisciplinary framework integrating chemical parameters, plant bioassays, and soil fauna avoidance tests to assess the ecological compatibility and potential reuse of 13 hydrocarbon-contaminated soils after bioremediation in two treatment plants in Northern Italy. All treated soils met the regulatory chemical limits established for reuse; however, the proposed framework goes beyond compliance by supporting potential reuse decisions based on ecological functionality. The framework was applied through a sequential three-phase approach: (i) phytotoxicity screening based on the germination index (GI), applying an "OR" criterion (GI > 90% in at least one test species, *Lepidium sativum* or *Cucumis sativus*); (ii) avoidance assays with *Eisenia fetida* and *Folsomia candida* based on Net Response (%); and (iii) assignment to ecological quality classes. In addition, we propose a list of potential green infrastructure applications associated with each ecological quality class. Most bioremediated soils showed no phytotoxic effects and were classified as high ecological quality. Multivariate analysis indicated that environmental variability was structured by texture, nutrient content, and pH rather than residual hydrocarbon concentrations. Hydrocarbon levels alone did not explain biological responses: *E. fetida* avoidance was associated with nutrient gradients and texture, while *C. sativus* germination was related to texture components. Microbial community composition, assessed through 16S rRNA sequencing, was strongly associated with nutrient gradients, reflecting integrated soil conditions rather than residual contamination. Overall, the results suggest that bioremediation may contribute to the improvement of soil ecological functionality beyond contaminant reduction. The proposed framework provides an ecological interpretation based on ecotoxicological endpoints and may support evidence-based reuse decisions aligned with the functional capacity of treated soils.

KEYWORDS
bioremediation, ecological assessment, hydrocarbon-contaminated soils, restoration ecology, soil ecological quality, soil reuse

1 Introduction

Bioremediation represents a sustainable and low-impact strategy for the management of contaminated soils, potentially contributing to the improvement of ecological functionality and the reintroduction of treated soils into circular economy pathways (1, 2). However, despite its growing application, the safe reuse of bioremediated soils still relies predominantly on chemical thresholds, which, although essential, often underestimate the ecological response of soil ecosystems and provide limited insight into the bioavailability of residual contaminants to biota. In most regulatory frameworks, soil quality assessment is primarily based on contaminant thresholds established through human health and environmental risk assessment procedures (Legislative Decree 152/2006, Part IV, Annex 5, Table 1, Column B (3)). These thresholds are generally designed to ensure an acceptable level of risks for specific land use scenarios and often rely on exposure modeling approaches (4). Although ecotoxicological information may contribute to the derivation of some regulatory values, post-remediation compliance is commonly assessed through contaminant concentrations rather than through direct measurements of biological responses or ecosystem functioning. Consequently, treated soils that comply with concentration-based criteria may still exhibit differences in biological functioning or habitat quality, highlighting the potential value of complementary ecological indicators (5, 6).

Soil contamination by petroleum hydrocarbons represents one of the most widespread forms of soil degradation associated with industrial activities, fuel storage, transport accidents and legacy pollution sites (7). Across Europe, large areas of land are affected by hydrocarbon-contaminated soils, posing long-term risks to ecosystem services, human health and land reuse potential (8). Hydrocarbons can persist in soil matrices due to their hydrophobic nature, strong sorption to organic matter and limited bioavailability, leading to chronic contamination even after remediation interventions (9, 10). In addition to their direct toxicity, petroleum hydrocarbons alter key soil properties, including porosity, water retention, nutrient cycling and redox conditions, thereby affecting biological functioning (11).

Over the past decades, bioremediation techniques have been increasingly adopted to treat hydrocarbon-contaminated soils, owing to their cost-effectiveness and lower environmental impact compared to physical or chemical treatments (12). These approaches rely on the metabolic activity of indigenous or stimulated microbial communities to degrade contaminants, often leading to substantial reductions in total petroleum hydrocarbons (13).

However, the achievement of chemical cleanup targets does not necessarily correspond to the recovery of soil ecological quality. Several studies have shown that soils meeting regulatory concentration limits may still exhibit impaired biological activity, altered community structures and reduced capacity to support soil organisms and vegetation, raising concerns about their actual suitability for reuse (14–17). This discrepancy can be explained by the fact that standard chemical analyses do not account for mixture effects, bioavailability, or sublethal toxicity. Residual contaminants, even at low concentrations, may interact synergistically or cumulatively, leading to biological effects that are not predictable from individual compound thresholds (18). Moreover, chemical measurements typically quantify total concentration rather than the biologically available fraction, which is more directly related to organism exposure and ecological impact (19).

In this context, soil quality assessment is progressively shifting from a purely chemical compliance-based approach toward an integrated evaluation of soil functionality. Current soil monitoring frameworks at the European level emphasize the need to incorporate biological indicators capable of capturing sub-lethal effects, cumulative stress and ecosystem-level responses (5, 6, 20). Recent initiatives, including the EU Soil Strategy for 2030 and the proposed Soil Monitoring Law, increasingly recognize the need to integrate biological indicators into soil assessment schemes to complement chemical monitoring and better reflect ecosystem functionality (5, 21). Biological indicators respond to contaminant bioavailability rather than total concentrations and provide early-warning signals of residual toxicity or functional imbalance that are often overlooked by chemical analyses alone (19). However, despite increasing regulatory attention to biological indicators, there is still a lack of integrative assessment frameworks capable of linking chemical thresholds, soil functionality, operational reuse criteria and only limited evidence is available regarding their application in full-scale bioremediation facilities. Most available studies remain confined to laboratory conditions or isolated endpoints, limiting their applicability to real remediation treatment plants (22). Consequently, there is still limited evidence supporting the operational integration of biological endpoints into reuse decision-making processes at industrial-scale remediation.

Among biological tools, ecotoxicological tests using soil-dwelling organisms have gained increasing relevance in the assessment of contaminated and remediated soils (23). Edaphic organisms play a fundamental role in soil structure formation, organic matter turnover and nutrient cycling, and their responses are closely linked to soil health (17). Earthworms such as *Eisenia fetida* are widely recognized as key indicators due to their sensitivity to a broad range of contaminants, their ecological relevance and standardized testing protocols (24). Earthworm avoidance behavior has been proposed as a rapid and integrative endpoint, reflecting habitat suitability and bioavailable toxicity rather than acute lethal effects (25).

Similarly, collembolans such as *Folsomia candida* represent essential components of soil mesofauna and are highly sensitive to changes in soil chemical and physical conditions. Their feeding activity, reproduction and mobility are closely associated with microbial communities and organic matter quality, making them particularly suitable indicators for post-remediation assessments (26, 27). Avoidance and reproduction tests with *F. candida* have been shown to detect residual toxicity in treated soils where chemical concentrations fall below regulatory thresholds, highlighting their value in evaluating functional recovery (24, 25).

Despite the recognized sensitivity and ecological relevance of these organisms, their application in post-bioremediation scenarios remains limited and fragmented, often confined to laboratory-scale studies or isolated endpoints. Moreover, the integration of soil fauna responses with microbial community structure and phytotoxicity assessments is still rarely applied in industrial-scale remediation contexts. This limits the ability to translate biological responses into operational criteria for soil classification and reuse decision-making (1, 28).

The present study addresses this gap by proposing an integrated framework to support the assessment of the suitability of biologically treated soils for landscape revegetation and urban green infrastructure applications. We applied this framework to 13 full-scale bioremediated soils originally contaminated with aliphatic hydrocarbons. The approach combines chemical–physical characterization, plant-based phytotoxicity assays, soil fauna avoidance tests (*E. fetida* and *F. candida*), and microbial community profiling. By linking biological endpoints to environmental gradients, the study aims to evaluate whether residual hydrocarbon concentration alone adequately reflects soil ecological functionality and reuse suitability.

As a first step, we developed a methodological framework for interpreting ecotoxicological data and proposing a classification approach for soil reuse. This framework was preliminarily evaluated through its application to a set of 13 bioremediated soils, allowing an initial assessment of its applicability and highlighting the importance of integrating biological responses into post-remediation evaluations.

Specific chemical, physical and microbiological parameters were subsequently examined to identify the factors most strongly influencing avoidance and attraction behaviors, thereby contributing to the interpretability of ecotoxicological results and supporting a more comprehensive evaluation of soil ecological functionality.

2 Materials and methods

2.1 Chemical analysis

Soil physicochemical properties were determined according to official reference methods. Soil pH (H_2O) was measured potentiometrically in a 1:2.5 soil-to-solution suspension following standard procedures (29). Total carbon (C_tot) and total nitrogen (N_tot) were determined by dry combustion using an elemental analyzer (Flash EA 1112 NCSoil, Thermo Fisher Scientific, Pittsburgh, PA, USA) after preparing and sieving at 0.2 mm the fine earth (ISO 10694:1995) (30). In carbonate-containing samples, organic carbon (C_org) was calculated by subtracting inorganic carbon derived from total carbonate content (ISO 10693:1995) (31) from total carbon. Total carbonate content ($CaCO_3$) was quantified by volumetric calcimetry through CO_2 evolution after HCl addition.

Total phosphorus (P_tot) was determined by acid digestion followed by spectrophotometric detection according to Official Method XV.1 (Supplemento Ordinario G.U. n° 248/1999). Organic phosphorus (P_org) was measured after thermal treatment and acid extraction (29), and available phosphorus (P_ass) was determined using the Olsen method (32).

Soil texture (sand, silt, clay fractions) was assessed using the pipette method based on sedimentation principles (pipette method; 33). Hydrocarbon concentration (C > 12 fraction, HC) was quantified in ppm according to standard procedures adopted by the remediation facility.

All analyses were performed on air-dried, 0.2–2 mm sieved soil samples. Results were expressed on a dry weight basis.

2.2 Phytotoxicity analysis

Seed germination and root elongation tests were conducted on *Lepidium sativum* L. (1753) and *Cucumis sativus* L. (1753), two species widely employed in phytotoxicity assessments due to their rapid germination and documented sensitivity to soil contaminants and physicochemical conditions. The use of two plant species was intended to reduce species-specific bias and provide a broader evaluation of potential phytotoxic effects (34). The tests were performed following previously described procedures (35). Each soil sample was divided into eight replicates (four for *L. sativum* and four for *C. sativus*, 10 g each). Water was added to reach 110% of the water holding capacity, and each replicate was suspended in 5 mL of distilled water and applied to Petri dishes lined with filter paper. Ten seeds were placed on the moistened filter paper, and the dishes were sealed and incubated at 25 °C for 72 h.

At the end of the incubation period, the Germination Index (GI%) was calculated as indicated in Equation (1):

$$GI\,\%\ (GERMINATION\ \ INDEX)\ (x) = \frac{Gs*Ls}{Gc*Lc}*100 \tag{1}$$

- Gs = Percentage of germinated seeds in the sample (%)
- Ls = Mean root length in the sample (mm)
- Gc = percentage of germinated seeds in the control (%)
- Lc = Mean root length in the control (mm)

2.3 Ecotoxicological analysis and quality classification

Earthworms: *Eisenia fetida* (Savigny, 1826) were incubated at controlled conditions of 20 ± 2 °C of temperature, and 16:8 h light-dark and collected from a vermicompost (50% sphagnum peat and 50% cattle dung). Earthworm cocoons were periodically separated from the culture to which earthworm cohorts synchronized for tests. Only adult worms with well-developed clitellae and fresh weight between 250–300 mg were randomly selected for the behavioral tests.

Collembolans: *Folsomia candida* (Willem, 1902) were cultured in glass Petri-dishes (∅ = 8.5 cm) on a moist substrate of plaster of Paris and activated charcoal (8:1) and maintained at temperature of 20 ± 2 °C, photoperiod regime of 16:8h light:dark and fed weekly with dried baker's yeast *(Saccharomyces cerevisae)*. Collembola eggs were periodically separated from the culture to obtain synchronized individuals for tests. Only adults 20 days after hatching were selected.

The two species were selected because they represent complementary ecological compartments of the soil fauna. *E. fetida* is a widely used indicator of soil habitat suitability and organic matter quality, whereas *F. candida* is representative of the mesofauna and is particularly sensitive to changes in soil physicochemical and microbiological conditions. Their combined use provides a broader assessment of ecological functionality than either species alone (17, 22, 26).

The ecological quality in terms of habitat functions was assessed performing the avoidance behavioral tests (30, 31) to evaluate the effects of soils on the behavior of *E. fetida* (Savigny, 1826) and *F. candida* (Willem, 1902) after 48 hours of exposure, in comparison with a control soil. Avoidance assays were conducted in transparent vessels divided into two compartments: one containing the test soil and the other LUFA 2.2 (Speyer, Germany) soil, which represent a standard soil reference for laboratory experiments. The properties of this soil include a pH = 5.5 ± 0.2 (0.01 M CaCl2), WHC = 44.5 ± 3.0 (g/100 g), C = 1.77 ± 0.2 (%), N = 0.17 ± 0.02, texture = 7.3 ± 1.2 (%) clay; 13.8 ± 2.7 (%) silt and 78.9 ± 3.5 (%) sand. After removal of the divider, ten organisms were placed in the center and exposed under a 16:8 h light/dark cycle. Responses after 48 hours were expressed as Net Response Percentage (NR%), which was calculated as indicated in Equation (2):

$$NR\%\ (NET\ \ RESPONSE)\ (x) = \frac{nc - nt}{N} * 100 \qquad (2)$$

- nc = number of organisms found in the standard soil after 48 h
- nt = number of organisms found in the test soil after 48 h
- N = total number of introduced organisms

The following criteria, set out in Table 1, shall be used to assess habitat functionality. The threshold values adopted in this study correspond to those proposed by UNI EN ISO 17512 (25) for the interpretation of habitat functionality based on avoidance responses.

Building upon the ISO habitat functionality thresholds (Table 1), a simplified three-class ecological quality scheme was proposed to facilitate the interpretation of avoidance responses according to the criteria set out in Table 2. The classification was intended as an operational tool differentiating treated soils showing no partial or marked evidence of functional limitation.

TABLE 1 **Association between net response threshold % and habitat functionality.**

Assessment of habitat functionality	
Limits of net response % (NR%)	Habitat functionality
NR%>80% tested using *E. fetida*	the functionality of the habitat is limited
NR%>70% tested using *F. candida*	the functionality of the habitat is limited

2.4 Microbiological analyses

Bacterial community composition was characterized by targeting the V5–V6 hypervariable regions of the 16S rRNA gene, following previously described protocols (Wang and Qian, 2009). Total genomic DNA was extracted from soil samples using the FastDNA™ Spin Kit (MP Biomedicals, USA) according to the manufacturer's instructions.

For community profiling, the V5–V6 region was amplified using primers F783/R1027 as previously reported (36).

PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA) and sequenced on an Illumina MiSeq platform with a 2x300 bp paired-end protocol (Illumina Inc., San Diego, CA, USA). Raw sequences were processed using the DADA2 pipeline to infer amplicon sequence variants (ASVs), remove chimeras, and generate a high-resolution ASV table for downstream analyses (37).

2.5 Statistical analysis

To investigate the relationships among soil physicochemical properties, microbial community structure, and biological responses at the end of the bioremediation process, an integrative multivariate statistical framework was applied to explore potential ecological associations among environmental variables, microbial community structure and biological responses. Considering the potential collinearity among environmental variables, dimensionality reduction techniques were used to reduce model complexity and avoid overfitting. All analyses were performed in R (version 4.4.1), using the packages vegan and stats (38).

2.5.1 Variables describing texture and nutrient gradients

Soil texture is described by three strongly interdependent variables (sand, silt, and clay). Therefore, a Principal Component

TABLE 2 **Association between net response limit value % and class.**

Ecological quality classification	
Limits of net response % (NR%)	Classes
both avoidance values exceed the NR% limits	C
only one of the avoidance values exceeds the NR% limits	B
both avoidance values are below the NR% limits	A

Analysis (PCA) was performed on centered and standardized variables (scale = TRUE) to summarize the main gradients of variation while reducing collinearity among predictors (39). PCA was implemented using the rda() function in the vegan package without constraints. The because first two principal components were retained because together they captured the majority of the texture-related variance and provided synthetic descriptors of soil structural variability (PC1Texture 64.1% and PC2Texture 35.9%). To describe soil nutrient balance, total carbon and nitrogen (expressed in g kg^{-1}) and total phosphorus were used to calculate molar ratios (C:N, C:P, and N:P) (40). Log-transformed molar ratios were subjected to PCA to derive a single synthetic variable summarizing the dominant nutrient gradient across treated soils. The first principal component, explaining the largest proportion of variance, was retained as a synthetic descriptor of soil nutrient status (PC1Elements).

2.5.2 Variables describing microbial community structure

After removing singleton ASVs, the abundance matrix was Hellinger-transformed to account for compositional structure and differences in sequencing depth (41). PCA was subsequently applied to the transformed matrix using the rda() function. The first two principal components (PC1Micro and PC2Micro) were retained to describe dominant gradients in microbial community structure. The PCA on the microbial community was used as an exploratory tool to summarize major patterns of community turnover and reduce the dimensionality of the ASV dataset prior to integration with environmental variables.

2.5.3 Integrated environmental ordination

The integrated ordination was designed to evaluate whether the main environmental gradients identified in the previous analyses were associated with biological responses. To avoid excessive model complexity relative to sample size, only the synthetic descriptors derived from previous PCAs (texture, nutrient balance and microbial structure) together with pH and hydrocarbon concentration were retained for the final analysis. The final PCA on standardized variables including pH, hydrocarbon concentration (HC), PC1Texture, PC2Texture, and PC1Elements (39) was carried out. Pearson correlation coefficients were calculated to verify that retained environmental descriptors were not strongly collinear ($|r| < 0.6$). This preliminary screening was used to reduce redundancy among predictors and limit multicollinearity in the subsequent analyses. Biological response variables, avoidance responses for *E. fetida* and *F. candida*, and germination indices for *C. sativus* and *L. sativum* were projected onto the environmental ordination space using permutation-based vector fitting (envfit function in vegan, 999 permutations) (42). Because bioremediated soils samples originated from two independent treatment facilities (PL1 and PL2), site identity was considered during the interpretation of ordination patterns as a potential source of environmental variation.

2.5.4 Regression analysis

To assess the relative contribution of individual gradients, simple linear models were fitted using hydrocarbon concentration (HC), PC1Elements, PC1Micro, and texture components as predictors of ecotoxicological responses. Due to the strong correlation between PC1Elements and PC1Micro ($r = -0.92$), these variables were not included simultaneously in the same model to avoid multicollinearity. Given the exploratory nature of the study and the limited sample size, model complexity was intentionally reduced, and regression results were interpreted primarily as indicators of potential ecological associations rather than evidence of causal relationships.

3 Results

3.1 Proposed decision framework

The proposed decision framework (Figure 1) was designed as a post-remediation evaluation tool specifically for treated soils that already comply with the Italian regulatory limits for reuse (Legislative Decree 152/2006, Part IV, Annex 5, Table 1, Column B). Within this context, the framework does not replace chemical compliance assessment but builds upon it to provide an ecological and functional evaluation of bioremediated soils suitability. Beyond assigning an ecological quality class, the workflow supports decision-making on whether treated soils may be considered for different reuse scenarios, including technical applications (e.g., infrastructure works) and ecological green application, with suggested end-uses associated with each quality class.

This approach addresses a key limitation of the current regulatory system, which primarily distinguishes between urban and industrial land uses without considering ecological functionality (Legislative Decree 152/2006, Part IV, Annex 5, Table 1, Column B). Therefore, treated soils are often downcycled into low-value applications (e.g., road sub-bases, embankments, guardrail backfilling), resulting in a loss of potential to support biological processes. The proposed framework introduces a function-based classification to better align the properties of bioremediate soils with appropriate reuse options.

The framework was preliminarily applied to all 13 bioremediated soils as a proof-of-concept assessment of its applicability in full-scale remediation contexts through a sequential, three-phase approach: (i) phytotoxicity screening; (ii) avoidance assays with soil invertebrates; and (iii) assignment to an ecological quality class (A–C).

All bioremediated soils met the regulatory chemical limits established for reuse. Phytotoxicity was evaluated using a Germination Index (GI) threshold of 90%, following Ambaye et al. (1). In line with an inclusive approach, the framework applies an "OR" criterion: treated soils were considered suitable to proceed if GI exceeded 90% in at least one test species (*L. sativum* or *C. sativus*), while treated soils showing GI < 90% in both species would be classified as unsuitable. This decision rule was adopted to account for species-specific differences in phytotoxicity responses and to avoid excluding bioremediated soils based on a response observed in a single plant species. Because the framework was conceived as a first-tier screening tool for treated soils already compliant with chemical criteria, exclusion was restricted to cases

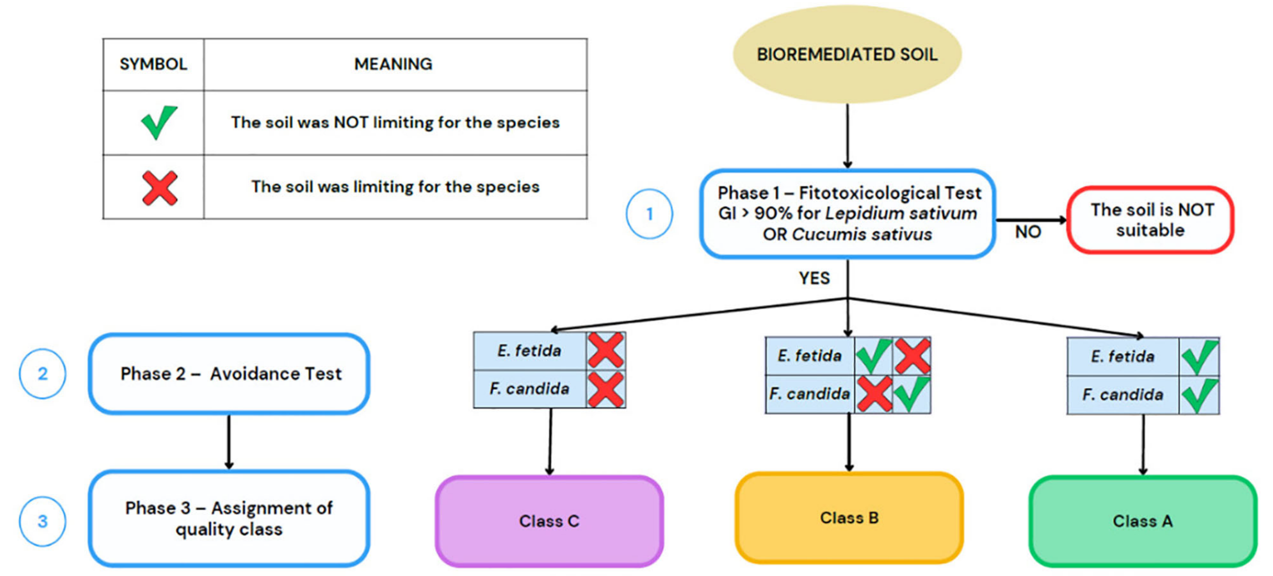

FIGURE 1
The workflow comprises sequential phases: Phase 1 - phytotoxicity screening (GI > 90% for *L. sativum* and *C. sativus*); Phase 2 - avoidance tests with *E. fetida* and *F. candida*; Phase 3 - assignment to ecological quality class (A–B–C).

showing consistent phytotoxic effects across both plant models. Under this criterion, none of the tested soils were excluded after Phase 1.

Phase 2 consisted of avoidance assays with *E. fetida* and *F. candida* (25). Behavioral responses were interpreted using species-specific Net Response Percentage (NR%) thresholds. The combined outcomes of these assays informed the final classification in Phase 3, assigning bioremediated soils to: Class A (no functional limitation), Class B (moderate functional restriction), or Class C (limited ecological suitability).

The ecological quality classes were interpreted in relation to the typology of urban Green Infrastructure (GI) proposed by Jones et al. (43), particularly with respect to the capacity to support biodiversity. Accordingly, Class C corresponds to GI types with negligible to low biodiversity support, Class B to medium and high levels, and Class A to very high biodiversity support. This linkage provides a direct connection between ecotoxicological responses and GI planning criteria, enabling the translation of classification results into potential reuse scenarios.

Importantly, avoidance tests are not used as a standalone exclusion criterion but represent the key step for differentiating ecological functionality among compliant and non-phytotoxic treated soils. Therefore, the framework integrates chemical, phytotoxicological, and behavioral endpoints into a tiered decision scheme that was designed to support suitability screening and function-based classification, ultimately guiding the selection of appropriate reuse options for each class.

Overall, although all treated soils complied with chemical standards, the sequential biological assessment revealed variability in ecological functionality, leading to differentiated quality classifications.

3.2 Chemical analysis

The thirteen bioremediated soils exhibited overall comparable chemical characteristics, with site-specific differences between PL1 and PL2 (Table 3).

All treated soils were slightly alkaline, with pH values ranging between 7.5 and 7.9. Total $CaCO_3$ content varied substantially, being consistently higher in PL2 samples (159–208 g kg^{-1}) compared to PL1 (96–115 g kg^{-1}), indicating a stronger calcareous buffering capacity in PL2 bioremediated soils (44).

Organic carbon content showed marked variability across samples. PL1 bioremediated soils ranged between 1.5–2.5% Corg, whereas PL2 samples reached higher values (up to 6.3% Corg). Total nitrogen followed a similar pattern, resulting in C/N ratios generally within agronomic equilibrium ranges (approximately 9 - 12) (40, 45). However, two samples showed higher C/N ratios (>14–15), which may indicate slower organic matter decomposition and potential accumulation of organic substrates. This interpretation is consistent with the higher organic carbon content observed in these treated soils (PL2-BIO 2 to PL2-BIO 6).

Total phosphorus (Ptot) ranged from 0.96 to 2.52 g kg^{-1}, with the highest concentrations recorded in PL2-BIO3 and PL2-BIO4. Available phosphorus (Pass) showed pronounced variability, particularly in PL2 samples, where values exceeded 200 mg kg^{-1} in two cases.

Texture analysis revealed structural differences between sites. PL1 bioremediated soils were predominantly sandy (sand content up to 768 g kg^{-1}), whereas PL2 bioremediated soils exhibited finer textures with higher silt and clay fractions, particularly in PL2-BIO3 to PL2-BIO6.

TABLE 3 Summary of the chemical and physical characteristics of the treated soils samples.

Name	pH H_2O	$CaCO_3$ g kg^{-1}	C tot %	C org %	N tot %	P tot g kg^{-1}	P org g kg^{-1}	P ass mg kg^{-1}	Sand g kg^{-1}	Silt g kg^{-1}	Clay g kg^{-1}	HC C>12 (ppm)
PL1-BIO1	7.5	96	2.87	1.72	0.14	1.25	0.25	24.64	768	164	68	600
PL1-BIO2	7.6	109	3.26	1.96	0.15	1.54	0.20	167.20	669	225	106	500
PL1-BIO3	7.6	97	2.68	1.51	0.18	1.60	0.45	81.01	489	325	186	550
PL1-BIO4	7.7	105	3.75	2.49	0.25	1.61	0.19	37.61	493	330	177	660
PL1-BIO5	7.9	107	2.84	1.56	0.14	1.15	0.23	29.32	476	342	182	609
PL1-BIO6	7.8	110	3.44	2.12	0.24	1.94	0.41	38.54	497	322	181	276
PL1-BIO7	7.7	115	3.70	2.32	0.27	1.99	0.33	40.42	486	328	186	449
PL2-BIO1	7.5	160	3.81	1.89	0.16	0.96	0.57	24.91	486	339	175	151
PL2-BIO2	7.9	170	6.51	4.47	0.28	1.20	0.33	55.94	576	269	155	229
PL2-BIO3	7.7	206	7.84	5.36	0.32	2.52	0.23	242.35	403	503	94	564
PL2-BIO4	7.5	200	8.18	5.78	0.36	2.47	0.27	239.16	441	461	98	564
PL2-BIO5	7.6	159	4.42	2.51	0.20	1.11	0.48	36.38	561	280	159	100
PL2-BIO6	7.8	208	8.84	6.34	0.30	1.65	0.29	73.31	417	487	96	750

Residual hydrocarbon concentrations (C>12) ranged between 100 and 750 mg kg^{-1}. Values above 500 mg kg^{-1} were observed in several samples from both sites, indicating the presence of residual hydrocarbons despite bioremediation.

Overall, the dataset indicates that variability among samples was not limited to hydrocarbon concentration but extended to nutrient balance, carbonate content, and texture components, suggesting the presence of multiple environmental gradients structuring the system. In addition, several of these gradients showed a clear site-related pattern, particularly between PL1 and PL2 samples.

3.3 Phytotoxicity analysis

The phytotoxicity assays revealed species-specific responses across the bioremediated soils (Figure 2).

For *L. sativum*, the Germination Index (GI%) values were generally high across all samples. Most treated soils showed GI values equal to or above the 90% threshold required to pass Phase 2 of the framework. Several samples exhibited GI values exceeding 120%, with peak responses reaching approximately 150–160%, indicating stimulation relative to the control (46, 47).

In contrast, *C. sativus* displayed greater sensitivity. While several treated soils maintained GI values above the 90% threshold, multiple samples fell below this limit, indicating potential residual phytotoxic effects according to the adopted criteria. Compared to *L. sativum*, *C. sativus* responses showed narrower margins above the threshold, and a higher frequency of values close to or below 90%.

Site-specific patterns were also observed. PL1 samples exhibited wider variability, including both strongly stimulatory and inhibitory responses. PL2 samples were more clustered within the 90–120% range, showing moderate stimulation and fewer marked inhibitory effects.

Overall, all treated soils complied with the phytotoxicity criterion required to proceed to Phase 3 of the normative framework. However, the differential sensitivity between plant species highlights the importance of using multiple test organisms to detect residual biological effects.

To evaluate the influence of the adopted decision rule, the phytotoxicity results were also interpreted using a more conservative AND criterion, requiring GI values above 90% in both plant species before proceeding to the subsequent assessment phase. Under this criterion, three tested soils (PL1.Bio1, PL2.Bio3 and PL2.Bio4) were flagged because GI values below the threshold were observed only in *Cucumis sativus*, whereas the remaining ten treated soils would have been classified identically under both decision rules. This comparison shows that the overall classification remained largely consistent under both approaches, differing only for three of the thirteen tested soils.

In the present study, the discrepant classifications were exclusively associated with the response of *C. sativus*, whereas *Lepidium sativum* exceeded the phytotoxicity threshold in all treated soils. These cases therefore reflect species-specific phytotoxicity responses rather than consistent evidence of ecological impairment. Such interspecific differences are well documented in phytotoxicity bioassays and support the use of multiple plant species when assessing contaminated or bioremediated soils, as plant responses may vary depending on both residual contaminants and soil physicochemical characteristics (34, 48). Consequently, the OR criterion was retained as an inclusive first-tier screening approach for chemically compliant treated soils, while species-specific reductions in GI were interpreted as precautionary signals to be considered in conjunction with the subsequent soil-fauna avoidance tests, thereby providing a more comprehensive assessment of soil ecological functionality.

3.4 Ecotoxicological analysis and quality classification

Avoidance assays revealed clear differences among treated soils and between test species (Figure 3). Net Response Percentage (NR %) values were interpreted using species-specific thresholds (80%

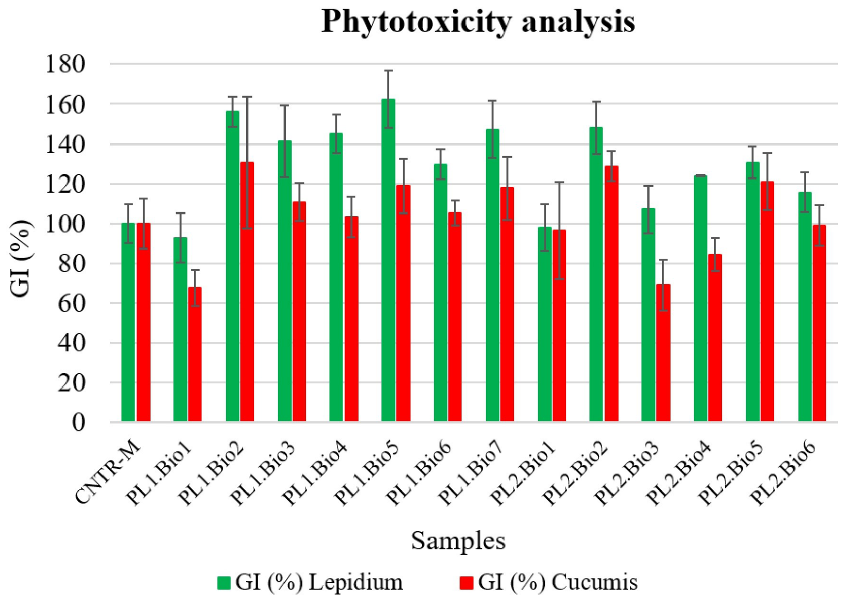

FIGURE 2
Phytotoxicity tests conducted with *Lepidium sativum* (green bars) and *Cucumis sativus* (red bars).

for *E. fetida*; 70% for *F. candida*). Responses varied across samples, with patterns differing between PL1 and PL2.

In PL2 bioremediated soils, avoidance thresholds were exceeded in only one case, and this was limited to *F. candida.* Most PL2 samples showed NR% values between −20% and +20%, indicating neutral responses. In several cases, NR% values approached -40%, suggesting attraction toward the tested soils rather than avoidance (24, 49).

In contrast, PL1 samples exhibited more pronounced responses. For *E. fetida*, avoidance exceeded the threshold in three samples (PL1-BIO1, PL1-BIO2, and PL1-BIO3). For *F. candida*, the threshold was exceeded only in PL1-BIO1. The remaining PL1 samples showed moderate or neutral responses. These results suggest greater functional heterogeneity among PL1 treated soils compared to PL2, where responses were more homogeneous and generally within non-limiting ranges.

Based on the combined responses of the two species, soils were assigned to ecological quality classes (Table 4). Most samples were classified as Class A, indicating no functional limitation. Four samples (PL1-BIO2, PL1-BIO3, PL2-BIO4) were assigned to Class B due to threshold exceedance in one species. Only one sample (PL1-BIO1) was classified as Class C, reflecting threshold exceedance in both species.

Overall, although all treated soils complied with chemical and phytotoxic criteria, avoidance assays provided additional ecological discrimination, revealing variability in habitat functionality not captured by earlier screening phases.

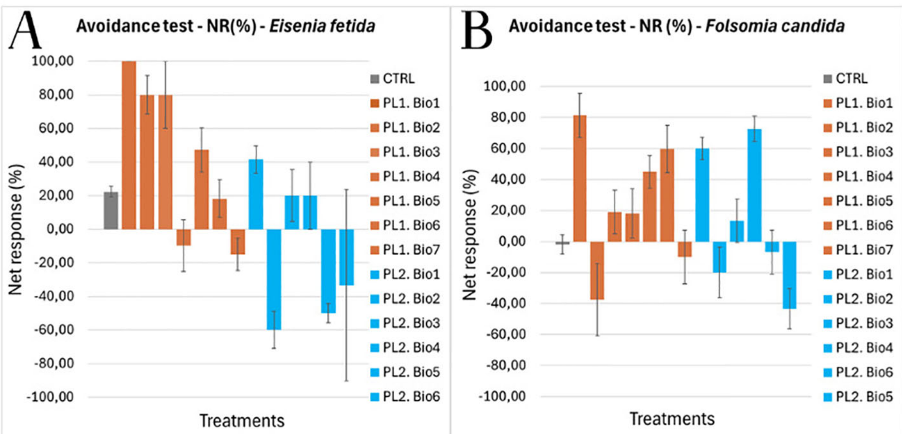

FIGURE 3
(A) Avoidance test conducted with *Eisenia fetida*: the blue bars represent samples from PL2, while the orange bars represent samples from PL. The gray bar represents control. **(B)** Avoidance test conducted with *Folsomia candida*: the blue bars represent samples from PL2, while the orange bars represent samples from PL. The gray bar represents control.

TABLE 4 **Summary of quality classes associated with each soil sample according to the framework (phase 3).**

Sample	Class A	Class B	Class C
PL1-BIO-1			X
PL1-BIO-2		X	
PL1-BIO-3		X	
PL1-BIO-4	X		
PL1-BIO-5	X		
PL1-BIO-6	X		
PL1-BIO-7	X		
PL2-BIO-1	X		
PL2-BIO-2	X		
PL2-BIO-3	X		
PL2-BIO-4		X	
PL2-BIO-5	X		
PL2-BIO-6	X		

3.5 Integrative statistical analysis: determining factors and site signatures

3.5.1 Ordination analysis

A first Principal Component Analysis (PCA) (Figure 4) was performed on standardized environmental descriptors (pH, hydrocarbon concentration, PC1Texture, PC2Texture, and PC1Elements) to identify the main gradients structuring soil variability after bioremediation.

The first two principal components explained 59.5% of the total variance (PC1: 30.9%; PC2: 28.6%), indicating that environmental variability was distributed across multiple axes rather than dominated by a single factor. PC1 was primarily associated with hydrocarbon concentration and texture-related components, representing a combined contamination–structure gradient. In contrast, PC2 was strongly associated with pH and inversely related to the nutrients component (PC1Elements), reflecting a chemical–nutrient balance gradient.

Importantly, within the range of concentrations observed in the present dataset, hydrocarbon concentrations were not among the dominant variables structuring the principal components. Instead, residual contamination was embedded within a broader multidimensional gradient integrating structural and physicochemical properties.

Sample distribution also reflected a degree of separation between the two treatment facilities (PL1 and PL2), suggesting that site-related characteristics may have contributed to the observed environmental gradients.

Permutation-based vector fitting (envfit) revealed species-specific associations with these gradients. Among avoidance endpoints, *E. fetida* avoidance showed a significant relationship with the environmental ordination ($r^2 = 0.51$, $p = 0.030$), with the vector primarily aligned along PC2. In contrast, *F. candida* did not show significant association. Although these associations should be interpreted as exploratory, they suggest that biological responses were structured along integrated environmental gradients.

Among phytotoxicity endpoints, *C. sativus* GI was significantly associated with environmental gradients ($r^2 = 0.45$, $p = 0.040$), reflecting combined alignment across PC1 and PC2. *L. sativum* did not show significant association.

These results indicate that biological responses were structured along integrated environmental gradients rather than along a single contamination axis.

A second Principal Component Analysis (PCA) (Figure 5) performed on Hellinger-transformed ASV data revealed structured variation in bacterial community composition across samples. The first axis (PC1) explained 28.8% of total variance and represented

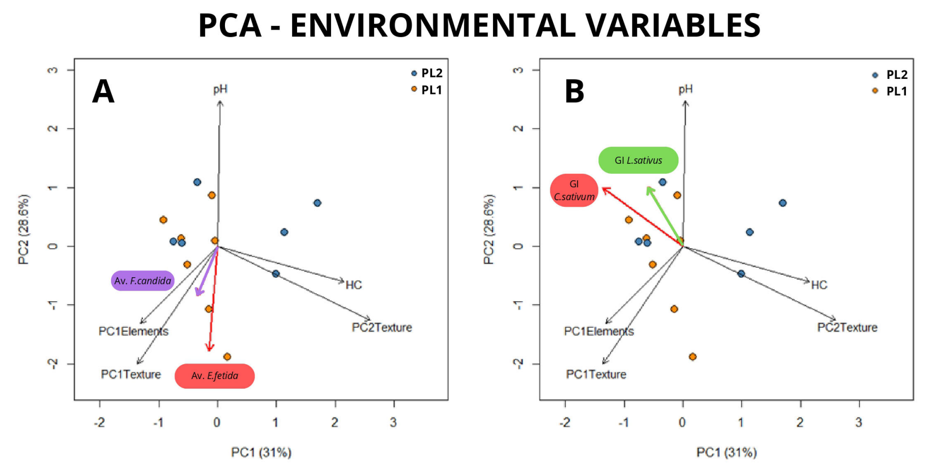

FIGURE 4
Principal Component Analysis (PCA) of standardized environmental descriptors (pH, hydrocarbon concentration, texture components, and stoichiometric component) across the 13 bioremediated soils. PC1 (30.9%) and PC2 (28.6%) together explained 59.5% of total variance. Arrows represent environmental loadings, and sample points correspond to individual treated soils. **(A)** Avoidance responses of *Eisenia fetida* (red arrow) and *Folsomia candida* (purple arrow). **(B)** Germination indices of *Cucumis sativus* (red arrow) and *Lepidium sativum* (green arrow). Biological endpoints were projected onto the ordination using permutation-based vector fitting (999 permutations).

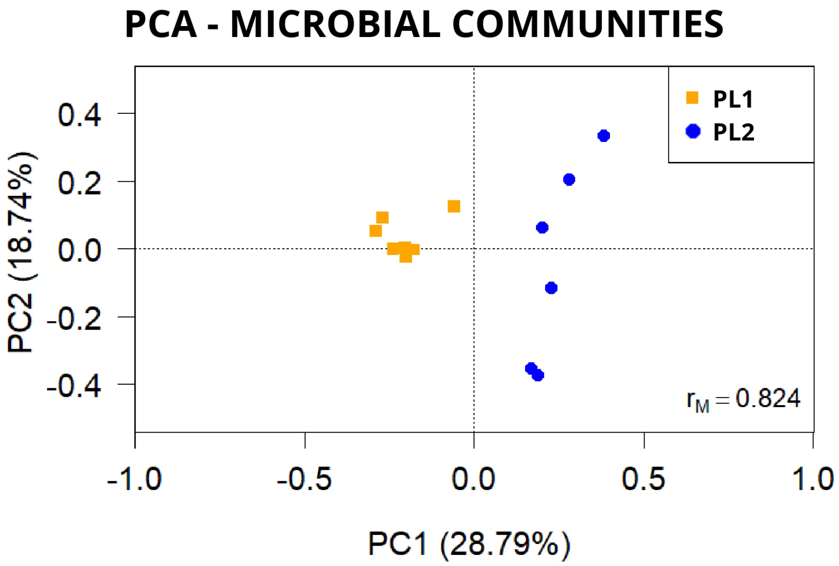

FIGURE 5
Principal Component Analysis (PCA) of Hellinger-transformed ASV data from the 13 bioremediated soils. PC1Micro (28.8%) represents the dominant gradient of community turnover. Sample separation reflects site-related differences and underlying physicochemical gradients.

the dominant gradient of community turnover among soils. This axis clearly separated PL1 and PL2 samples, indicating consistent site-related structuring of bacterial assemblages.

PC1Micro showed a strong negative correlation with the stoichiometric component derived from environmental PCA (PC1Elements; $r = -0.92$), indicating that bacterial community composition closely mirrored the main chemical–nutrient gradient structuring the soil system.

In contrast, correlation between PC1Micro and hydrocarbon concentration was moderate ($r = -0.37$), suggesting that residual contamination alone did not represent the primary driver of community composition.

The second microbial axis (PC2Micro) accounted for a smaller proportion of variance and showed moderate associations with texture-related components, indicating that soil physical structure contributed to secondary differentiation of microbial assemblages.

Overall, microbial community structure reflected integrated physicochemical gradients rather than hydrocarbon concentration alone.

3.5.2 Cross-system integration

The integrative statistical framework provided an exploratory comparison between environmental gradients, microbial community structure, and ecotoxicological responses.

The first PCA (Figure 4) revealed that hydrocarbon concentration did not dominate soil variability but was embedded within a multidimensional gradient integrating texture and physicochemical properties. Within this ordination space, permutation-based vector fitting identified species-specific associations.

Among avoidance assays, *E. fetida* was significantly associated with the environmental structure ($r^2 = 0.51$, $p = 0.030$), primarily aligned along the chemical–stoichiometric gradient (PC2). In contrast, *F. candida* did not show a significant association with the environmental ordination.

Among phytotoxicity assays, *C. sativus* showed a significant association with environmental gradients ($r^2 = 0.45$, $p = 0.040$), while *L. sativum* did not.

To further evaluate the relative contribution of individual environmental descriptors, simple linear models were fitted.

For *E. fetida* avoidance, hydrocarbon concentration alone did not significantly explain response variability. In contrast, the stoichiometric component (PC1Elements) significantly explained 38% of the variance ($p = 0.026$), whereas the microbial component (PC1Micro) showed a moderate but non-significant association ($R^2 = 0.20$, $p = 0.125$).

For *C. sativus* GI, hydrocarbon concentration, pH, PC1Texture, and PC1Micro were not significant predictors. However, the second texture component (PC2Texture) significantly explained 39.6% of the variance in germination index ($p = 0.021$), indicating structural control on plant response.

Across endpoints, hydrocarbon concentration did not emerge as the strongest explanatory variable when compared to integrated chemical and physical gradients. Given the limited sample size, these relationships should be interpreted as indicative of potential ecological associations rather than as evidence of generalizable causal mechanisms.

3.6 Characterization of the microbial community

Amplicon sequencing (Figure 6) revealed a diverse bacterial community across the bioremediated soils, with several genera exceeding 3% relative abundance in at least one sample. Community composition varied substantially among samples, indicating site-specific structuring.

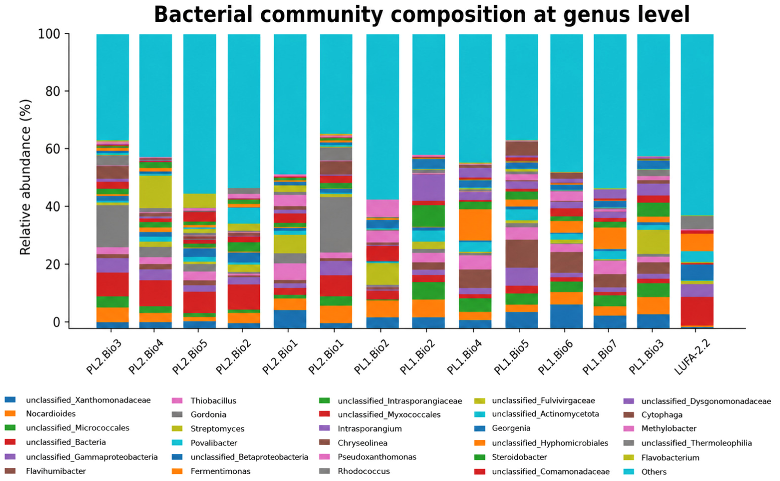

FIGURE 6
Relative abundance of dominant bacterial genera (>3% relative abundance in at least one sample) in bioremediated soils and the reference soil (LUFA 2.2). Stacked bar plots illustrate shifts in bacterial community composition at the genus level based on 16S rRNA gene sequencing (V5–V6 region). Genera exceeding 3% relative abundance in at least one sample are shown individually, whereas less abundant taxa were grouped under "Others".

Actinobacteria were consistently represented across samples, particularly genera such as *Gordonia*, *Nocardioides*, and *Streptomyces*. *Gordonia* reached high relative abundance in specific samples (e.g., PL2-Bio3: 13.5%; PL2-Bio6: 17.6%) but remained low in others.

Nocardioides maintained relatively stable abundance (2–6%) across most samples.

Streptomyces reached up to 7.8% in selected samples and were broadly distributed.

Proteobacteria and Bacteroidota were also prominent. Unclassified members of Xanthomonadaceae and Gammaproteobacteria frequently ranged between 3–10%, particularly in PL1-series samples. Genera belonging to Bacteroidota, including *Flavihumibacter* and *Flavobacterium*, exceeded 6–9% in several PL1 samples.

Thiobacillus was detected at relatively high abundance (4–5%) in selected samples (e.g., PL1-Bio2, PL1-Bio6).

The LUFA 2.2 reference soil exhibited a distinct composition, characterized by higher relative abundance of unclassified_Bacteria (11%) and unclassified Thermoleophilia (4.3%), and comparatively lower representation of genera observed in treated soils.

Within the proposed framework, microbial community profiling was not used as a criterion for ecological classification. Instead, it was included as a complementary line of evidence to support the interpretation of environmental gradients and biological responses.

Overall, microbial composition differed between PL1 and PL2 samples, reflecting variability in physicochemical background and treatment history including differences associated with the two remediation facilities.

4 Discussion

The proposed workflow provides a structured approach for exploring the suitability of post-bioremediation treated soils for landscape revegetation and green infrastructure applications. The framework is structured as a sequential, tiered decision process, including: phytotoxicity screening based on germination responses and avoidance assays with soil invertebrates, leading to the assignment of ecological quality classes.

This stepwise approach was designed to support the identification of suitability for reuse, and the differentiation of treated soils according to their ecological functionality. In this way, the framework supports the allocation of bioremediated soils to reuse options with different ecological values.

The results highlight that ecotoxicological responses are not solely driven by residual contaminant concentrations, but are influenced by a combination of factors, including organic matter content, nutrient availability, and other physicochemical properties of the treated soils. This is particularly relevant for disentangling the effects of contaminants from those related to substrate characteristics, which can independently affect organism behavior and plant performance. As a consequence, the integration of multiple biological endpoints within the proposed workflow allows a more robust interpretation of ecological compatibility, avoiding the risk of attributing observed effects exclusively to contamination levels.

4.1 Plant bioassays reveal species-specific sensitivity

Plant-based bioassays highlighted a clear species-specific pattern in post-bioremediation soils. *L. sativum* showed a broadly tolerant response across samples, with germination index values often close to or above control, whereas *C. sativus* displayed wider variability and a higher frequency of inhibitory responses (Figure 2). This divergence is consistent with evidence that seed germination and root elongation endpoints can vary substantially among test species and that cucumber is frequently selected as a sensitive sentinel in short-term phytotoxicity/root-elongation assays. These differences support the use of multi-species assays to avoid underestimating residual phytotoxicity when only one plant model is used (34, 50).

A second relevant aspect is that GI values >100% (i.e., apparent stimulation) are not uncommon in phytotoxicity screening and can reflect improved nutrient availability, reduced osmotic stress, or other soil extract properties relative to the control substrate (51). Importantly, international standards explicitly note that differences in nutrient levels between test and control soils may influence early-growth endpoints and can even generate misleading patterns if the control is substantially richer or poorer than the test matrix. For this reason, "stimulatory" values should be discussed cautiously, framed as relative performance in the chosen test conditions rather than interpreted as unequivocal enhancement of plant fitness (52).

In our dataset, the statistical signal for *C. sativus* is coherent with this ecological interpretation: germination variability was significantly associated with the texture-related gradient (PC2Texture), while hydrocarbon concentration was not a significant predictor (Figure 4). From a mechanistic perspective, texture governs water retention, aeration, and the balance between water and oxygen availability during imbibition and early root expansion; therefore, coarse vs. fine soil structure can shape germination outcomes independently of residual contaminant concentrations (53).

This finding is consistent with the hypothesis that post-remediation biological performance may be influenced by soil physical functionality in addition to contaminant concentration.

4.2 Avoidance tests capture subtle differences not detectable chemically

Avoidance assays (Figure 3) provided a sensitive behavioral endpoint capable of detecting differences among tested soils that were not predictable from hydrocarbon concentration alone. In the present study, most samples fell within the range indicative of habitat compatibility, and in several cases net attraction was observed. Behavioral avoidance tests are designed to detect sub-lethal stress and habitat preference rather than acute toxicity, and they are widely recognized as integrative indicators of soil quality because they respond to the bioavailable fraction of stressors and to the overall physicochemical suitability of the substrate (25, 54).

Within the concentration range observed in this study, an interesting outcome of the integrative statistical analysis was that hydrocarbon concentration (C>12) did not significantly explain avoidance responses when considered alone. Instead, *E. fetida* avoidance was significantly associated with the stoichiometric gradient (PC1Elements), which summarized variation in C: N:P balance (Figure 4). This suggests that nutrient balance and chemical equilibrium, rather than residual hydrocarbon levels per se, structured earthworm behavioral responses (55). Soil faunae are known to respond strongly to pH, organic matter quality, and nutrient stoichiometry, which influence microbial activity, food resource quality, and burrowing conditions (56). Therefore, the observed association suggests that post-bioremediation soils may exert ecological effects through different nutrient balance rather than through direct contaminant toxicity under the conditions tested in this study.

In contrast, *F. candida* did not show a statistically significant alignment with the environmental ordination. This difference between species reinforces the importance of multi-organism bioassays. Earthworms and collembolans occupy distinct ecological niches and differ in exposure pathways, feeding strategies, and sensitivity thresholds. Avoidance responses in earthworms often integrate soil structure, moisture, and chemical conditions, whereas collembolans may respond more directly to microhabitat properties and microbial composition (25, 54). The absence of a strong signal for *F. candida* therefore does not indicate ecological neutrality, but rather species-specific sensitivity patterns.

Overall, these findings challenge the assumption that residual hydrocarbon concentration is the primary determinant of ecological compatibility in remediated soils. Instead, avoidance behavior appears to reflect multidimensional gradients of soil functionality, particularly nutrient balance and physicochemical equilibrium. This supports the conceptual shift from contaminant-centered assessment toward functional ecological evaluation in post-bioremediation contexts.

4.3 Microbial structure reflects physicochemical gradients and site specificity

In the context of the present study, microbial community profiling was included primarily as a complementary source of ecological information rather than as a direct component of the classification framework.

The 16S rRNA-based profiling revealed diverse and structured bacterial communities across the bioremediated soils, with substantial differences in relative abundance patterns among samples. Actinobacteria such as *Gordonia*, *Nocardioides*, and *Streptomyces* were recurrently detected, alongside representatives of Proteobacteria and *Bacteroidota* (Figure 6). These taxa are commonly reported in hydrocarbon-impacted or remediated soils and are frequently associated with the degradation of complex organic compounds and hydrocarbons (57, 58).

However, the statistical analysis indicates that microbial community composition was more strongly associated with the stoichiometric gradient (PC1Elements; $r = -0.92$ with PC1Micro) than with residual hydrocarbon concentration ($r = -0.37$) (Figure 5). This suggests that, at the end of the biopile treatment, bacterial assemblages were structured primarily by nutrient balance and soil physicochemical conditions rather than by contaminant concentration alone.

The presence of genera typically linked to organic matter turnover and nutrient cycling, including *Flavobacterium*, *Flavihumibacter*, and members of Xanthomonadaceae, further supports the interpretation that microbial communities reflect integrated soil quality gradients rather than a purely contamination-driven signature (59, 60).

Importantly, the inclusion of microbial principal components in regression models did not substantially improve the prediction of avoidance or germination responses compared to physicochemical descriptors. This indicates that microbial structure mirrors the environmental gradients influencing biological endpoints but does not provide an independent predictive signal within the present dataset.

4.4 From contamination metrics to functional soil assessment

The integrative analysis combining chemical descriptors, biological endpoints and microbial community structure provide an integrated perspective of post-bioremediation soil functionality. Across datasets, residual hydrocarbon concentration did not emerge as the dominant explanatory variable within the concentration range and treatment context examined in this study. Instead, biological endpoints were aligned along multidimensional gradients integrating nutrient balance, pH, and texture-related components.

This pattern is particularly evident in statistical models. For *E. fetida*, avoidance behavior was more strongly associated with the stoichiometric gradient than with hydrocarbon concentration. Similarly, *C. sativus* germination was significantly related to texture-related components (PC2Texture), while hydrocarbon concentration alone did not significantly explain variability. These findings indicate that soil biological responses at the end of the biopile treatment were structured primarily by integrated soil quality parameters rather than by residual contaminant levels (61).

The strong correlation between the main microbial axis (PC1Micro) and the stoichiometric component further supports this interpretation. Microbial community structure mirrored the same chemical gradients influencing plant and invertebrate responses, suggesting that soil organisms and microbial assemblages responded coherently to underlying physicochemical conditions (62). However, microbial structure did not provide independent predictive power in regression models, reinforcing the view that microbial composition functions as a parallel indicator of ecological state rather than as an independent driver of the tested endpoints.

From an applied perspective, these results challenge the assumption that compliance with hydrocarbon concentration limits is sufficient to guarantee ecological compatibility. While contaminant reduction remains a necessary condition for soil reuse, it does not fully capture the functional dimension of soil ecological compatibility. Instead, nutrient balance, soil structure, and pH appear to shape habitat suitability and plant performance more strongly in post-remediation contexts (61). It should also be noted that several environmental and microbial gradients reflected differences between the two treatment facilities, suggesting that site-specific factors may have contributed to the observed patterns.

The assessment framework proposed in this study integrates these dimensions into a sequential decision pathway. Chemical compliance ensures regulatory safety, phytotoxicity screening identifies residual inhibitory effects on vegetation, and avoidance tests refine the classification by detecting subtle habitat-level responses. The multivariate analysis is consistent with the rationale underlying such an integrated approach, suggesting that soil functionality emerges from the interaction of multiple gradients rather than from contaminant concentration alone.

Overall, the findings support a shift from a contaminant-centered evaluation toward a functional soil quality framework. In post-bioremediation scenarios, the ecological compatibility of bioremediated soils should be assessed through the combined interpretation of chemical, physical and biological indicators, ensuring that reused decisions reflect ecosystem-level functionality rather than solely regulatory compliance. Importantly, the high proportion of Class A soils suggests that bioremediation, when properly managed, may support ecological compatibility across the selected biological endpoints. beyond simple contaminant reduction. The results suggest that treated soils may transition from a regulatory liability toward a reusable resource, provided that functional indicators confirm biological compatibility beyond simple contaminant reduction.

5 Conclusion

This study suggests that residual hydrocarbon concentration alone may not adequately explain biological responses in post-bioremediation soils. Across the samples investigated, avoidance behavior and plant germination were more strongly associated with multidimensional gradients integrating soil physicochemical properties, including nutrient stoichiometry, pH and texture, rather than with hydrocarbon concentration alone.

Plant bioassays revealed species-specific sensitivity patterns. *L. sativum* generally showed tolerance or stimulation, whereas *C. sativus* responded more clearly to environmental gradients related to soil texture. Similarly, avoidance behavior in *E. fetida* was significantly associated with the stoichiometric gradient, indicating that soil nutrient balance and physicochemical context play a stronger role in shaping organism responses after remediation.

Microbial community structure reflected the same chemical gradients influencing plant and invertebrate responses, suggesting that microbial assemblages mirror integrated soil conditions rather than residual contamination per se. Most treated soils were classified as Class A within the proposed framework, suggesting a generally favorable response across the selected biological endpoints. Within the concentration range observed, residual hydrocarbons did not represent the dominant ecological constraint.

Overall, these results support the integration of functional biological endpoints alongside contaminant-based evaluation when assessing post-bioremediation soils. Integrating chemical thresholds with plant bioassays, soil fauna tests and microbial indicators improves the interpretation of soil ecological functionality, providing a more realistic basis for reuse decisions.

From a regulatory perspective, the incorporation of biological indicators into post-remediation evaluation protocols could facilitate the assessment of soil functionality beyond compliance with contaminant limits, helping to evaluate whether bioremediated soils are not only chemically acceptable but also ecologically compatible. The framework should therefore be considered a proof-of-concept approach intended to support ecological interpretation of post-remediation soils rather than a fully validated classification system. Because the framework was evaluated on a limited number of full-scale bioremediated soils, further applications across different remediation contexts will be necessary to assess its robustness and transferability. Future research should focus on defining ecological thresholds for biological endpoints and assessing the long-term functional stability of treated soils under field conditions.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA1484031.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

DR: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. LF: Data curation, Formal analysis, Methodology, Writing – review & editing. DA: Investigation, Writing – review & editing. LR: Writing – review & editing, Conceptualization. FP: Writing – review & editing, Data curation, Software. VS: Writing – review & editing, Resources. SV: Conceptualization, Supervision, Writing – review & editing, Methodology. AF: Conceptualization, Funding acquisition, Investigation, Resources, Supervision, Writing – review & editing.

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Conflict of interest

Authors VS was employed by company Sistemi ambientali Srl.

The remaining author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declared that generative AI was used in the creation of this manuscript. Artificial intelligence (AI) tools were used exclusively to support language editing and text revision. The authors retained full responsibility for the content, scientific interpretation, and conclusions of the manuscript.

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