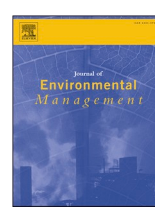
Journal of Environmental Management 393 (2025) 127239

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman



Research article

Assessment of phyto-compatibility after soil remediation: Insights from plant growth, physiological, and metabolomic analyses

Davide Marzi[a,b], Valentina Mazzurco-Miritana[a,c,*], Laura Passatore[a], Massimo Zacchini[a,b], Fabrizio Pietrini[a,b], Serena Carloni[a], Eleonora Peruzzi[d], Mariana Louro[e], Marta Sousa Silva[e], Carlos Cordeiro[e], Isabel Nogués[a]

[a] *Research Institute on Terrestrial Ecosystems, National Research Council (IRET-CNR), SP 35d, km 0.7, Montelibretti, 00010, Rome, Italy*
[b] *National Biodiversity Future Center (NBFC), 90133, Palermo, Italy*
[c] *Department of Energy Technology & Renewables, Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), via Anguillarese 301, 00123, Rome, Italy*
[d] *Research Institute on Terrestrial Ecosystems, National Research Council (IRET-CNR), via Moruzzi 1, 56124, Pisa, Italy*
[e] *Laboratório de FTICR e Espectrometria de Massa Estrutural, Instituto de Biosistemas & Ciências Integrativas (BioISI), Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal*

A R T I C L E I N F O

Keywords:
Bioaugmentation
Bioremediation
Lepidium sativum
Microorganisms-enriched biochar
Phytoremediation
Phytotoxicity

A B S T R A C T

Soil contamination from heavy metals and organic pollutants represents a significant global concern. In this context, biochar and microbial communities have been identified as promising remediation tools. Indeed, the structural characteristics of biochar facilitate contaminant immobilization, while the presence of microbial communities promotes their biodegradation, thereby enhancing soil recovery. Besides, phytoremediation has been successfully applied to restore contaminated soils. Historically, the success of soil remediation has been predominantly contingent on chemical parameters. However, recent approaches have focused on soil health, fertility, and ecological function after remediation. In this framework, through phytotoxicity tests, this study investigates the phyto-compatibility of a soil contaminated with hydrocarbons and copper after the treatment with 5 distinct remediation strategies: i) natural attenuation (S), ii) treatment with biochar (SB), iii) treatment with microorganism-enriched biochar (SBB), iv) treatment with biochar and phytoremediation (SBP), and v) treatment with microorganism-enriched biochar and phytoremediation (SBBP). Moreover, chlorophyll fluorescence and untargeted metabolomics analyses were performed in plants to get a more comprehensive understanding of responses of plants grown on remediated soil. The results of this study demonstrated significant variations among the plants treated with soil recovered from the different remediation strategies. Compared to other treatments, SBB promoted *L. sativum* plants growth showing limited induction of stress markers. However, a certain degree of photoinhibition was observed in all treatments, highlighting the importance of characterizing the phyto-compatibility of remediated soils.

1. Introduction

Soil contamination represents a significant global issue, with both heavy metals and organic compounds responsible for causing extensive environmental pollution. Considerable efforts have been made in recent times to remediate polluted soils by a variety of strategies and methods (Zhang et al., 2013; Cheng et al., 2016; Rouhani et al., 2025). In this context, biochar, a vegetal black carbon produced by the pyrolysis of biomass, is receiving considerable attention in the field of soil remediation due to its physico-chemical properties (Palmeggiani et al., 2021). Among these properties, the surface area and adsorption capacity of the pore structure make biochar a suitable medium for immobilizing toxic compounds, including heavy metals (Dai et al., 2019). Similarly, soil microbial communities have been specifically studied in recent years for exploiting their biological properties for the bioremediation of polluted substrates (Mukherjee et al., 2022; Zheng et al., 2022; Abbas et al., 2024). In addition, their pivotal role in many fundamental soil processes, including organic matter breakdown, nutrient turnover and

* Corresponding author. Research Institute on Terrestrial Ecosystems, National Research Council (IRET-CNR), SP 35d, km 0.7, Montelibretti, 00010, Rome, Italy.
E-mail address: valentina.mazzurco@enea.it (V. Mazzurco-Miritana).

https://doi.org/10.1016/j.jenvman.2025.127239
Received 7 August 2025; Received in revised form 4 September 2025; Accepted 6 September 2025
Available online 10 September 2025
0301-4797/© 2025 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

primary productivity, is a key factor to consider when addressing environmental issues (Malkawi and Kapiel, 2024). The potentiality of applying microorganisms on biochar for accelerating the biodegradation of petroleum hydrocarbons in soil has been tested in scientific literature (Guo et al., 2022; Fang et al., 2024) with positive results. On the other hand, phytoremediation is a well-established green technology that exploits the capacity of selected plant species to uptake and accumulate significant amounts of contaminants, thereby facilitating their removal from soils (Kafle et al., 2022).

Following the implementation of remediation strategies, it is essential to employ assessment methods to ascertain whether the desired remediation objectives have been met and whether environmental restoration has been achieved. Assessing the effectiveness of remediation is essential to support soil management and reuse following treatment (Kuppusamy et al., 2017; Xu et al., 2024). Nevertheless, in general, to determine the effectiveness of soil restoration, only soil chemical parameters as contaminant levels, cation exchange capacity (CEC) and pH are considered. Recent research highlighted a more comprehensive approach to the remediation process that considers not only the removal or immobilization of contaminants, but also the quality of the resulting soil, its fertility, structure, ecological functionality and biodiversity (He et al., 2024). In this context, Dorn and Salanitro (2000) emphasized the importance of establishing biological criteria for the assessment of soil quality following remediation. This approach would facilitate the identification of potential ecological risks associated with the soil. Phytotoxicity tests represent valuable biological tools to assess the quality and toxicity of a soil sample as well as the effectiveness of remediation (Zawierucha et al., 2022). They are based on the utilization of representative *in vivo* models able to provide information about the potential hazards that a contaminated site poses for plants and the environment (de la Parra et al., 2022). Contaminant availability to living organisms is influenced by important properties of the soil. Alterations in these properties (i.e. soil structure, organic matter, CEC water retention, pH) during the remediation process have been demonstrated to exert influence on the phytotoxicity associated with contaminated soil, even in absence of any alteration in contaminant concentration (Cipullo et al., 2019; Kwak et al., 2019).

Among plant species, *Lepidium sativum* L. is frequently used in laboratory tests to assess phytotoxicity (Iannilli et al., 2024). *Lepidium*, commonly referred to as garden cress, is an edible annual herb with fast growth rates. The high sensitivity of *Lepidium* to heavy metals and polycyclic aromatic hydrocarbons makes it a suitable organism for the assessment of soil contamination through biological tests. Cress has been used to assess the phytotoxicity of contaminated soil in several studies (Passatore et al., 2022; Pietrini et al., 2023; Iannilli et al., 2024). Furthermore, cress is frequently recommended for use in a variety of biotoxicity tests such as ISO 11269–2:2012 (2012). Plant performances in phytotoxicity tests are usually assessed through biometric measurements and sometime by chlorophyll fluorescence or biomass nutrient content (Mendes et al., 2021; Zhang et al., 2025). Photosynthetic processes are among the main targets of toxic compounds and the reliability of chlorophyll fluorescence parameters for describing the photosystem efficiency in plants grown in contaminated soil has been proven (Pietrini et al., 2023). Further insights into the processes underlying the plant toxicity response can be obtained through the metabolite analysis of plant biomass. Indeed, using an untargeted metabolomics approach in plants provides information, not only on the numbers of identified metabolites, but also linking the changes in metabolites to the observed phenotypes in terms of stress tolerance, nutrient uptake, photosynthesis, and responses to environmental stimuli (Maia et al., 2023). Plant metabolomics is especially valuable in evaluating changes in metabolite profiles that occur under different conditions.

In a previous study, soil contaminated with hydrocarbons and copper (Cu) was treated with different remediation strategies including soil amended with biochar alone, addition of microorganism-enriched biochar to soil, biochar and phytoremediation, and a combination of microorganism-enriched biochar with phytoremediation (Mazzurco-Miritana et al., 2025). In addition to soil analysis, the efficacy of each approach in reducing toxicity and restoring soil fertility was assessed by phytotoxicity assays. To get an in-depth evaluation of the toxicity process, *Lepidium* plants resulting from the assay were characterized through morpho-physiological and untargeted metabolomic analysis. Based on existing knowledge, few studies have undertaken comprehensive phytotoxicological assessments of soils following diverse remediation interventions within a multidisciplinary framework. The simultaneous integration of phytotoxicity tests, physiological evaluations, and metabolomic analyses of plants grown in remediated soils is crucial for understanding the ecological impact and effectiveness of remediation strategies. Such an approach not only enhances the reliability of soil health evaluations but also creates opportunities for developing predictive models, optimizing remediation protocols, and advancing sustainable land management practices.

2. Material and methods

2.1. Soil and leachate collection after remediation treatments

The soil employed in the phytotoxicity test was previously subjected to the following remediation process: i) natural attenuation (S), ii) treatment with biochar (SB), iii) treatment with microorganism-enriched biochar (SBB), iv) treatment with biochar and phytoremediation (SBP), v) treatment with microorganism-enriched biochar and phytoremediation (SBBP) (Mazzurco-Miritana et al., 2025). Upon conclusion of the trial (9 months), samples of hydrocarbons- and Cu-contaminated soil subjected to 5 different conditions (Fig. 1) were collected, in addition to soil leaching solutions, to be assayed for the residual toxicity by phytotoxicity tests.

2.2. Phytotoxicity tests

Phytotoxic effects associated with soil and leaching solutions recovered after remediation treatments were tested using 2 different standard tests. The toxicity assay on the leaching solution collected from the mesocosms was based on the US EPA OPPTS 850.4200 guidelines (1996) (US EPA, 1996), modified as previously described (Passatore et al., 2022). *Lepidium sativum* L. seeds, obtained from Ingegnoli seed company (Milano, Italy), were incubated for 72 h in darkness at 25 ± 1 °C (Eyela incubator, Tokyo, Japan) in Petri dishes filled with deionized water (control) or with the leaching solutions from each mesocosm conditions (pooled sample of the replicates). The percentage of seed sprouting and root growth were assessed to calculate the germination index (GI %) according to (APAT, 2004).

The phytotoxicity of the different soils was assessed through a

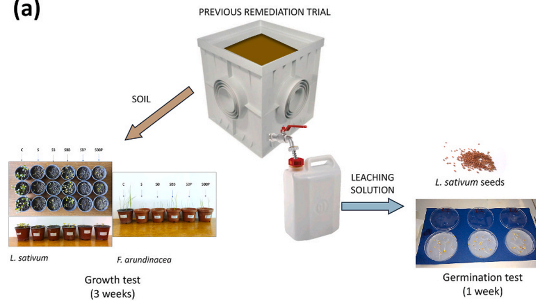


Fig. 1. Schematic view of the soil and leachate recovered from previous remediation trials including natural attenuation (S), treatment with biochar (SB), treatment with microorganism-enriched biochar (SBB), treatment with biochar and phytoremediation (SBP), treatment with microorganism-enriched biochar and phytoremediation (SBBP) in mesocosms (Mazzurco-Miritana et al., 2025).

growth assay based on European Standard ISO 11269-2:2012 (2012). A monocotyledonous (*Festuca arundinacea* Schreb.) and a dicotyledonous (*Lepidium*) plant species were tested in parallel. Seeds were untreated and derived from commercially available certified material (Ingegnoli seed company, Milano, Italy). As control, a commercial peat substrate was used (Natural Torf, produced by Vialca Srl, Uzzano, PT, Italy). The test was conducted by using 4 replicates for each soil sample (C, S, SB, SBB, SBP, SBBP), carried out in 8 cm diameter plastic pots and sowing 10 seeds in each pot (Fig. 1). Pots were maintained during 21 d in a growth chamber (temperature 21 ± 1 °C, 16 L/8 D photoperiod, 70 ± 5 % humidity, 350 ± 50 μmol $m^{-2}s^{-1}$ PPFD) and loosely covered with plastic sheet until at least 50 % of seeds have germinated. Soil's humidity was kept at 70 % of its water retention potential. At emergence of the first 5 seedlings in control pots (3 d for *Lepidium* and 5 d for *Festuca*), the number of germinated seeds in each pot was recorded and seedlings were thinned to obtain a total of 5 evenly spaced representative individuals in each pot. At the end of the 21 d, after physiological measurements, the aerial biomass of plantlets was cutoff and weighed. *Lepidium* samples were immediately frozen for metabolomic analyses. The fresh weight of the aboveground biomass per pot at harvest and the relative growth inhibition in relation to C treatment (difference between the biomass of control and the biomass of the treatment with respect to control) were recorded as endpoints.

2.3. Chlorophyll fluorescence parameters and leaf pigment content

At the end of the test (21 d after sowing), and before plant growth analysis, measurements of chlorophyll fluorescence were performed on *Lepidium* plants to evaluate the photosynthetic performance. Specifically, the maximal quantum yield of PSII photochemistry (F_v/F_m) and the effective quantum yield of PSII photochemistry (ΦPSII) were determined through a MAXI-Imaging-PAM (Walz, Germany) on well-developed leaves. Fv/Fm values were measured in leaves subjected to 30 min of darkness, while ΦPSII was determined in leaves acclimated for at least 5 min to 310 μmol m^{-2} s^{-1} PPFD to ensure steady-state photosynthetic activity. Parameters were calculated following (Di Baccio et al., 2017). Total chlorophyll content was evaluated using SPAD-502 chlorophyll meter readings (Minolta Camera Co., Osaka, Japan), taken from the same leaves previously analyzed for chlorophyll fluorescence. Measurements were conducted with a minimum of 2 representative fully expanded leaves per pot. Two SPAD measurements were acquired from the broadest portion of the leaf lamina, carefully excluding major veins. Two SPAD readings per leaf were averaged to obtain representative value. Chlorophyll content (μg cm^{-2}) was estimated from SPAD values via the following equation (Cerovic et al., 2012):

Chlorophyll content = (99 x SPAD value) / (144 – SPAD value)

2.4. Metabolomic analyses by FT-ICR-MS

Frozen leaf samples of *Lepidium* plants resulting from the phytotoxicity assay were lyophilized and ground, collecting 4 biological replicates per treatment. Extraction of metabolites was carried out as previously described (Maia et al., 2023; Nogues et al., 2023). Approximately 50 mg of frozen leaf tissue was extracted in 1 mL of 50 % methanol/water (LC-MS grade; Merck), and the resulting extract was subsequently diluted 1:1000 in methanol. Samples were analyzed by direct infusion through a syringe pump, at 2 mL min^{-1} on a 7-T SolariX XR Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) equipped with an Apollo II ESI ion source and a ParaCell (Brüker Daltonics). Leucine enkephalin (YGGFL, Sigma Aldrich) was added to each sample as an internal calibration standard ([M+H]+ = 556.276575 m/z) at a concentration of 0.1 μg mL^{-1}. Each sample was supplemented with 0.1 % (v/v) formic acid (Sigma Aldrich, MS grade). Spectra were acquired in positive mode (ESI+), at 4M acquisition size, between 100 and 1500 m/z, as previously described (Maia et al., 2023). The accumulation time was 0.1 s and 100 transients were accumulated for each spectrum, zero-filled, and apodised (half sine) and magnitude processed. Online calibration was conducted utilizing the monoisotopic m/z value of leucine enkephalin. Bruker Compass data Analysis 5.0 software (Brüker Daltonics, Bremen, Germany) was used to process and retrieve the mass lists, considering peaks with a minimum signal-to-noise ratio of 4. Spectral alignment across samples was carried out using Metaboscape 5.0 (Brüker Daltonics, Bremen, Germany) employing the T-ReX 2D algorithm for MRMS single spectra and normalizing peak intensities to the total spectral signal. Variables appearing in only 1 of the 15 samples and data for the internal standard (leucine enkephalin, neutral mass 555.2693063368 Da) were removed from the data set.

The number of shared and unique metabolites of plants grown in each soil type was quantified and visualized using an intersection plot, following the method described by Lex et al. (2014). Molecular formulae for each isotopic cluster were determined using the SmartFormula function in Metaboscape, removing formulae containing chloride and fluoride. Metabolite annotation was conducted utilizing the Human Metabolome Database (HMDB, accessed on December 14, 2023) and LOTUS (accessed on December 14, 2023), both of which were integrated into MetaboScape 5.0 with a maximum mass deviation threshold of 1 ppm (Rutz et al., 2022; Wishart et al., 2022). Compounds' "superclass" was assigned according to the ChemOnt chemical taxonomy (Djoumbou Feunang et al., 2016) and for lipids the "class" was also reported.

2.5. Statistical analysis

Statistical analysis on morpho-physiological traits was performed using GraphPad Prism 8. Differences between the effects of different treatments were compared using one-way analysis of variance (ANOVA) followed by Tukey's test was used to analyze. Significant differences ($p \leq 0.05$) between groups are indicated by different letters. Data are expressed as means ± standard error (SE).

Statistical analysis of metabolomics data was conducted using the statistical analysis (single factor) module implemented in MetaboAnalyst (https://new.metaboanalyst.ca) (Xia et al., 2009). Missing values were imputed by assigning one-fifth of the lowest intensity observed for each m/z across all samples. Subsequently, the data were subjected to Pareto scaling prior to conducting unsupervised principal component analysis (PCA). To distinguish between plants grown in different soil types, partial least squares discriminant analysis (PLS-DA) models were constructed. The variable importance in projection (VIP) scores, which were derived as a weighted sum of the squared correlations between the PLS-DA components and the original variables, were used to assess metabolite's importance.

3. Results and discussion

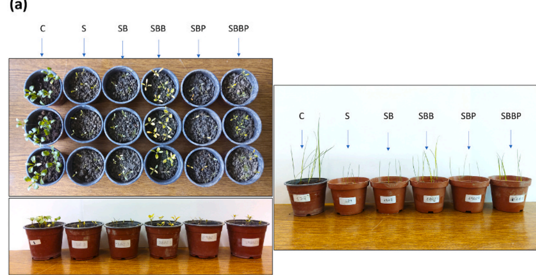
3.1. Phytotoxicity tests on leaching solutions and on treated soils

To investigate the phyto-compatibility of soil after treatment with different remediation strategies, including biochar (SB), microorganism-enriched biochar (SBB), biochar and phytoremediation (SBP), microorganism-enriched biochar and phytoremediation (SBBP), soil and leachate were recovered from mesocosm upon each treatment and experimental control (natural attenuation, S) (Fig. 1). Mobility of contaminants in the soil was assessed through the germination test on the leaching solution collected from the mesocosms. Results indicated a slight inhibition of germination for the S, SB, and SBB treatments, with the GI being 98.7 ± 3.5 %, 96.5 ± 2.1 %, and 92.79 ± 9.02 %, respectively. Nevertheless, the inhibition effect remained below 20 %, a threshold below which the data can be considered negligible. As for the treatments with the presence of plants (SBP and SBBP), a slight stimulation of germination occurred compared to the control (the GI was 105.91 ± 1.08 % and 110.08 ± 3.7 %, respectively), still below the 20 %

threshold. These findings suggest that the collected solution did not exhibit consistent toxicity, which is in line with the measured pollutant concentration in the leaching solution: C > 12 concentration in the range 40.90–72.80 mg L^{-1} and total Cu in the range 0.60–1.22 mg L^{-1} (Mazzurco-Miritana et al., 2025).

The endpoints recorded at the end of the ISO European Standard n. 11269-2:2012, 2012 plant growth test on treated soil matrices (21 d) are summarized in Table 1 for *Lepidium* and *Festuca*. Regarding biomass per pot, both the *Festuca* and *Lepidium* phytotoxicity tests showed a similar trend among treatments (Table 1, Fig. 2). SBB treatment (microorganism-enriched biochar) resulted in significantly higher biomass production compared to other treatments ($p \leq 0.05$). In contrast, strong growth inhibition was observed in plants grown on S (soil resulting from natural attenuation process), resulting in 85 % and 90 % biomass reduction for *Lepidium* and *Festuca*, respectively. The number of *Lepidium* and *Festuca* seeds that germinated 3 and 5 d, respectively, after sowing (when at least 50 % of seeds in control germinated), was lower in all treatments compared to control (Table 1). These results are consistent with the residual contaminant concentrations measured in soil samples after the treatments (Table S1). Indeed, the microorganisms-enriched biochar amendment (SBB), was shown to reduce hydrocarbon concentrations and to decrease the most bioavailable and toxic fraction of copper (Cu) in soil, as described in Mazzurco-Miritana et al. (2025). The lower hydrocarbon content and Cu bioavailability is likely to have influenced the toxicity of the soil matrix, allowing higher plant growth compared to other treatments. The same effect was observed by Steliga & Kluk. (2021) following a two-phases bioremediation treatment (bioremediation with strains of bacteria, fungi and yeasts, and phytoremediation using the plant *Melilotus officinalis*). The cited study used soil contaminant analyses and toxicological assays (ostracods-, bacteria- and plant-based assays) to evaluate the effectiveness of the applied treatments. The higher plant biomass in SBB could also be in part attributable to the elevated available P present in this soil in comparison to other treatments, a consequence of the remediation treatment. It is noteworthy that, during the process of soil remediation, the presence of plants (P) in synergy with biochar amendment, with or without microorganisms' addition (SBBP, SBP), resulted in a soil where *Lepidium* and *Festuca* plants exhibited a higher degree of inhibition compared to SBB. However, also the SBBP and SBP remediation treatments have led to a reduction in hydrocarbon concentrations and Cu bioavailability in soil recovered from previous treatment. The presence of allelopathic compounds released by *Melilotus* plants used during the soil remediation process may explain this result (Mikhailova et al., 2022; Yu et al., 2022). As demonstrated by the results outlined above and according to other studies, toxicological tests highlight effects of the bioremediation treatments that are not immediately apparent from the sole soil contaminant analysis but that greatly influence soil quality and its future utilization (Conte et al., 2021; Guerin, 2022; Tripathi et al., 2024). As previously noted by Baud-Grasset et al. (1993), the growth test on the soil showed greater effectiveness than the germination test on the leaching solution in evaluating the effects of bioremediation treatments,

(a)



(b)

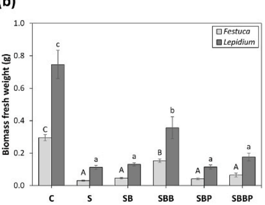


Fig. 2. (a) Pictures of the pot used for phytotoxicity assays with *Lepidium* (left) and *Festuca* (right) on soil resulting from different remediation treatments: natural attenuation (S), biochar amendment (SB); microorganisms-enriched biochar (SBB); SB plus plants (SBP); SBB plus plants (SBBP). Commercial peat substrate was used as control (C). The photos were taken at the end of the testing period (21 days). (b) Fresh weight of the aboveground biomass collected from *Lepidium* and *Festuca* plants grown for 21 days on contaminated soil subject to different treatments (C, S, SB, SBB, SBP, SBBP). Mean data are shown (n = 4, ±S.E.), different letters correspond to statistical differences among the soil treatments within each plant species (Tukey's test, $p \leq 0.05$).

indicating that the contaminants remained largely bound to the soil matrix and were not easily released into the water phase.

3.2. Physiological parameter measurements

As the effect of treatments on relative growth inhibition (Table 1) was similar in *Lepidium* and *Festuca* plants cultivated on recovered soil remediated with different strategies, subsequent physiological parameter measurements focused exclusively on *Lepidium* plants. Therefore, to evaluate the phytotoxicity and to study the spatial variability of photosynthetic performance, variations of chlorophyll fluorescence image and its leaf content were measured in *Lepidium* plants at the end of the trial (Fig. 3). Fig. 3 shows an image illustrating chlorophyll fluorescence metrics, including the maximum quantum yield of PSII photochemistry (Fv/Fm) and the effective quantum yield of PSII photochemistry (ΦPSII) in the leaves of Lepidium plants used in this study. As a quick and non-invasive method, chlorophyll fluorescence has been extensively used to examine the impact of contaminants on plants, offering insights into the functionality of the photosynthetic system (Mazzurco-Miritana et al., 2025). Chlorophyll content in leaves is a key

Table 1
Endpoints recorded for the growth test with *Lepidium* and *Festuca.* Commercial peat substrate was used as control (C). The tested treatments were natural attenuation (S), biochar amendment (SB); microorganisms-enriched biochar (SBB); SB plus plants (SBP); SBB plus plants (SBBP). The relative growth inhibition was calculated as the difference between the biomass of the control and the biomass of the treatment Mean data (n = 4 ± SE) are shown. The data of biomass weight have been elaborated with one way ANOVA Test. Different letters correspond to statistical different values (Tukey's test, $p \leq 0.05$).

Treatment	*Lepidium sativum*			*Festuca arundinacea*		
	Fresh weight of the biomass per pot (g)	Relative growth inhibition	Germinated seeds 3 d after sowing	Fresh weight of the biomass per pot (g)	Relative growth inhibition	Germinated seeds 5 d after sowing
C	0.747(±0.04) a	0 %	9.50	0.296(±0.02) a	0 %	5.50
S	0.113(±0.01) c	85 %	5.25	0.031(±0.00) c	90 %	2.25
SB	0.131(±0.01) c	82 %	1.50	0.046(±0.00) c	84 %	2.50
SBB	0.356(±0.03) b	52 %	1.00	0.154(±0.01) b	48 %	4.75
SBP	0.115(±0.01) c	85 %	7.00	0.042(±0.01) c	86 %	6.00
SBBP	0.176(±0.01) c	76 %	1.00	0.065(±0.01) c	78 %	4.75

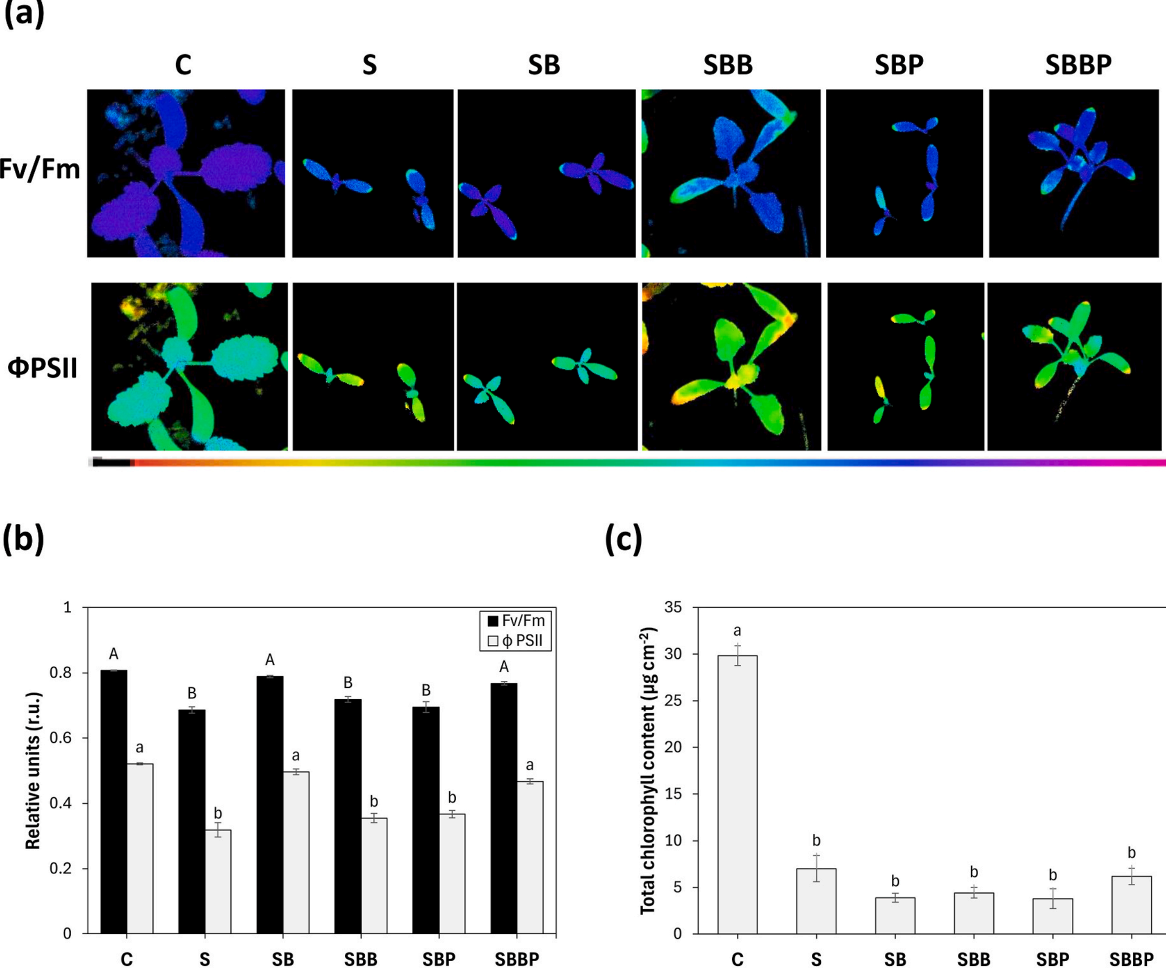


Fig. 3. (a) Images of chlorophyll fluorescence parameters in *Lepidium* plants grown for 21 days on contaminated soil subject to different treatments: natural attenuation (S), biochar amendment (SB); microorganisms-enriched biochar (SBB); SB plus plants (SBP); SBB plus plants (SBBP). Commercial peat substrate was used as control (C). Maximal quantum yield of PSII photochemistry (F_v/F_m) and the effective quantum yield of PSII photochemistry (ΦPSII) are measured with an Imaging-PAM M-series system. The false color code depicted at the base of the images ranges from 0.000 (black) to 1.000 (pink). (b, c) Changes in the maximal quantum yield of PSII photochemistry in dark adapted leaves (F_v/F_m), effective quantum yield of PSII photochemistry (ΦPSII) in light adapted leaves (b) and total chlorophyll content (c) in *Lepidium* plants grown for 21 days on contaminated soil subjected to different treatments., in light-adapted leaves (ΦPSII). The vertical bars represent mean values ($n = 4$) ± S.E. The data were subjected to one-way analysis of variance (ANOVA) and different letters indicate significant differences in accordance with Tukey's test ($p \leq 0.05$).

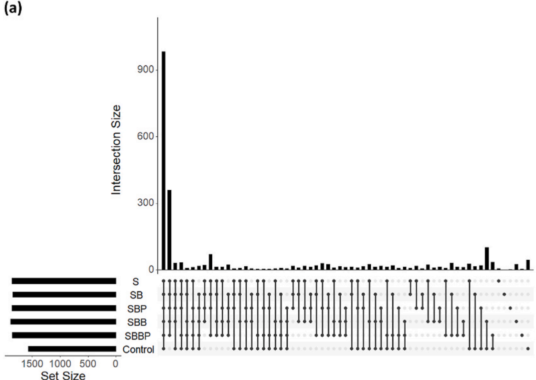
determinant of the capacity for photosynthesis and biomass production (Dai et al., 2009). The results of the present study revealed that *Lepidium* plants grown on soil recovered after remediation exhibited clear signs of stress, as evidenced in a significant decrease in chlorophyll content in all treatment groups when compared to the control plants (Fig. 3). The decrease in chlorophyll levels observed in treated plants was accompanied by a simultaneous decline in stem and leaf biomass, underscoring the potential of chlorophyll content as a reliable physiological parameter for toxicity evaluation, as similarly documented in studies involving various metals (Pietrini et al., 2015, 2023; Chandra and Kang, 2016) and organic pollutants (Kummerová et al., 2006; Pietrini et al., 2022).

Analysis of pigment fluorescence provided a more accurate representation of the eco-physiological state of *Lepidium* plants at the conclusion of this study. Chlorophyll fluorescence images (Fig. 3a) revealed a remarkable asymmetric distribution of light use and photosynthetic performance within both dark- and light-acclimated leaves (F_v/F_m and ΦPSII, respectively) across the 4 treatment groups (S, SBBP, SBP and SB) compared to the control plants (C), which showed a homogeneous fluorescence signal distribution. These results indicate that, within the parameters of the present study, treatments influenced photosynthetic performance with a decrease of photosynthetic performance at the edges of older leaves. In this situation, increased fluorescence emission is concentrated along the margins of mature leaves, whereas youngest leaves remain intact. As reported by Sánchez-Moreiras et al. (2020), this pattern may indicate an early senescence as suggested by the images of fluorescence distribution in *Arabidopsis thaliana* plants exposed to other organic compounds. On the contrary, SBB treatment showed an atypical pattern of decreased PSII performance (F_v/F_m and ΦPSII), where younger leaves displayed lower values than the older ones (Fig. 3b). Some authors have reported that this scenario could reflect a variation in the oxidative environment of plants (Martínez-Peñalver et al., 2011; Sánchez-Moreiras et al., 2020). This impacts specific sites intimately associated with the photosynthetic system, causing greater damage to leaves that are actively synthesizing. Results illustrated in Fig. 3b indicate that measurements of maximum quantum yield of PSII photochemistry (F_v/F_m) and effective quantum yield of PSII photochemistry (ΦPSII) were comparable to the control in plants cultivated on SBBP and SB, while they were significantly lower in plants treated with S, SBB and SBP. Both photosynthetic fluorescence indices, F_v/F_m and ΦPSII, are widely considered to be indicators of plant stress (Guidi et al., 2019; Pietrini et al., 2010). Lower values of these parameters indicate that stress is affecting the ability of plant's

photosynthetic machinery to capture and utilize light energy effectively, which ultimately impacts plant growth and development (Tian et al., 2017). These results showed that in S and SBP treatments, low F_v/F_m and ΦPSII values were associated with a decreased plant biomass and chlorophyll content (Figs. 2 and 3c). This finding suggests that the plant's ability to perform photosynthesis was compromised at the onset of the treatment, preventing normal plant growth and development. Conversely, in SB and SBBP treatments, elevated F_v/F_m and ΦPSII values, along with low plant biomass and chlorophyll content, suggests a discrepancy in how plants are using light energy for photosynthesis (Figs. 2 and 3). In fact, while elevated F_v/F_m and ΦPSII values are indicative of efficient light capture and photochemistry, the low plant biomass suggests that this efficiency is not being converted into observable increases in plant growth and development. This could be due to various factors, such as limitations in carbon fixation or in other metabolic processes that take place downstream of photosynthesis (Swoczyna et al., 2022). Finally, regarding the SBB treatment, an initial period of plant growth was followed by a phase in which the treatment effect led to a reduction of fluorescence parameters F_v/F_m, ΦPSII and chlorophyll content (Figs. 2 and 3). This situation may have resulted in physical damage to the antenna complex, leading to chronic photoinhibition (Sánchez-Moreiras et al., 2020).

3.3. Metabolomic analysis in *Lepidium* leaves

To get further insights into alterations induced in *Lepidium* plants grown on the different soil types (Fig. 1), untargeted metabolomics analysis using FT-ICR-MS was performed. A total of 2429 metabolites were identified. The number of metabolites within each treatment was comparable (S: 1323; SB: 1335; SBP: 1370; SBB: 1299; SBBP: 1343), except for the control which showed a reduced compound content (C: 1003). Among the identified metabolites, 982 were detected across all samples, whereas 360 were present in plants grown on contaminated soil but not in the control group (Fig. 4a). The control group exhibited the greatest number of unique metabolites, while the SB group showed the fewest. Moreover, molecular formulas were assigned to 1997 compounds among which 827 metabolites were successfully annotated using HMDB and LOTUS (Table S2). Overall, 437 metabolites annotated by name were present in all samples, including important metabolites and bioactive compounds, such as ascorbic acid, shikimic acid, quinic acid, phenolic compounds (sinapic acid, ferulic acid, proglobeflowery acid, syringic acid), and fatty acids (myristic acid, linoleic acid, oleic acid, lauric acid, geranic *acid*, tridecanoic acid). Some of these compounds have been previously identified in *Lepidium* plants (Painuli et al., 2022). On the other hand, 166 metabolites were exclusively detected in plants grown on contaminated soil, including syringin and ferulic acid, which play a major role in response to abiotic stress (Šamec et al., 2021; Zhou et al., 2024).



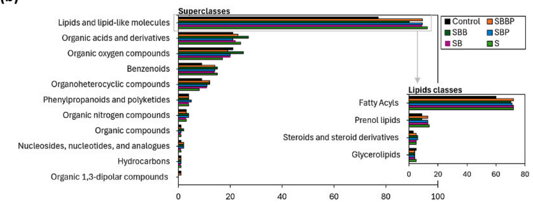


Fig. 4. (a) Intersection plot showing common and exclusive metabolites in *Lepidium* plants grown in six different experimental conditions: natural attenuation (S), biochar amendment (SB); microorganisms-enriched biochar (SBB); SB plus plants (SBP); SBB plus plants (SBBP); control (C) (b) Compound classification into superclasses. The insert shows the distribution of compounds within the different lipids' classes.

Metabolite analysis showed that, within the superclass, most of compounds consisted of lipids and lipid-like molecules, organic acids, organic oxygen compounds and their derivatives (Fig. 4b). Among lipids classes, treated plants showed a higher number of fatty acyls and prenol lipids compared to the control. Indeed, it has been suggested that the plant lipidome is mostly affected in response to stress (Mahood et al., 2023). This phenomenon can be attributed to changes in cellular membranes and signaling pathways. Moreover, among treated plants, SBB accumulated a lower amount of lipids and lipid-like molecules. Prenol lipids have been demonstrated to fulfil significant defense functions, including the protection against environmental stresses and attacks by pathogens (Satish et al., 2020). As reported in Ma et al. (2025), lipids upregulation, primarily prenol lipids and fatty acyls, was observed in wheat leaves in response to heat stress. Thus, with lower amounts of these lipids' classes, our results may suggest that control and SBB-treated plants exhibited a lower level of stress in comparison to other treatments throughout the 21 d of growth.

Other authors have observed changes in the metabolic profile of plants under stress conditions. Guo et al. (2020) found 53 metabolites as potential biomarkers for the presence of perfluorooctanesulfonic acid (PFOS) in leaves of *Arabidopsis* plants grown on PFOS-contaminated soil using LC-Q-TOF. They also found activation of the phenylpropanoid pathway leading to the formation of several polyphenols as a defense against ROS. Ascorbate was also activated. Cu-toxic effects on *Citrus grandis* leaves' metabolites were studied by Huang et al. (2021). Authors stated that 59 upregulated and 52 downregulated compounds were detected in Cu-toxic leaves. Furthermore, Cu-induced leaf toxicity was linked to the accumulation of ammonium (NH_4^+) and a reduction in nitrogen metabolism.

To evaluate the validity of the analysis and explore the similarity of metabolic profiles between different treatments, PCA and hierarchical cluster analysis (HCA) were performed. PCA analysis (Fig. 5a) indicated that most of the components identified in plants grown on contaminated and treated soil exhibited significant overlap, while they were clearly distinguishable from the control samples. Among the plants grown on contaminated soil, those grown on SBB defined a distinguishable group, exhibiting a slight degree of overlap with the remaining samples. Results of the hierarchical clustering analysis demonstrated that the control samples appear in a separated group, while the plants grown on contaminated soil are grouped together (Fig. 5b). The dendrograms generated by the hierarchical clustering analysis revealed the presence of 3 major clusters. The first cluster consisted of control samples, the second cluster encompassed all SBB samples and samples from other treatments (SB and SBBP), while the third cluster included the remaining samples. These findings suggest that metabolic analysis of *Lepidium* leaves can effectively differentiate soil type as a primary factor and remediation treatment as the secondary factor.

PLS-DA maximizes the separation between the plants grown in different conditions and identified the metabolites that contributed the most for this separation (Fig. 5c). The control appears in a well-defined and separated group. Among the important metabolites identified by PLS-DA (VIP scores) to differentiate the group of samples analyzed, erucamide and phytol were the most important ones.

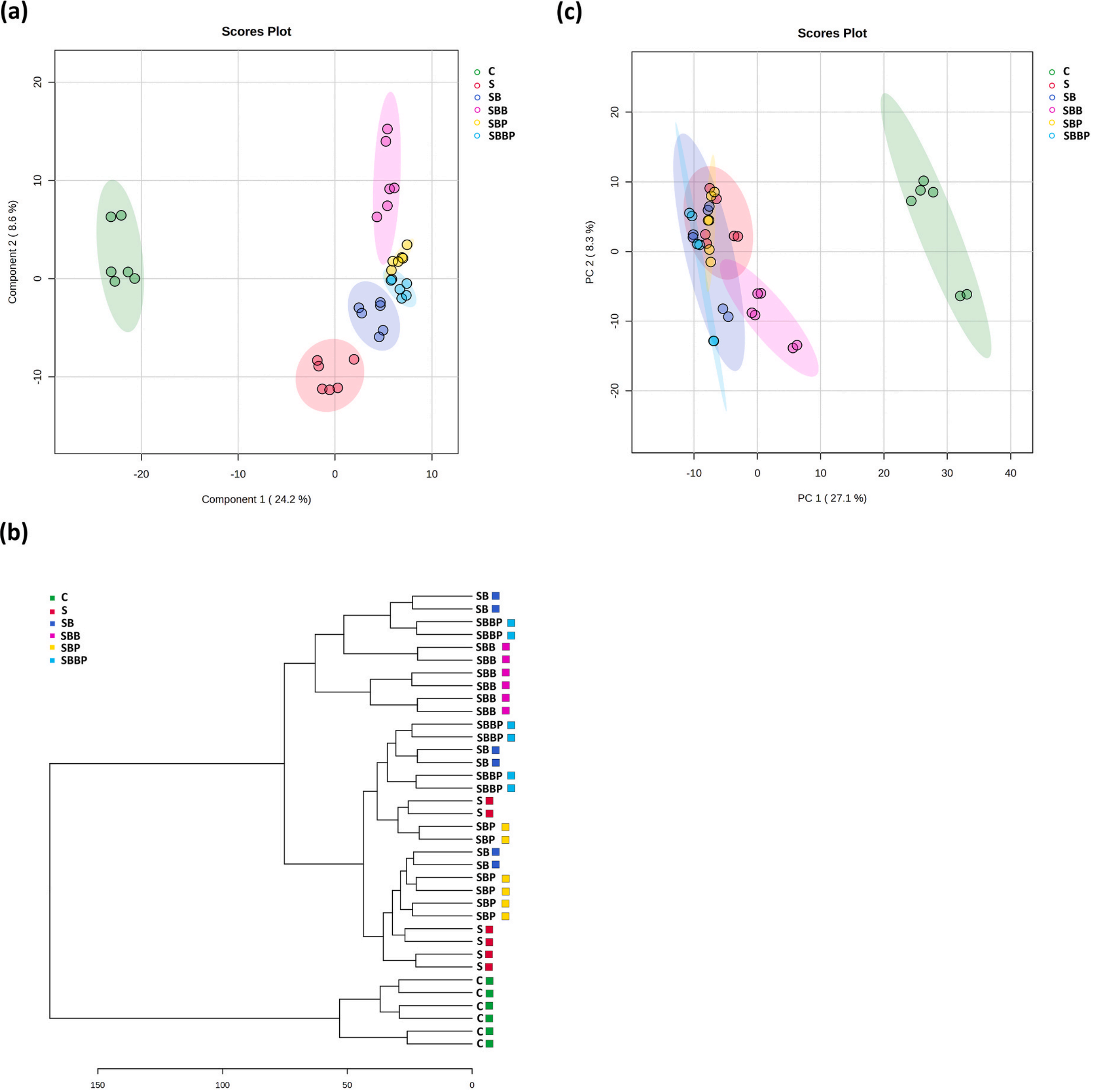


Fig. 5. Plant metabolites from *Lepidium* plants grown in six different experimental conditions were analyzed through (a) principal component analysis (PCA), (b) hierarchical clustering analysis (HCA) of untargeted metabolomics, (c) partial least squares discriminant analysis (PLS-DA) for the separation of the six soil treatments. Natural attenuation (S), biochar amendment (SB); microorganisms-enriched biochar (SBB); SB plus plants (SBP); SBB plus plants (SBBP); commercial peat substrate (C).

Erucamide belongs to the group of monounsaturated fatty acid amides with a hydrocarbon chain of 22 carbon atoms and has been isolated from animals (Hamberger and Stenhagen, 2003), plants (Lohani et al., 2024) and microorganisms (Xie et al., 2021). Alkylamides, or fatty acid amides, are a category of nitrogen-containing fat-soluble fatty acid derivatives, many of which have strong biological effects and act as lipid-based signaling compounds.

Widely distributed in plants, alkamides are secondary metabolites that include more than 200 related compounds. They have been identified across ten plant families, including Aristolochiaceae, Asteraceae, Brassicaceae, Convolvulaceae, Euphorbiaceae, Menispermaceae, Piperaceae, Poaceae, Rutaceae, and Solanaceae. Erucamide has been also found in *Cinnamomum tamala* leaves together with other fatty acid amides such as Palmitoleamide, heptadecenamide, oleamide, eicosenamide (Lohani et al., 2024). Fatty acid amides released in plant root exudates, such as oleamide and erucamide, can play a role in significant interactions plants-microbes, improving nitrogen turnover in rhizosphere bacteria (Sun et al., 2016). In this regard, it has been reported that the duckweed species *Spirodela polyrrhiza* and *Lemna minor* release erucamide through their root exudates as a chemical signal that enhances the nitrogen removal efficiency of the denitrifying bacterium *Pseudomonas fluorescens* (Lu et al., 2024). However, how these fatty acid amides are secreted and the way they influence microbial metabolic activity remain unexplored (Sun et al., 2016). VIP scores analysis showed that erucamide is highly represented in plants grown on control soil, decreases in soil subject to natural attenuation (S) and further decreases in SB, SBB, SBP, SBBP, suggesting a negative interaction between the presence of plants and/or biochar and the synthesis of this compound.

Phytol, an isoprenoid alcohol ester, is linked to chlorophyll, the main pigment responsible for photosynthesis in plants. Throughout the ageing process of leaves and under conditions of stress, a significant quantity of phytol is released through the breakdown of chlorophyll. Furthermore, chlorophyll undergoes continuous renewal. Nonetheless, the catalytic pathway of phytol in plants is not yet well understood (Gutbrod et al., 2019; Lippold et al., 2012). Interestingly, Ischebeck et al. (2006) demonstrated that Arabidopsis seedlings incorporate phytol into

chlorophyll, tocopherol, and lipid esters, providing important insights in connection with this. Furthermore, as a response to senescence or chlorotic stress (e.g. nitrogen deprivation), tocopherol and phytyl ester accumulate in thylakoids and plastoglobules (Ischebeck et al., 2006; Vidi et al., 2006; Gaude et al., 2007).

In this study, phytol is highly present in plants grown on contaminated soil subject to natural attenuation (S), decreases in SB, further decreases in control soil and SBP, while is almost undetectable in SBB and SBBP. The results obtained demonstrate the deleterious effect that contaminated soil has on chlorophyll, a phenomenon that is mitigated in SBB and SBBP. However, a low phytol content may reflect not only a low degree of chlorophyll degradation but also a high use of phytol for the synthesis of other compounds. It could be hypothesized that SBB and SBBP soils induce a lesser degree of chlorophyl degradation in comparison to contaminated soils, on account of the fact that these treatments are efficacious in the remediation of soil. Conversely, the observation that the phytol content is even lower in SBB and SBBP soil than in the control soil may be attributable to the utilization of phytol as a precursor of other compounds belonging to the category of fatty acid phytyl esters. Indeed, 2-oxophytanic acid has been detected in leaves of *Lepidium*, supporting our hypothesis. Conversely, phytol levels in control leaves may be indicative of a more rapid turnover of chlorophyll *a* biosynthesis and degradation in these leaves. This hypothesis is supported by the finding that total chlorophyll content is higher in control leaves. On the other hand, a correlation was identified between elevated levels of degradation products as pheophorbide and a plant response aimed at augmenting biomass production (Villette et al., 2019). Furthermore, Zhao et al. (2022) discovered that pheophorbide contributes to the growth of wheat seedlings following the inoculation with *Bacillus* sp. *strain wp-6* under salt stress conditions. In the present study, levels of pheophorbide a, a degradation product of chlorophyll, were found to be elevated in both control and SBB leaves in comparison to other treatments, which exhibited lower plant biomass. Pheoporbide, another product of chlorophyll degradation, also resulted to be among the 30 molecules that explain separation between the different samples (VIP-scores).

4. Conclusions

This study investigates phytotoxicological effects induced in plants grown on soil recovered after various remediation treatments. The remediation approaches included: natural attenuation (S), treatment with biochar (SB), treatment with microorganism-enriched biochar (SBB), treatment with biochar and phytoremediation (SBP), and treatment with microorganism-enriched biochar and phytoremediation (SBBP). These were applied to soil contaminated by hydrocarbons and Cu.

Phytotoxicological assays and physiological analysis showed that the indices of plants cultivated in remediated soil, such as plant biomass production and photosynthetic efficiency, were reduced compared to plants grown on uncontaminated control soil. However, these parameters were generally higher in plants grown on remediated soils compared to S, as in the case of SB, SBB and SBBP. Further insights were provided by metabolomic analyses. The results of this study identified unique and shared metabolites in plants, and demonstrated that SBB had reduced the accumulation of lipids and lipid-like molecules. The latter constitutes the portion of the metabolome that is more responsive to stress.

Altogether, these findings indicate that the treatment with bioactivated biochar exhibited enhanced capacity for supporting plant growth in comparison to the other treatments over the course of the 21 d growth period. This study emphasized that, for a comprehensive evaluation of remediation strategies, it is critical to implement rigorous and standardized assessment protocols to quantify the removal of contaminants from soil and to substantiate the re-establishment of soil functionality and integrity. The analysis of contaminants within the remediated matrix alone does not provide a representative indication of soil quality recovery. The present study underlines the necessity of a comprehensive evaluation of the phyto-compatibility of soil following remediation, in order to support the efficient and sustainable application of remediation strategies.

CRediT authorship contribution statement

Davide Marzi: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Valentina Mazzurco-Miritana:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Laura Passatore:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Massimo Zacchini:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Fabrizio Pietrini:** Writing – original draft, Investigation, Formal analysis, Data curation. **Serena Carloni:** Investigation, Formal analysis. **Eleonora Peruzzi:** Investigation, Formal analysis. **Mariana Louro:** Investigation, Formal analysis, Data curation. **Marta Sousa Silva:** Writing – review & editing. **Carlos Cordeiro:** Writing – review & editing, Supervision, Conceptualization. **Isabel Nogués:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Investigation, Formal analysis, Data curation, Conceptualization.

Funding

No funding was received for this study.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

D.M. was supported by the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4 - Call for tender No. 3138 of December 16, 2021, rectified by Decree n.3175 of December 18, 2021 of Italian Ministry of University and Research funded by the European Union – NextGenerationEU; Project code CN_00000033, Concession Decree No. 1034 of June 17, 2022 adopted by the Italian Ministry of University and Research, CUP B83C22002930006, Project title "National Biodiversity Future Center - NBFC". The authors also acknowledge the support of the Short-term mobility Program for Scientists/Researchers from Italian and Foreign Institutions (2023) of the National Research Council of Italy (CNR), (prot. n. 206551 dated July 04, 2023).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2025.127239.

Data availability

Data will be made available on request.

References

Abbas, H.M.M., Rais, U., Altaf, M.M., Rasul, F., Shah, A., Tahir, A., Nafees-Ur-Rehman, M., Shaukat, M., Sultan, H., Zou, R., Khan, M.N., Nie, L., 2024. Microbial-inoculated biochar for remediation of salt and heavy metal contaminated soils. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2024.176104.

APAT, 2004. Proposal of a Technical Guidance on Analysis Methods for Soil and Contaminated Sites. Use of Ecotoxicological and Biological Indicators (Proposta di Guida Tecnica su Metodi di Analisi per il Suolo e i Siti Contaminati. Utilizzo di Indicatori Biologici ed Ecotossicologici). RTI CTN_TES 1/2004, Rome, Italy. APAT.

Baud-Grasset, F., Baud-Grasset, S., Safferman, S.I., 1993. Evaluation of the bioremediation of a contaminated soil with phytotoxicity tests. Chemosphere 26. https://doi.org/10.1016/0045-6535(93)90187-A.

Cerovic, Z.G., Masdoumier, G., Ghozlen, N.B., Latouche, G., 2012. A new optical leaf-clip meter for simultaneous non-destructive assessment of leaf chlorophyll and epidermal flavonoids. Physiol. Plantarum 146. https://doi.org/10.1111/j.1399-3054.2012.01639.x.

Chandra, R., Kang, H., 2016. Mixed heavy metal stress on photosynthesis, transpiration rate, and chlorophyll content in poplar hybrids. For. Sci. Technol. 12. https://doi.org/10.1080/21580103.2015.1044024.

Cheng, M., Zeng, G., Huang, D., Lai, C., Xu, P., Zhang, C., Liu, Y., 2016. Hydroxyl radicals based advanced oxidation processes (AOPs) for remediation of soils contaminated with organic compounds: a review. Chem. Eng. J. 284. https://doi.org/10.1016/j.cej.2015.09.001.

Cipullo, S., Negrin, I., Claveau, L., Snapir, B., Tardif, S., Pulleyblank, C., Prpich, G., Campo, P., Coulon, F., 2019. Linking bioavailability and toxicity changes of complex chemicals mixture to support decision making for remediation endpoint of contaminated soils. Sci. Total Environ. 650. https://doi.org/10.1016/j.scitotenv.2018.09.339.

Conte, A., Chiaberge, S., Pedron, F., Barbafieri, M., Petruzzelli, G., Vocciante, M., Franchi, E., Pietrini, I., 2021. Dealing with complex contamination: a novel approach with a combined bio-phytoremediation strategy and effective analytical techniques. J. Environ. Manag. 288. https://doi.org/10.1016/j.jenvman.2021.112381.

Dai, Y., Shen, Z., Liu, Y., Wang, L., Hannaway, D., Lu, H., 2009. Effects of shade treatments on the photosynthetic capacity, chlorophyll fluorescence, and chlorophyll content of Tetrastigma hemsleyanum Diels et Gilg. Environ. Exp. Bot. 65. https://doi.org/10.1016/j.envexpbot.2008.12.008.

Dai, Y., Zhang, N., Xing, C., Cui, Q., Sun, Q., 2019. The adsorption, regeneration and engineering applications of biochar for removal organic pollutants: a review. Chemosphere. https://doi.org/10.1016/j.chemosphere.2019.01.161.

de la Parra, S., González, V., Solórzano Vives, P., Curiel-Alegre, S., Velasco-Arroyo, B., Rad, C., Barros, R., Tamayo-Ramos, J.A., Rumbo, C., 2022. Comparative toxicological assessment of three soils polluted with different levels of hydrocarbons and heavy metals using in vitro and in vivo approaches. Environ. Pollut. 315. https://doi.org/10.1016/j.envpol.2022.120472.

Di Baccio, D., Pietrini, F., Bertolotto, P., Pérez, S., Barcelò, D., Zacchini, M., Donati, E., 2017. Response of Lemna gibba L. to high and environmentally relevant concentrations of ibuprofen: removal, metabolism and morpho-physiological traits for biomonitoring of emerging contaminants. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2016.12.191.

Djoumbou Feunang, Y., Eisner, R., Knox, C., Chepelev, L., Hastings, J., Owen, G., Fahy, E., Steinbeck, C., Subramanian, S., Bolton, E., Greiner, R., Wishart, D.S., 2016. ClassyFire: automated chemical classification with a comprehensive, computable taxonomy. J Cheminform 8. https://doi.org/10.1186/s13321-016-0174-y.

Dorn, P.B., Salanitro, J.P., 2000. Temporal ecological assessment of oil contaminated soils before and after bioremediation. Chemosphere 40. https://doi.org/10.1016/S0045-6535(99)00304-5.

Fang, X., Zhang, M., Zheng, P., Wang, H., Wang, K., Lv, J., Shi, F., 2024. Biochar-bacteria-plant combined potential for remediation of oil-contaminated soil. Front. Microbiol. 15. https://doi.org/10.3389/fmicb.2024.1343366.

Gaude, N., Bréhélin, C., Tischendorf, G., Kessler, F., Dörmann, P., 2007. Nitrogen deficiency in Arabidopsis affects galactolipid composition and gene expression and results in accumulation of fatty acid phytyl esters. Plant J. 49. https://doi.org/10.1111/j.1365-313X.2006.02992.x.

Guerin, T.F., 2022. Phytotoxicity complements chemical assessment for re-use and re-purposing of refinery wastes for soil amendment purposes after bioremediation. J. Environ. Manag. 317. https://doi.org/10.1016/j.jenvman.2022.115257.

Guidi, L., Lo Piccolo, E., Landi, M., 2019. Chlorophyll fluorescence, photoinhibition and abiotic stress: does it make any difference the fact to be a C3 or C4 species? Front. Plant Sci. https://doi.org/10.3389/fpls.2019.00174.

Guo, Q., He, Z., Liu, X., Liu, B., Zhang, Y., 2020. High-throughput non-targeted metabolomics study of the effects of perfluorooctane sulfonate (PFOS) on the metabolic characteristics of A. thaliana leaves. Sci. Total Environ. 710. https://doi.org/10.1016/j.scitotenv.2019.135542.

Guo, S., Liu, X., Tang, J., 2022. Enhanced degradation of petroleum hydrocarbons by immobilizing multiple bacteria on wheat bran biochar and its effect on greenhouse gas emission in saline-alkali soil. Chemosphere 286. https://doi.org/10.1016/j.chemosphere.2021.131663.

Gutbrod, K., Romer, J., Dörmann, P., 2019. Phytol metabolism in plants. Prog. Lipid Res. 74. https://doi.org/10.1016/j.plipres.2019.01.002.

Hamberger, A., Stenhagen, G., 2003. Erucamide as a modulator of water balance: new function of a fatty acid amide. Neurochem. Res. 28. https://doi.org/10.1023/A:1022364830421.

He, L., Xu, Yan, Zhang, M., Gul, S., Zhang, X., Zhong, H., Tang, Y., Dong, D., Xu, Yong, Liu, D., Bolan, N., Wang, H., 2024. Effect of remediation technologies on soil fertility in heavy metal(loid)-contaminated soils: a critical review. Crit. Rev. Environ. Sci. Technol. 54. https://doi.org/10.1080/10643389.2024.2315009.

Huang, H.Y., Ren, Q.Q., Lai, Y.H., Peng, M.Y., Zhang, J., Yang, L.T., Huang, Z.R., Chen, L. S., 2021. Metabolomics combined with physiology and transcriptomics reveals how Citrus grandis leaves cope with copper-toxicity. Ecotoxicol. Environ. Saf. 223. https://doi.org/10.1016/j.ecoenv.2021.112579.

Iannilli, V., D'Onofrio, G., Marzi, D., Passatore, L., Pietrini, F., Massimi, L., Zacchini, M., 2024. Lithium toxicity in Lepidium sativum L. seedlings: exploring Li accumulation's impact on germination, root growth, and DNA integrity. Environments - MDPI 11. https://doi.org/10.3390/environments11050093.

Ischebeck, T., Zbierzak, A.M., Kanwischer, M., Dörmann, P., 2006. A salvage pathway for phytol metabolism in Arabidopsis. J. Biol. Chem. 281. https://doi.org/10.1074/jbc.M509222200.

ISO 11269-2:2012, 2012. Soil Quality — Determination of the Effects of Pollutants on Soil Flora — Part 2: Effects of Contaminated Soil on the Emergence and Early Growth of Higher Plants. Germany.

Kafle, A., Timilsina, A., Gautam, A., Adhikari, K., Bhattarai, A., Aryal, N., 2022. Phytoremediation: mechanisms, plant selection and enhancement by natural and synthetic agents. Environ. Adv. 8. https://doi.org/10.1016/j.envadv.2022.100203.

Kummerová, M., Krulová, J., Zezulka, Š., Tříska, J., 2006. Evaluation of fluoranthene phytotoxicity in pea plants by Hill reaction and chlorophyll fluorescence. Chemosphere 65. https://doi.org/10.1016/j.chemosphere.2006.01.052.

Kuppusamy, S., Thavamani, P., Venkateswarlu, K., Lee, Y.B., Naidu, R., Megharaj, M., 2017. Remediation approaches for polycyclic aromatic hydrocarbons (PAHs) contaminated soils: technological constraints, emerging trends and future directions. Chemosphere 168. https://doi.org/10.1016/j.chemosphere.2016.10.115.

Kwak, J. Il, Nam, S.-H., Kim, S.W., Bajagain, R., Jeong, S.-W., An, Y.-J., 2019. Changes in soil properties after remediation influence the performance and survival of soil algae and earthworm. Ecotoxicol. Environ. Saf. 174. https://doi.org/10.1016/j.ecoenv.2019.02.079.

Lex, A., Gehlenborg, N., Strobelt, H., Vuillemot, R., Pfister, H., 2014. UpSet: visualization of intersecting sets. IEEE Trans. Vis. Comput. Graph. 20. https://doi.org/10.1109/TVCG.2014.2346248.

Lippold, F., vom Dorp, K., Abraham, M., Hölzl, G., Wewer, V., Yilmaz, J.L., Lager, I., Montandon, C., Besagni, C., Kessler, F., Stymne, S., Dörmann, P., 2012. Fatty acid phytyl ester synthesis in chloroplasts of Arabidopsis. Plant Cell 24. https://doi.org/10.1105/tpc.112.095588.

Lohani, H., Kumar, A., Bidarakundi, V., Agrawal, L., Haider, S.Z., Chauhan, N.K., 2024. Identification of Fatty acids, amides and cinnamic acid derivatives in Supercritical-CO2 extracts of Cinnamomum tamala leaves using UPLC-Q-TOF-MSE combined with chemometrics. Molecules 29. https://doi.org/10.3390/molecules29163760.

Lu, Y., Kronzucker, H.J., Yu, M., Shabala, S., Shi, W., 2024. Nitrogen-loss and carbon-footprint reduction by plant-rhizosphere exudates. Trends Plant Sci. https://doi.org/10.1016/j.tplants.2023.09.007.

Ma, D., Hu, H., Feng, J., Xu, B., Du, C., Yang, Y., Xie, Y., Wang, C., 2025. Metabolomics and transcriptomics analyses revealed overexpression of TaMGD enhances wheat plant heat stress resistance through multiple responses. Ecotoxicol. Environ. Saf. 290. https://doi.org/10.1016/j.ecoenv.2025.117738.

Mahood, E.H., Bennett, A.A., Komatsu, K., Kruse, L.H., Lau, V., Rahmati Ishka, M., Jiang, Y., Bravo, A., Louie, K., Bowen, B.P., Harrison, M.J., Provart, N.J., Vatamaniuk, O.K., Moghe, G.D., 2023. Information theory and machine learning illuminate large-scale metabolomic responses of Brachypodium distachyon to environmental change. Plant J. 114. https://doi.org/10.1111/tpj.16160.

Maia, M., Figueiredo, A., Cordeiro, C., Sousa Silva, M., 2023. FT-ICR-MS-based metabolomics: a deep dive into plant metabolism. Mass Spectrom. Rev. https://doi.org/10.1002/mas.21731.

Malkawi, H.I., Kapiel, T.Y.S., 2024. Microbial biotechnology: a key tool for addressing climate change and food insecurity. European Journal of Biology and Biotechnology 5. https://doi.org/10.24018/ejbio.2024.5.2.503.

Martínez-Peñalver, A., Reigosa, M.J., Sánchez-Moreiras, A.M., 2011. Imaging chlorophyll a fluorescence reveals specific spatial distributions under different stress conditions. Flora: morphology, Distribution. Functional Ecology of Plants 206. https://doi.org/10.1016/j.flora.2011.02.004.

Mazzurco-Miritana, V., Passatore, L., Zacchini, M., Pietrini, F., Peruzzi, E., Carloni, S., Rolando, L., Garbini, G.L., Barra Caracciolo, A., Silvani, V., Moscatelli, M.C., Marabottini, R., Massaccesi, L., Marinari, S., Nogués, I., 2025. Promoting the remediation of contaminated soils using biochar in combination with bioaugmentation and phytoremediation techniques. Sci. Rep. 15. https://doi.org/10.1038/s41598-025-93879-5.

Mendes, P.M., Ribeiro, J.A., Martins, G.A., Lucia, T., Araujo, T.R., Fuentes-Guevara, M. D., Corrêa, L.B., Corrêa, É.K., 2021. Phytotoxicity test in check: proposition of methodology for comparison of different method adaptations usually used worldwide. J. Environ. Manag. 291. https://doi.org/10.1016/j.jenvman.2021.112698.

Mikhailova, S.I., Andreeva, V.Y., Zinner, N.S., Gulik, E.S., Suchkova, S.A., Belousov, M. V., 2022. Toxic properties and allelopathic activity of Melilotus officinalis (L.) Pall. Acta Biol Sib 8. https://doi.org/10.14258/abs.v8.e03.

Mukherjee, S., Sarkar, B., Aralappanavar, V.K., Mukhopadhyay, R., Basak, B.B., Srivastava, P., Marchut-Mikołajczyk, O., Bhatnagar, A., Semple, K.T., Bolan, N., 2022. Biochar-microorganism interactions for organic pollutant remediation: challenges and perspectives. Environ. Pollut. 308. https://doi.org/10.1016/j.envpol.2022.119609.

Nogues, I., Passatore, L., Bustamante, M.Á., Pallozzi, E., Luz, J., Traquete, F., Ferreira, A. E.N., Sousa Silva, M., Cordeiro, C., 2023. Cultivation of Melilotus officinalis as a source of bioactive compounds in association with soil recovery practices. Front. Plant Sci. 14. https://doi.org/10.3389/fpls.2023.1218594.

Painuli, S., Quispe, C., Herrera-Bravo, J., Semwal, P., Martorell, M., Almarhoon, Z.M., Seilkhan, A., Ydyrys, A., Rad, J.S., Alshehri, M.M., Daştan, S.D., Taheri, Y., Calina, D., Cho, W.C., 2022. Nutraceutical profiling, bioactive composition, and biological applications of Lepidium sativum L. Oxid. Med. Cell. Longev. https://doi.org/10.1155/2022/2910411.

Palmeggiani, G., Lebrun, M., Simiele, M., Bourgerie, S., Morabito, D., 2021. Effect of biochar application depth on a former mine technosol: impact on metal(loid)s and alnus growth. Environments - MDPI 8. https://doi.org/10.3390/environments8110120.

Passatore, L., Pietrini, F., Carloni, S., Massimi, L., Giusto, C., Zacchini, M., Iannilli, V., 2022. Morpho-physiological and molecular responses of Lepidium sativum L. seeds induced by bismuth exposure. Sci. Total Environ. 831. https://doi.org/10.1016/j.scitotenv.2022.154896.

Pietrini, F., Iannilli, V., Passatore, L., Carloni, S., Sciacca, G., Cerasa, M., Zacchini, M., 2022. Ecotoxicological and genotoxic effects of dimethyl phthalate (DMP) on Lemna minor L. and Spirodela polyrhiza (L.) Schleid. plants under a short-term laboratory assay. Sci. Total Environ. 806. https://doi.org/10.1016/j.scitotenv.2021.150972.

Pietrini, F., Iori, V., Cheremisina, A., Shevyakova, N.I., Radyukina, N., Kuznetsov, V.V., Zacchini, M., 2015. Evaluation of nickel tolerance in amaranthus paniculatus l Plants by measuring photosynthesis oxidative status antioxidative response and metal-binding molecule content. Environ. Sci. Pollut. Control Ser. 22. https://doi.org/10.1007/s11356-014-3349-y.

Pietrini, F., Passatore, L., Carloni, S., Massimi, L., Astolfi, M.L., Giusto, C., Zacchini, M., 2023. Bismuth exposure affects morpho-physiological performances and the ionomic profile in garden cress (*Lepidium sativum* L.) plants. Front. Environ. Sci. 11. https://doi.org/10.3389/fenvs.2023.1221573.

Pietrini, F., Zacchini, M., Iori, V., Pietrosanti, L., Bianconi, D., Massacci, A., 2010. Screening of poplar clones for cadmium phytoremediation using photosynthesis, biomass and cadmium content analyses. Int. J. Phytoremediation 12. https://doi.org/10.1080/15226510902767163.

Rouhani, A., Hejcman, M., Trögl, J., 2025. A review of soil pollution by potentially toxic elements and remediation strategies in copper mining areas in Iran. Int. J. Environ. Sci. Technol. https://doi.org/10.1007/s13762-024-05800-8.

Rutz, A., Sorokina, M., Galgonek, J., Mietchen, D., Willighagen, E., Gaudry, A., Graham, J.G., Stephan, R., Page, R., Vondrášek, J., Steinbeck, C., Pauli, G.F., Wolfender, J.L., Bisson, J., Allard, P.M., 2022. The LOTUS initiative for open knowledge management in natural products research. eLife 11. https://doi.org/10.7554/eLife.70780.

Šamec, D., Karalija, E., Šola, I., Vujčić Bok, V., Salopek-Sondi, B., 2021. The role of polyphenols in abiotic stress response: the influence of molecular structure. Plants. https://doi.org/10.3390/plants10010118.

Sánchez-Moreiras, A.M., Graña, E., Reigosa, M.J., Araniti, F., 2020. Imaging of chlorophyll a fluorescence in natural compound-induced stress detection. Front. Plant Sci. https://doi.org/10.3389/fpls.2020.583590.

Satish, L., Shamili, S., Yolcu, S., Lavanya, G., Alavilli, H., Swamy, M.K., 2020. Biosynthesis of secondary metabolites in plants as influenced by different factors. In: Swamy, M.K. (Ed.), Plant-Derived Bioactives: Production, Properties and Therapeutic Applications. Springer, Singapore, Singapore. https://doi.org/10.1007/978-981-15-1761-7_3.

Steliga, T., Kluk, D., 2021. Assessment of the suitability of melilotus officinalis for phytoremediation of soil contaminated with petroleum hydrocarbons (TPH and PAH), Zn, Pb and Cd based on toxicological tests. Toxics 9. https://doi.org/10.3390/toxics9070148.

Sun, L., Lu, Y., Kronzucker, H.J., Shi, W., 2016. Quantification and enzyme targets of fatty acid amides from duckweed root exudates involved in the stimulation of denitrification. J. Plant Physiol. 198. https://doi.org/10.1016/j.jplph.2016.04.010.

Swoczyna, T., Kalaji, H.M., Bussotti, F., Mojski, J., Pollastrini, M., 2022. Environmental stress - what can we learn from chlorophyll a fluorescence analysis in woody plants? A review. Front. Plant Sci. https://doi.org/10.3389/fpls.2022.1048582.

Tian, Y., Ungerer, P., Zhang, H., Ruban, A.V., 2017. Direct impact of the sustained decline in the photosystem II efficiency upon plant productivity at different developmental stages. J. Plant Physiol. 212. https://doi.org/10.1016/j.jplph.2016.10.017.

Tripathi, V., Gaur, V.K., Kaur, I., Srivastava, P.K., Manickam, N., 2024. Unlocking bioremediation potential for site restoration: a comprehensive approach for crude oil degradation in agricultural soil and phytotoxicity assessment. J. Environ. Manag. 355. https://doi.org/10.1016/j.jenvman.2024.120508.

US EPA, 1996. Ecological Effects Test Guidelines OPPTS 850.4200: Seed Germination/Root Elongation Toxicity Test.

Vidi, P.-A., Kanwischer, M., Baginsky, S., Austin, J.R., Csucs, G., Dörmann, P., Kessler, F., Bréhélin, C., 2006. Tocopherol cyclase (VTE1) localization and vitamin E accumulation in chloroplast Plastoglobule Lipoprotein particles. J. Biol. Chem. 281. https://doi.org/10.1074/jbc.M511939200.

Villette, C., Maurer, L., Delecolle, J., Zumsteg, J., Erhardt, M., Heintz, D., 2019. In situ localization of micropollutants and associated stress response in Populus nigra leaves. Environ. Int. 126. https://doi.org/10.1016/j.envint.2019.02.066.

Wishart, D.S., Guo, A.C., Oler, E., Wang, F., Anjum, A., Peters, H., Dizon, R., Sayeeda, Z., Tian, S., Lee, B.L., Berjanskii, M., Mah, R., Yamamoto, M., Jovel, J., Torres-Calzada, C., Hiebert-Giesbrecht, M., Lui, V.W., Varshavi, Dorna, Varshavi, Dorsa, Allen, D., Arndt, D., Khetarpal, N., Sivakumaran, A., Harford, K., Sanford, S., Yee, K., Cao, X., Budinski, Z., Liigand, J., Zhang, L., Zheng, J., Mandal, R., Karu, N., Dambrova, M., Schiöth, H.B., Greiner, R., Gautam, V., 2022. Hmdb 5.0: the Human Metabolome database for 2022. Nucleic Acids Res. 50. https://doi.org/10.1093/nar/gkab1062.

Xia, J., Psychogios, N., Young, N., Wishart, D.S., 2009. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucleic Acids Res. 37. https://doi.org/10.1093/nar/gkp356.

Xie, Y., Peng, Q., Ji, Y., Xie, A., Yang, L., Mu, S., Li, Z., He, T., Xiao, Y., Zhao, J., Zhang, Q., 2021. Isolation and identification of antibacterial bioactive compounds from Bacillus megaterium L2. Front. Microbiol. 12. https://doi.org/10.3389/fmicb.2021.645484.

Xu, L., Zhao, F., Xing, X., Peng, J., Wang, J., Ji, M., Li, B.L., 2024. A review on remediation technology and the remediation evaluation of heavy metal-contaminated soils. Toxics 12. https://doi.org/10.3390/toxics12120897.

Yu, H., He, Y., Zhang, W., Chen, L., Zhang, J., Zhang, X., Dawson, W., Ding, J., 2022. Greater chemical signaling in root exudates enhances soil mutualistic associations in invasive plants compared to natives. New Phytol. 236. https://doi.org/10.1111/nph.18289.

Zawierucha, I., Malina, G., Herman, B., Rychter, P., Biczak, R., Pawlowska, B., Bandurska, K., Barczynska, R., 2022. Ecotoxicity and bioremediation potential assessment of soil from oil refinery station area. J. Environ. Health Sci. Eng. 20. https://doi.org/10.1007/s40201-021-00780-0.

Zhang, Y., Dong, X., Jiang, Z., Cao, B., Ge, S., Hu, M., 2013. Assessment of the ecological security of immobilized enzyme remediation process with biological indicators of soil health. Environ. Sci. Pollut. Control Ser. 20. https://doi.org/10.1007/s11356-012-1455-2.

Zhang, Z., Ma, R., Tao, Y., Ma, H., Jiang, X., Wang, Z., Yang, Y., 2025. Changes of photosynthetic characteristics, stomatal microstructure and proline metabolism in wheat seedlings under different combined treatments of zinc, iron and copper. Ecotoxicol. Environ. Saf. 302. https://doi.org/10.1016/j.ecoenv.2025.118661.

Zhao, Y., Zhang, F., Mickan, B., Wang, D., Wang, W., 2022. Physiological, proteomic, and metabolomic analysis provide insights into bacillus sp.-mediated salt tolerance in wheat. Plant Cell Rep. 41. https://doi.org/10.1007/s00299-021-02788-0.

Zheng, X., Xu, W., Dong, J., Yang, T., Shangguan, Z., Qu, J., Li, X., Tan, X., 2022. The effects of biochar and its applications in the microbial remediation of contaminated soil: a review. J. Hazard Mater. 438. https://doi.org/10.1016/j.jhazmat.2022.129557.

Zhou, Xiangru, Gong, F., Dong, J., Lin, X., Cao, K., Xu, H., Zhou, Xiaofu, 2024. Abscisic acid affects phenolic acid content to increase tolerance to UV-B stress in Rhododendron chrysanthum pall. Int. J. Mol. Sci. 25. https://doi.org/10.3390/ijms25021234.