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Managing Post-Phytoremediation Biomass Within a Circular Economy Framework: Multitrophic Ecotoxicological Assessment of Biomass, Derived Biochar and Their Leachable Fractions

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Featured Application

The proposed multitrophic ecotoxicological assessment framework can be directly applied by environmental practitioners, waste management operators, and decision-makers to evaluate the safety of phytoremediation-derived biomass and its conversion products prior to environmental use. The results demonstrate that pyrolysis can serve as an effective risk mitigation strategy, enabling the transformation of contaminated biomass into a more stable material suitable for soil application under controlled conditions. At the same time, the study highlights the necessity of including leachate-based aquatic toxicity tests to avoid underestimation of environmental risks. The approach supports risk-based decision-making and contributes to the development of safe circular economy pathways for biomass valorization in soil remediation systems.

Abstract

Phytoremediation is a sustainable approach for the remediation of heavy metal–contaminated soils; however, the management of contaminated biomass generated during this process remains an insufficiently addressed challenge. Such biomass constitutes a secondary waste stream that may release mobile pollutants and pose environmental risks. In this study, an integrated ecotoxicological assessment framework was applied to evaluate phytoremediation-derived biomass and its transformation products obtained via pyrolysis. Two types of woody biomass with different heavy metal contents and their corresponding biochars produced at 700 °C were investigated. A multitrophic battery of bioassays combining direct exposure assays using terrestrial organisms (higher plants, *Eisenia fetida*, and soil microbial activity) with leachate-based assays using aquatic organisms (*Lemna minor*, *Daphnia magna*, and *Aliivibrio fischeri*) was applied. Untreated biomass exhibited high to extreme toxicity in aquatic systems (toxic units, TU >100) and significant phytotoxic effects. Pyrolysis substantially reduced contaminant mobility and ecotoxicity of leachates, resulting in lower toxicity (TU typically <15) and no significant effects on plant growth, earthworm survival, or soil microbial functional diversity. Residual toxicity was linked to elevated pH and trace amounts of thermally generated organic substances. These results demonstrate that pyrolysis effectively reduces the environmental risk of contaminated biomass and supports the use of multitrophic ecotoxicological testing for safe waste valorization within circular economy strategies.

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1. Introduction

Soil pollution remains one of the primary environmental challenges worldwide, significantly affecting ecosystem functioning, agricultural productivity and human health. It directly interferes with the achievement of several of the Sustainable Development Goals (SDGs) including food security (SDG2), clean water (SDG6), terrestrial (SDG15) and aquatic (SDG14) biodiversity [1]. In particular, soil pollution is attributed to industrial activities, mining, waste processing and agriculture [2]. In 2018, nearly 650,000 contaminated sites with a total area of nearly 180,000 km^2 were registered in the EU-28. Of these, about 19% may require remediation. Meanwhile, it is estimated that there are about 2.8 million potentially contaminated sites in the EU-28 [3].

In response to these challenges, remediation technologies have evolved from conventional physicochemical approaches (including excavation of contaminated soil, thermal desorption coupled with vapor treatment, or the pump-and-treat method for remediating contaminated groundwater) toward more sustainable and nature-based solutions [4]. Among these, phytoremediation has gained increased attention as a cost-effective and environmentally friendly strategy for both soil and water treatment [5,6]. However, despite its advantages, phytoremediation introduces a critical and often overlooked secondary problem: the generation of large quantities of biomass, in many cases representing hazardous waste [7].

Biomass derived from phytoremediation processes frequently contains elevated concentrations of heavy metals and organic contaminants. If it is not properly managed, this material may pose a significant risk of secondary environmental contamination during storage, processing or disposal. While several management options exist, including biological treatments (e.g., composting) and thermochemical processes (e.g., combustion, gasification, and pyrolysis), there remains a lack of consensus regarding their environmental safety and suitability within circular economy frameworks [8].

Pyrolysis has emerged as a promising pathway for transforming contaminated biomass. This process leads to a simultaneous substantial reduction in the waste volume and production of value-added products, primarily biochar. At the same time, pyrolysis induces significant physicochemical transformations, including the concentration of non-volatile elements such as heavy metals and the formation of new organic compounds, including polycyclic aromatic hydrocarbons and volatile organic compounds. These transformations may alter the environmental risk profile of the resulting material in complex and not fully predictable ways [9,10].

Biochar is an easily modifiable, highly porous, and carbon-rich material with a large specific surface area [11]. Due to its properties, a number of applications for biochar are being explored, mainly in the environmental sector. Among other applications, it can be used in water treatment for the adsorption of a variety of pollutants or biogenic substances [12], as a catalyst for the degradation of organic compounds [13], or as a compost additive [14]. Nonetheless, the soil is typically the final disposal site for biochar [15]. Biochar may improve soil physicochemical properties, including enhancing water-holding capacity (WHC) [16], increasing nutrient retention [17] and immobilizing soil pollutants [18]. Most notably, biochar can stimulate growth and increase the yield and productivity of plants [19].

On the other hand, improper use of those materials could result in adverse environmental effects [20,21]. Following its application, biochar can negatively influence living organisms due to the release process of accumulated contaminants [22]. The issue mag-

nifies when plants are used for bioremediation of soil contaminated with heavy metals (HMs). In contrast to organic compounds HMs are non-biodegradable [10]. Through phytoaccumulation, HMs are transported and accumulated in plant tissues, and metal concentrations may reach level of thousands of mg/kg in the dry weight of the plant, as their phytovolatilization is negligible [6,23]. During pyrolysis, the bioavailable metal fraction decreases considerably, although the total concentration of metals in biochar increases and the problem remains [24,25].

Aside from heavy metals, other contaminants may be present in biochar. The most common include carcinogenic and persistent pollutants, namely polycyclic aromatic hydrocarbons (PAHs), formed during thermal treatment of biomass [26,27]. Besides them, volatile organic compounds (VOCs), triggering microbial responses and mimicking the action of plant hormones, are also fairly common [28]. Improper management of biochar may result in secondary contamination with the aforementioned pollutants, potentially leading to toxic effects. Therefore, rigorous evaluation of biochar for potential ecotoxicological impacts is essential to ensure environmental safety.

Although numerous studies have addressed the physicochemical properties of biochar and its potential applications, comparatively little attention has been devoted to the integrated ecotoxicological evaluation of biomass derived specifically from phytoremediation processes and their transformation products. Existing ecotoxicological studies on contaminated biomass and biochars have predominantly focused either on physicochemical characterization, contaminant concentrations, or single-organism bioassays [8–11,24]. Moreover, most investigations have evaluated biochars intended for agricultural or remediation purposes without simultaneously considering the environmental behavior of the original phytoremediation-derived biomass and its transformed products [10,11,15]. As a result, limited information is available regarding how thermochemical conversion alters ecotoxicological risk across different exposure pathways and trophic levels.

To address this gap, the present study introduces an integrated ecotoxicological assessment framework for evaluating post-phytoremediation biomass and its derived biochar. The novelty of the present work lies in the integration of standardized leaching procedures [29] with a multitrophic ecotoxicological framework encompassing both direct soil exposure and leachate-based assays. Furthermore, this study simultaneously evaluates phytoremediation-derived biomass and corresponding biochars using a comparative risk-oriented approach, enabling assessment not only of contaminant concentrations but also of contaminant mobility and biological responses across terrestrial and aquatic compartments. Such integration enables a more comprehensive evaluation of potential environmental risks associated with both solid materials and their mobile fractions.

The specific objectives of this study were:

- To compare the physicochemical properties and contaminant mobility in phytoremediation-derived biomass and corresponding biochars;
- To assess their ecotoxicological effects using a suite of bioassays representing different trophic levels and environmental compartments;
- To identify key factors responsible for observed toxicity patterns and their transformation during pyrolysis.

We hypothesize that pyrolysis significantly reduces the ecotoxicological risk of phytoremediation-derived biomass not through a reduction in total contaminant concentrations, but through the immobilization of heavy metals and degradation of labile organic toxicants, thereby decreasing contaminant mobility, bioavailability and associated biological effects. Although non-volatile metals may become concentrated during pyrolysis due to mass reduction, their incorporation into more stable mineral and carbonaceous structures is expected to reduce environmental availability. At the same time, we assume

that residual toxicity in biochar leachates may be governed by physicochemical factors such as alkalinity and trace organic by-products rather than metal mobility.

The presented approach directly contributes to current developments in soil pollution management by addressing the critical, yet underexplored, stage of post-remediation biomass handling. By linking ecotoxicological assessment with circular economy strategies, this work supports the development of safe and sustainable pathways for the valorization of remediation residues.

2. Materials and Methods

2.1. Biomass and Biochar Characterization

Two types of biomass derived from birch (*Betula pendula*) wood were investigated. The first type, referred to as 'biomass' (BM), originated from trees grown in uncontaminated soil in Miasteczko Śląskie, Poland. The second type, termed 'contaminated biomass' (BMC), was obtained from trees growing on a contaminated smelter site within the same city locality (50°30′07.0″ N, 18°55′31.6″ E). This site has been the subject of several studies reporting elevated levels of heavy metals, notably Zn and Pb, in topsoil layers [30–32].

Both feedstock materials were pyrolyzed and processed into biochar' (BC) and 'contaminated biochar' (BCC), respectively. The detailed pyrolysis procedure and physicochemical characteristics of the obtained materials have been described in our previous work [33]. Briefly, pyrolysis was performed in a laboratory muffle furnace using covered ceramic crucibles under oxygen-limited conditions. The furnace was preheated to the target temperature (700 °C), after which the samples were introduced and thermally treated for 10 min. Two ceramic crucibles were processed simultaneously, each containing 10 g of biomass. The crucibles were sealed with lids to minimize the access of oxidizing gases and maintain oxygen-limited conditions during thermal conversion. Following pyrolysis, an average of approximately 2.1 g of biochar was obtained from 10 g of biomass, corresponding to a mean yield of approximately 21%. Until further analysis, biomass samples were stored in paper envelopes and biochars in glass containers. For toxicity assessments, all samples were crumbled and sieved through a 4 mm mesh sieve.

Preparation of Leachates

Toxicity tests on aquatic organisms were carried out using aqueous leachates of biomass and biochar samples. The leaching procedure was carried out in accordance with EN 12457-2 [34]. 25 g of each sample was placed in a bottle and topped up with 250 mL of distilled water to obtain a mixture with a liquid/solid ratio (L/S) of 10. The closed bottles were then placed in a shaker and stirred at 170 rpm for 24 h. The mixtures were then centrifuged for 30 min at 10,000 rpm, and the supernatants were filtered through quality MN 617 filter papers (Macherey-Nagel, Düren, Germany).

2.2. Physicochemical Analyses

To establish the relationship between the toxicity of biomass and biochar samples and their physicochemical parameters, as well as the differences between them, several physicochemical analyses were performed. Heavy metal contents in solid samples were determined by digestion with a mixture of nitric acid and hydrogen peroxide and analysis using Optima™ 8300 ICP-OES (PerkinElmer, Inc., Waltham, MA, USA). The pH of leachate samples was measured using a Multi 3401 pH meter (WTW, Wuppertal, Germany). Total organic carbon (TOC) was determined using spectrometer NANOCOLOR UV/VIS II (Macherey-Nagel, Düren, Germany) and spectrophotometric test (TOC, test no. 0-78) (Macherey-Nagel, Düren, Germany). Zinc (Zn, test no. 114566), lead (Pb, test no. 114833), phenol (test no. 100856), and total nitrogen (TN, test no. 114763) were de-

termined using spectrometer Spectroquant NOVA 60TM (Merck, Darmstadt, Germany) and spectrophotometric tests (Merck, Darmstadt, Germany). Chlorides, sulfates and phosphates were analyzed using Ion Chromatography Dionex Aquion (Thermo Fisher Scientific, Warsaw, Poland).

2.3. Toxicity Tests on Aquatic Organisms (Assessment of Leachates)

Toxicity tests were conducted on leachate samples across a concentration range of 50%, 25%, 12.5%, 6.25%, and 3.125% (*v*/*v*), using test-specific media as diluents and controls. All experiments were conducted in triplicate (n = 3) unless stated otherwise. Test conditions, including temperature, photoperiod, illumination regime, and medium characteristics, were maintained according to the corresponding OECD or ISO protocols to ensure stable environmental conditions throughout the exposure period.

2.3.1. *Lemna minor* Growth Inhibition Test

The duckweed growth inhibition test was conducted in accordance with OECD 221 [35] on *L. minor*. The tests were performed using plants from our own stock culture purchased from a local aquatic plant supplier (Invital, Kopaczów, Poland). Both stock culture and test plants were cultivated in a climate chamber under a light/dark cycle of 16/8 h, at 23 °C, and 70% humidity. Illumination conditions were provided by Osram Fluora L36W fluorescent lamps (Osram, Munich, Germany) and were maintained at a constant intensity of 40–50 µmol $m^{-2}s^{-1}$ throughout cultivation and testing to ensure uniform plant growth conditions. Steinberg's medium served as the culture medium, and also as both the control solution and diluent.

The test solutions were transferred to multiwell plates (Bionovo, Warsaw, Poland), and in each replicate, 20 mL of solution and four plants with a total of 9 fronds were used. The test duration was 7 days, after which the number of fronds per replicate was recorded. The average growth rate for the control solution and each tested concentration was calculated using Equation (1), and then the percentage inhibition of growth for each solution was determined using Equation (2).

$$\mu_{i-j} = \frac{\ln(N_j) - \ln(N_i)}{t}, \qquad \frac{1}{\mathrm{d}} \tag{1}$$

where terms are defined as follows:

μ_{i-j}—average specific growth rate from time i to j [d^{-1}];
$N_i(j)$—the number of fronds in time i (j); t is the time between i and j.

$$\%I_r = \frac{\mu_C - \mu_T}{\mu_C}, \qquad \% \tag{2}$$

where terms are defined as follows:

$\%I_r$—inhibition in average specific growth rate [%];
μ_C—the µ value of the control group;
μ_T—the µ value of the treatment group.

Based on the inhibition percentage for each dilution, EC50 values were calculated using a linear interpolation method [36].

2.3.2. *Daphnia magna* Acute Immobilization Test

Toxicity tests on daphnids were conducted following OECD 202 [37] using the Daphtoxkit F Magna kit (Microbiotest Inc., Ghent, Belgium). To hatch the test organisms, the ephippia were rinsed with aerated mineral water and then placed in the same medium on Petri dishes. The dishes were continuously illuminated for 72 h. Aerated mineral water was used throughout hatching and testing procedures to maintain adequate oxygen availability

(>3 mg L^{-1}) and stable exposure conditions according to OECD 202 recommendations. Two hours prior to the tests, the organisms were fed powdered spirulina.

The test was conducted in plastic plates with 10 mL of test solution and five juvenile organisms (not older than 24 h) per replicate. Dilutions were prepared with aerated drinking quality mineral water. After 48 h of the test vessels' incubation in darkness, dead or immobilized organisms were counted and the percentage inhibition was calculated according to Equation (3).

$$\%E = \frac{n - n_T}{\mathrm{n}}, \qquad \% \tag{3}$$

where terms are defined as follows:

$\%E$—the percentage of immobilized or dead organisms at the end of the study [%];
n—the number of organisms at the start of the study (5 in each replicate);
n_T—the number of mobile organisms at the end of the study.

Based on these values, it was possible to calculate toxic effects in terms of EC50, or concentrations of leachate that cause 50% immobilization, using a linear interpolation method [36].

2.3.3. *Aliivibrio fischeri* Luminescent Bacteria Inhibition Test

Toxicity test against *A. fischeri* was carried out using a Microtox M500 Analyzer (Modern Water, York, UK), following ISO 11348-3 [38]. The Whole Effluent Toxicity (WET) EPA procedure was used to examine the leachates' toxicity. Solution of 20 g L^{-1} NaCl served as control sample and diluent. Leachate dilutions and controls were tested in 1 mL volumes in round glass cuvettes, continuously cooled to 15 °C. 10 µL of bacterial suspension was added to each cell and the solutions were vortexed (Bio-mix, Warsaw, Poland). The decrease in the luminescence after an exposure time of 15 min was measured. Because BM and BMC leachates were brownish in color, a color-correction procedure was applied. The absorbance of diluted samples was measured at 490 nm to compensate for the luminescence absorption effect associated with color samples. Bioluminescence inhibition calculations were performed using MicrotoxOmni software v 2.1. provided by the Microtox system manufacturer. IC50 values were determined using the linear regression method; detailed information was provided in Supplementary Information—(Equations (S1)–(S6)).

2.3.4. Toxicity Classification of Leachate Samples

Toxicity results obtained for individual bioassays were converted into toxic units (*TUs*) to enable standardized comparison of ecotoxicological responses among different test organisms and endpoints. *TU* values provide an integrated measure of sample toxicity, where higher values indicate stronger toxic effects.

For bioassays in which median effect concentrations (EC_{50} or IC_{50}) could be determined, *TU* values were calculated according to Equation (4) [39].

$$TU = \frac{100}{EC_{50}(IC_{50})} \tag{4}$$

However, in some cases the observed effects did not reach 50% even in least diluted samples, preventing calculation of EC_{50} values by dose–response modeling. In such situations, *TU* values were estimated according to Equation (5) if the toxic effect of the undiluted sample was in the range of 20–50%. If the toxic effect in that sample was <20%, then the sample was considered to be non-toxic [39].

$$TU = 0.02 \cdot E \tag{5}$$

where terms are defined as follows:

E—the highest observed effect.

The use of two equations therefore reflects differences in response characteristics rather than different toxicity concepts. Equation (4) was applied when sufficient toxicity was observed to determine EC_{50} values, whereas Equation (5) was used for samples producing only partial effects below the EC_{50} threshold.

The resulting *TU* values were interpreted according to the toxicity classification system proposed by Persoone et al. [39], where $TU < 0.4$ indicates no toxicity, $TU = 0.4–1$ slight toxicity, $TU = 1–10$ acute toxicity, $TU = 10–100$ high toxicity, and $TU > 100$ very high toxicity (Table 1).

Table 1. Toxicity classification in relation to toxic effect or EC50 (IC50) and TU values [39].

Condition	*TU* Value [–]	Toxicity Classification
$E < 20\%$	<0.4	no toxicity
$20\% \leq E < 50\%$	0.4–1	low toxicity
$10\% < EC_{50}$ $(IC_{50}) \leq 100\%$	1–10	average toxicity
$1\% < EC_{50}$ $(IC_{50}) \leq 10\%$	10–100	high toxicity
EC_{50} $(IC_{50}) \leq 1\%$	≥100	extreme toxicity

2.4. Toxicity Tests on Terrestrial Organisms (Direct Assessment of Biochar Material)

Toxicity tests on terrestrial organisms utilized OECD reference soil [40]), comprising 10% of sphagnum peat (fraction < 2 mm), 20% kaolin clay, up to 70% quartz sand, and 0.3–1% calcium carbonate, ensuring a pH of 6.0 ± 0.5. The water-holding capacity (WHC) of artificial soil was determined prior to the experiments and used for moisture adjustment in all terrestrial assays [41]. Soil moisture conditions were maintained throughout testing by periodic gravimetric control and supplementation with distilled water where required. The tests were performed with a biomass or biochar concentration of 0.01%, 0.1% and 1% (*w*/*w*) for phytotoxicity and earthworm acute toxicity tests and 1% (*w*/*w*) for earthworm avoidance test. The selected application rates (0.01%, 0.1%, and 1% *w*/*w*) were chosen to reflect environmentally relevant and practically applicable doses of biochar and biomass in soil systems. These concentrations are consistent with ranges commonly recommended by commercial biochar producers for soil amendment, where typical application rates correspond to approximately 0.1–1% (*w*/*w*), depending on soil type and intended use. The inclusion of a lower concentration (0.01% *w*/*w*) enabled the assessment of potential effects at minimal exposure levels and provided insight into dose–response relationships.

All experiments were conducted in triplicate ($n = 3$) unless stated otherwise.

2.4.1. Seedling Emergence and Early Growth of Higher Plants Test

Phytotoxicity tests were conducted based on OECD 208 [42]. The soil was mixed with the test samples and then with distilled water to achieve 60% WHC. The pots were filled with 440 g of wet soil. Monocotyledonous *Triticum aestivum* (wheat) and the dicotyledonous plants *Lepidium sativum* (garden cress) and *Cucumis sativus* (cucumber) were used. The selected plant species were used as standardized ecotoxicological indicators due to their sensitivity and widespread use in phytotoxicity assessment and were intended to evaluate biological responses and soil habitat function rather than agricultural suitability. The seeds of the respective plant species were soaked in distilled water 24 h before the start of each test. Each pot was sown with either nine seeds of garden cress or wheat, or seven seeds of cucumber. Test containers were incubated in a climate chamber for 21 days, under conditions of 23 °C and 70% humidity with a 16 h light/8 h dark cycle. To maintain stable exposure conditions, soil moisture was periodically controlled gravimetrically by weighing pots and adjusting water content with distilled water to maintain the target level of 60%

WHC throughout the experimental period. The moisture content was adjusted following OECD 208 [42] recommendations, as this level provides favorable conditions for seed germination and plant growth while maintaining adequate soil aeration and preventing waterlogging. At the end of the test, plants were cut at the ground level and cleaned. The effects of biomasses or biochars on plants growth were accessed by comparing the stem height or dry weight (after drying at 65 °C) to those of control plants. The percentage effects were calculated based on Equation (6).

$$\%E = \frac{p_C - p_T}{p_C}, \qquad \% \tag{6}$$

where terms are defined as follows:

$\%E$—stem/dry biomass growth inhibition/stimulation [%];
p_C—the parameter (stem height or dry biomass weight) of control;
p_T—the parameter (stem height or dry biomass weight) of the treatment group.

2.4.2. Tests Against the Earthworms *Eisenia fetida*

Because biochar materials may substantially modify soil physical properties, particularly WHC, moisture adjustment was considered an important experimental factor in the present study. Previous studies have shown that increased biochar application rates may reduce water availability in artificial soils and consequently affect earthworm responses independently of the intrinsic toxicity of the tested material [43]. Therefore, moisture conditions were adjusted to minimize confounding effects associated with altered water availability and to enable assessment of toxic responses related primarily to the tested biomass and biochar materials. Both *E. fetida* assays were conducted using artificial soil adjusted to 85% WHC. Because biomass and biochar amendments exhibited different water-holding capacities, treatment-specific water volumes were applied to maintain comparable moisture availability across all variants.

Both earthworms' toxicity assays were conducted using organisms from our own breeding. The organisms were obtained from a registered culture of *E. fetida* (vet no. aPL14188401, Kępa Oborska, Poland) and were stored in a 50 L perforated plastic container under controlled, constant culture conditions: 20 °C and 16 h/8 h of day/night cycle.

Before each test, an adequate number of animals with visible clitellum were transferred from breeding to an incubation container filled with artificial soil saturated to 85% WHC. After 24 h, conditioned organisms were quicky washed and placed in a container with moistened paper towels for another 24 h, to allow gut-voiding. Only organisms weighing between 250 and 600 mg were used for testing. In both tests, each replicate used 10 suitable specimens.

Acute Toxicity Test

The test was performed according to the OECD 207 standard [40]. A total of 750 g of wet artificial soil was placed in each test container. Test containers were incubated under constant illumination. Soil moisture was periodically monitored and adjusted to maintain the target WHC values throughout the exposure period. The mortality of organisms was assessed after 7 and 14 days and calculated using Equation (7).

$$\%M = \frac{n - n_T}{n}, \qquad \% \tag{7}$$

where terms are defined as follows:

$\%M$—the percentage of dead organisms at the end of the study [%];
n—the number of organisms at the start of the study (10 in each replicate);
n_T—the number of alive organisms at the end of the study.

Avoidance Test

The avoidance test followed the ISO 17512-1 standard [44]. Two-chamber test containers were used, each including a control and treatment section with 350 g of wet artificial soil. Incubation was carried out in a 16 h/8 h light/dark cycle. After 48 h, the sections were separated and the number of individuals in each section was counted. The avoidance rate for each repetition was calculated based on Equation (8). Worms cut by the partition and separated into two sections were recorded as 0.5 for both compartments.

$$\%A_R = \frac{n_C - n_T}{n_C + n_T}, \qquad \% \tag{8}$$

where terms are defined as follows:

$\%A_R$—the avoidance rate [%];
n_C—the number of organisms in the control section;
n_T—the number of organisms in the treatment section.

2.4.3. Soil Microbial Functional Diversity

The functional diversity of soil microbial communities was assessed using the Biolog® EcoPlates™ system (Biolog Inc., Hayward, CA, USA), following the general procedure [45,46]. Soil suspensions (10 g soil in 90 mL 0.9% NaCl) were prepared from samples obtained after the plant growth tests and serially diluted. Each well of an EcoPlate™ (Biolog Inc., Hayward, CA, USA) was inoculated with 120 µL of suspension and incubated at room temperature in the dark. Color development was measured spectrophotometrically at 590 nm in intervals until the plateau of microbial metabolic activity was reached.

The average well color development (*AWCD*) was calculated from raw optical density values based on Equation (9) [47].

$$AWCD = \frac{\sum_{i=1}^{N} CW_i}{N} \tag{9}$$

where N = 31 substrates, CW_i is the color intensity per well.

Logistic modeling was applied to estimate maximum metabolic activity (A_{max}) and the time to half-maximum activity (t_{50}). Diversity indexes such as Shannon (H′) and Gini–Simpson (GS) were derived to provide an integrated description of functional diversity [48,49]. Detailed calculation formulas, the complete set of indexes, and accompanying statistical protocols are presented in Supplementary Information (Section S2).

2.4.4. Risk and Leaching Reduction Factors

To quantify the effect of pyrolysis on environmental risk reduction and contaminant mobility, three indicators were applied: the risk reduction factor (*RRF*), the leachate hazard index (*LHI*), and the leaching reduction factor (*LRF*).

The leachate hazard index (*LHI*) was calculated using a normalization–aggregation approach commonly applied in environmental assessment indexes (e.g., water and soil quality indexes), where multiple physicochemical parameters are transformed into dimensionless values and combined into a single metric [50–52]. Selected parameters of leachates, including heavy metals (Pb and Zn), total organic carbon (TOC) and phenolic compounds, were normalized relative to their maximum observed values across all samples. The normalized values were then aggregated using an arithmetic mean, according to Equation (10):

$$LHI = \frac{\sum \frac{C_i}{C_{max}}}{n} \tag{10}$$

where C_i is the measured value of parameter i, C_{max} is the maximum observed value of this parameter across the dataset, and n is the number of parameters included in the index (4).

This approach enables the integration of heterogeneous data into a unified, dimensionless indicator suitable for comparative analysis. All parameters were equally weighted.

The risk reduction factor (*RRF*) was calculated as the ratio of the maximum toxic unit (*TU*) value obtained for biomass to that of the corresponding biochar, according to Equation (11):

$$RRF = \frac{TU_{biomass}}{TU_{biochar}} \quad (11)$$

The use of maximum TU values reflects a conservative, worst-case scenario approach in the assessment of ecotoxicological risk.

The leaching reduction factor (*LRF*) was calculated as the ratio of LHI values for biomass and the corresponding biochar, according to Equation (12):

$$LRF = \frac{LHI_{biomass}}{LHI_{biochar}} \quad (12)$$

Higher values of *RRF* and *LRF* indicate a greater reduction in ecotoxicological risk and contaminant mobility, respectively, as a result of pyrolysis.

2.4.5. Statistical Analysis

Prior to statistical analysis, data were tested for normality using the Shapiro–Wilk test. Depending on data distribution and endpoint characteristics, differences between groups were evaluated using either one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) post hoc test or the Kruskal–Wallis test followed by multiple comparisons of mean ranks. Statistical significance was set at $p < 0.05$. Detailed statistical results and pairwise *p*-value matrices are provided in the Supplementary Information. All statistical analyses were performed using Statistica 13.0 (TIBCO Software Inc., Palo Alto, CA, USA) and graphical outputs were prepared accordingly. Detailed results of the statistical analyses, including exact *p*-values, are provided in Supplementary Material (Tables S3–S11).

3. Results

3.1. Physicochemical Characteristics of Biomass, Biochar, and Their Leachates

The feedstocks differed considerably in initial metal content. The contaminated biomass (BMC) contained nearly five times more zinc (1580 mg kg^{-1}) and over eighty times more lead (1863 mg kg^{-1}) than the uncontaminated biomass (BM) (Table 2). Higher retention values indicate greater immobilization of metals within the solid biochar matrix following thermal treatment.

Table 2. Heavy metals content in biomass and biochar (ICP-OES analysis). Metal retention (%) was calculated as the relative proportion of heavy metals retained in biochar compared with the corresponding initial biomass material.

Parameter		Unit	Material			
			BM	BMC	BC	BCC
Metal content	Zn	mg kg^{-1}	308	1580	740	6230
	Pb		23	1863	50	9590
Metal retention	Zn	%	–	–	50	83
	Pb		–	–	46	100

Similar trends were observed in leachate composition. Compared with BM, BMC leachates contained approximately 11-fold higher Zn concentrations (42.1 vs. 3.93 mg L^{-1}) and nearly 4-fold higher Pb concentrations (6.87 vs. 1.85 mg L^{-1}). Differences in dissolved

organic constituents were less pronounced, with TOC increasing by approximately 10% and phenolic compounds remaining within a comparable range between both biomass samples (Table 3). These results indicate that contamination status primarily influenced metal mobility rather than dissolved organic content.

Table 3. Selected properties of leachates [mg L^{-1}].

Parameter	Material			
	BM	BMC	BC	BCC
TOC	2620 ± 151	2887 ± 76	48 ± 7	51 ± 4
TN	<10 *	<10 *	<10 *	<10 *
P-PO_4	55.07 ± 1.14	33.78 ± 2.78	15.60 ± 1.57	4.81 ± 0.83
Cl^-	17.17 ± 0.20	22.10 ± 0.45	12.26 ± 1.69	15.82 ± 0.01
SO_4^{2-}	18.78 ± 0.32	57.87 ± 2.12	5.53 ± 0.59	22.13 ± 0.05
Zn	3.93 ± 0.04	42.10 ± 0.06	<0.20 *	<0.20 *
Pb	1.85 ± 0.05	6.87 ± 0.01	<0.10 *	0.58 ± 0.00
Phenols	138.20 ± 5.82	111.20 ± 0.96	<0.025 *	<0.025 *
pH	4.62 ± 0.03	4.98 ± 0.13	9.72 ± 0.02	10.03 ± 0.08

*—detection limit.

Pyrolysis at 700 °C strongly influenced elemental composition, causing both absolute enrichment and enhanced metal retention efficiency. Metal concentrations increased to 740 mg kg^{-1} Zn and 50 mg kg^{-1} Pb in BC, and 6230 mg kg^{-1} Zn and 9590 mg kg^{-1} Pb in BCC, corresponding to retention rates of 50–83% for Zn and 46–100% for Pb. Differences between feedstocks indicate that the degree of metal immobilization depends on the form of binding within plant tissues and on feedstock chemistry.

Marked changes were also observed in leachate composition (Table 3).

Biomass leachates were acidic (pH ≈ 4.6–5.0), rich in dissolved organic matter (TOC ≈ 2600–2900 mg L^{-1}) and phenols (110–138 mg L^{-1}). Biochar leachates, in contrast, were strongly alkaline (pH 9.7–10.0) and showed a >50-fold reduction in TOC (≈50 mg L^{-1}) and phenols (<0.025 mg L^{-1}). Lead and zinc concentrations dropped sharply after pyrolysis, especially for BCC, where only 0.58 mg L^{-1} Pb was detected, while Zn concentrations remained below the quantification limit (<0.2 mg L^{-1}).

Pyrolysis substantially reduced contaminant mobility irrespective of the initial contamination level. In contaminated biochar-derived leachates, Pb concentrations decreased by approximately 92%, while Zn concentrations fell below the detection limit (>99% reduction). Similar reductions were observed for dissolved organic constituents, with TOC decreasing by approximately 98% and phenolic compounds falling below detection limits. These results demonstrate that thermal conversion reduced not only total contaminant mobility but also minimized differences between uncontaminated and contaminated materials.

These data confirm that high-temperature pyrolysis effectively reduces the leachable fraction of both organic and inorganic contaminants, though it simultaneously increases the alkaline character of the resulting material.

3.2. Toxicity of Leachates Toward Aquatic Organisms

The ecotoxicological responses reported in this section originate from leachate-based assays assessing the toxicity of mobile contaminant fractions toward aquatic organisms.

Aquatic tests revealed important differences between untreated biomass- and biochar-derived leachates (Figure 1). Quantitative differences between uncontaminated and contaminated materials were most pronounced for aquatic toxicity endpoints. Relative to BM, BMC leachates showed approximately 4-fold higher toxicity toward *D. magna* (TU 27.5 vs. 6.3) and more than 5-fold higher toxicity toward *A. fischeri* (TU 145.3 vs. 28.5). In contrast,

differences observed for *L. minor* were substantially smaller (TU 53.3 vs. 45.4), suggesting organism-specific sensitivity to contamination-associated changes in leachate composition.

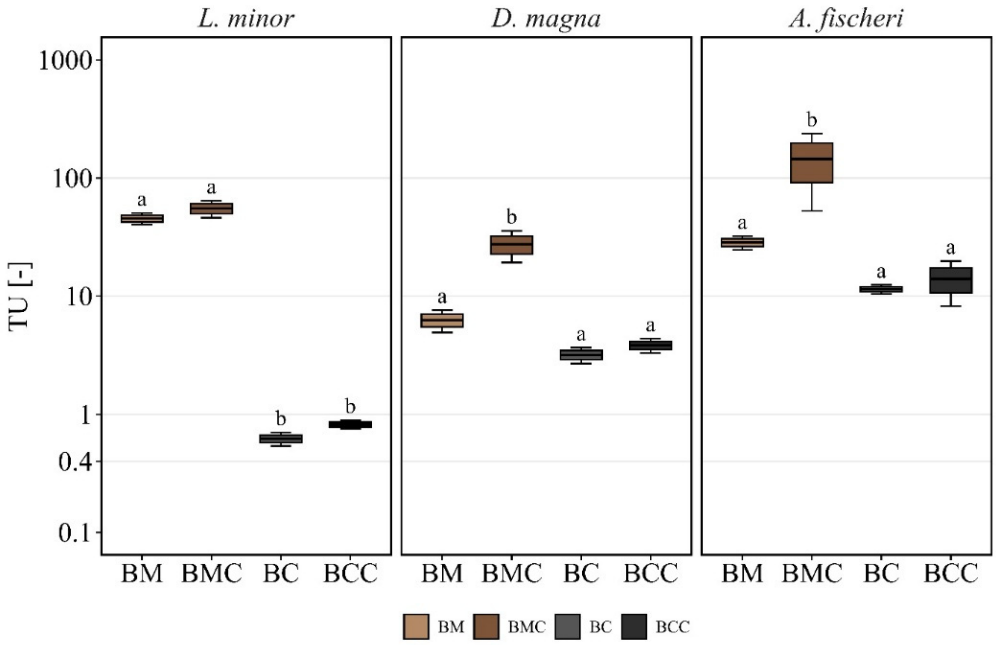

Figure 1. TU values of biomass samples and biochar leachates toward *L. minor*, *D. magna* and *A. fischeri*. Different lowercase letters indicate statistically significant differences between sample variants within each bioassay. Bars sharing at least one letter are not significantly different. Differences were assessed using one-way ANOVA followed by the LSD test at $\alpha = 0.05$.

Following pyrolysis, the toxicity of the corresponding biochar leachates toward *L. minor* decreased substantially, with both BC and BCC leachates showing only minor inhibitory effects ($TU \leq 1$), corresponding to low-to-no toxicity according to the applied *TU* classification system (Table 1). In the *D. magna* test, biomass leachates caused moderate to high toxicity (*TU* 6.3 for BM leachates and 27.5 for BMC leachates), whereas biochar leachates yielded much lower effects ($TU \approx 3$ for both BC and BCC derived leachates). Toxicity differences between uncontaminated and contaminated materials became substantially reduced. For example, *TU* values for *A. fischeri* differed by only approximately 22% between BC and BCC derived leachates (11.5 vs. 14.0), whereas the corresponding difference between BM and BMC derived leachates exceeded 400%. Similar convergence was observed for *D. magna* and *L. minor*, indicating that pyrolysis reduced not only the overall toxicity of leachates but also the influence of initial contamination status on ecotoxicological responses.

Among all tested bioindicators, *A. fischeri* was the most sensitive. The BMC leachate reached extreme toxicity (*TU* 145 $\pm$ 93), while BM-derived leachates were fivefold less toxic (*TU* 28 $\pm$ 4). Pyrolysis led to significant toxicity reduction in biochar leachates, though both biochar-derived leachate samples retained high *TU* values (BCC derived leachates 14 $\pm$ 6; BC leachates 11 $\pm$ 1). According to the classification by Persoone et al., 2003 [39] (Table 1), BMC leachate was extremely toxic, while all other materials were highly toxic (Table 4).

To better elucidate the drivers of observed leachate toxicity, correlation analysis between toxic unit (TU) values and selected physicochemical parameters was performed (Table 5).

Table 4. Toxicity classification of biomass- and biochar-derived leachates.

Organism	*TU* [-]			
	BM	**BMC**	**BC**	**BCC**
L. minor	45.40 ± 5.17	53.29 ± 9.10	0.62 ± 0.08	0.82 ± 0.07
D. magna	6.28 ± 1.35	27.53 ± 8.23	3.18 ± 0.49	3.84 ± 0.53
A. fischeri	28.49 ± 3.74	145.28 ± 92.64	11.48 ± 1.01	14.03 ± 5.81
Toxicity classification	High toxicity	Extreme toxicity	High toxicity	High toxicity

Table 5. Pearson correlation coefficients between toxic units (*TUs*) and selected physicochemical parameters in leachates.

Parameter	*TU*		
	D. magna	***L. minor***	***V. fischeri***
Pb	0.991	0.82	0.992
Zn	0.999	0.73	0.999
Phenols	0.545	0.965	0.553
TOC	0.716	0.999	0.723
pH	−0.625	−0.985	−0.634

Strong correlations were observed between toxic units (TUs) and selected parameters, with differences depending on the test organism.

Overall, the results show that heavy-metal contamination amplified the toxicity of biomass leachate in all aquatic assays, whereas pyrolysis largely mitigated these effects.

3.3. Effects on Terrestrial Plants

Unlike aquatic assays based on leachate exposure, terrestrial tests evaluated direct biological responses to biomass and biochar amendments incorporated into soil.

Phytotoxicity tests revealed distinct, species-specific responses (Figures 2–4).

Lepidium sativum (garden cress) exhibited the highest sensitivity to both BM and BMC, with significant stem growth inhibition (>40%) at all tested doses (0.01–1%). Plant dry biomass production of *L. sativum* was additionally reduced by up to 60% in treatments amended with 1% (*w*/*w*) biomaterial.

Cucumis sativus (cucumber) was the most tolerant species: no significant differences in stem height or dry mass were found relative to controls. For *Triticum aestivum* (wheat), biomass treatments did not affect stem elongation but caused a >30% reduction in dry biomass for BMC at 0.1% and 1%.

In contrast, neither of the biochars (BC and BCC) caused significant phytotoxic effects in any plant species. Stem and biomass values remained within control ranges, except for a minor, non-significant inhibition (~20%) of wheat biomass in the BC 0.1% series. The transformation of biomass to biochar eliminated the phytotoxic effects observed for fresh feedstocks. This change coincided with a substantial reduction in dissolved organic matter (TOC) and phenolic compounds in the corresponding leachates, suggesting a possible association between reduced organic content and lower phytotoxic responses. However, the specific compounds responsible for the observed effects were not directly identified in the present study. Therefore, the proposed mechanism should be considered indirect and based on observed correlations and literature evidence rather than direct chemical verification.

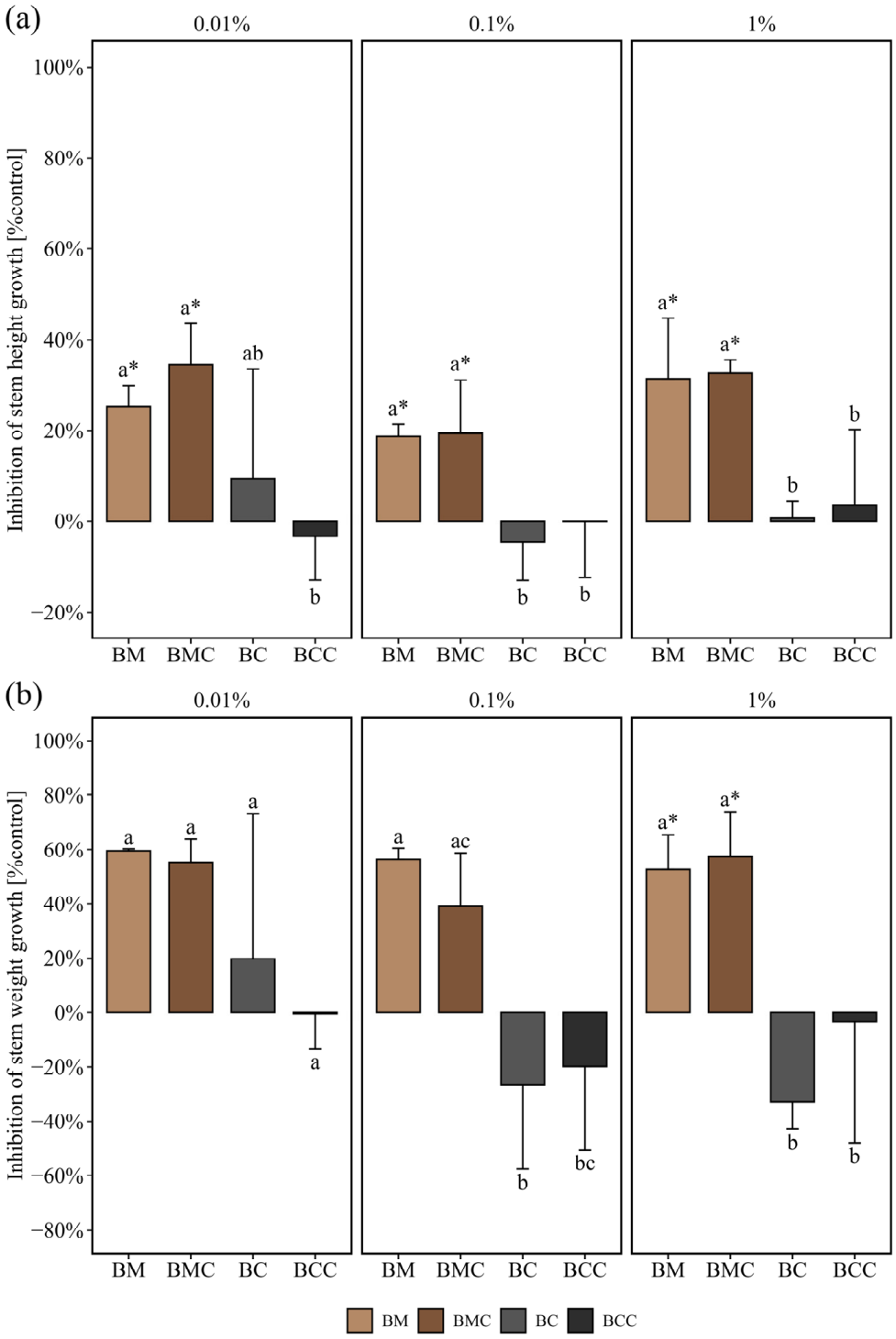

Figure 2. Toxicity of biomass samples and biochars to *L. sativum*: (**a**) stem height growth inhibition and (**b**) biomass growth inhibition. Different lowercase letters indicate statistically significant differences between sample variants within each concentration and endpoint. Bars sharing at least one letter are not significantly different. Asterisks indicate statistically significant differences relative to the control. For stem growth inhibition, differences were assessed using the Kruskal–Wallis test followed by multiple comparisons of mean ranks; for biomass growth inhibition, one-way ANOVA followed by the LSD test was used. Differences were considered significant at $\alpha = 0.05$.

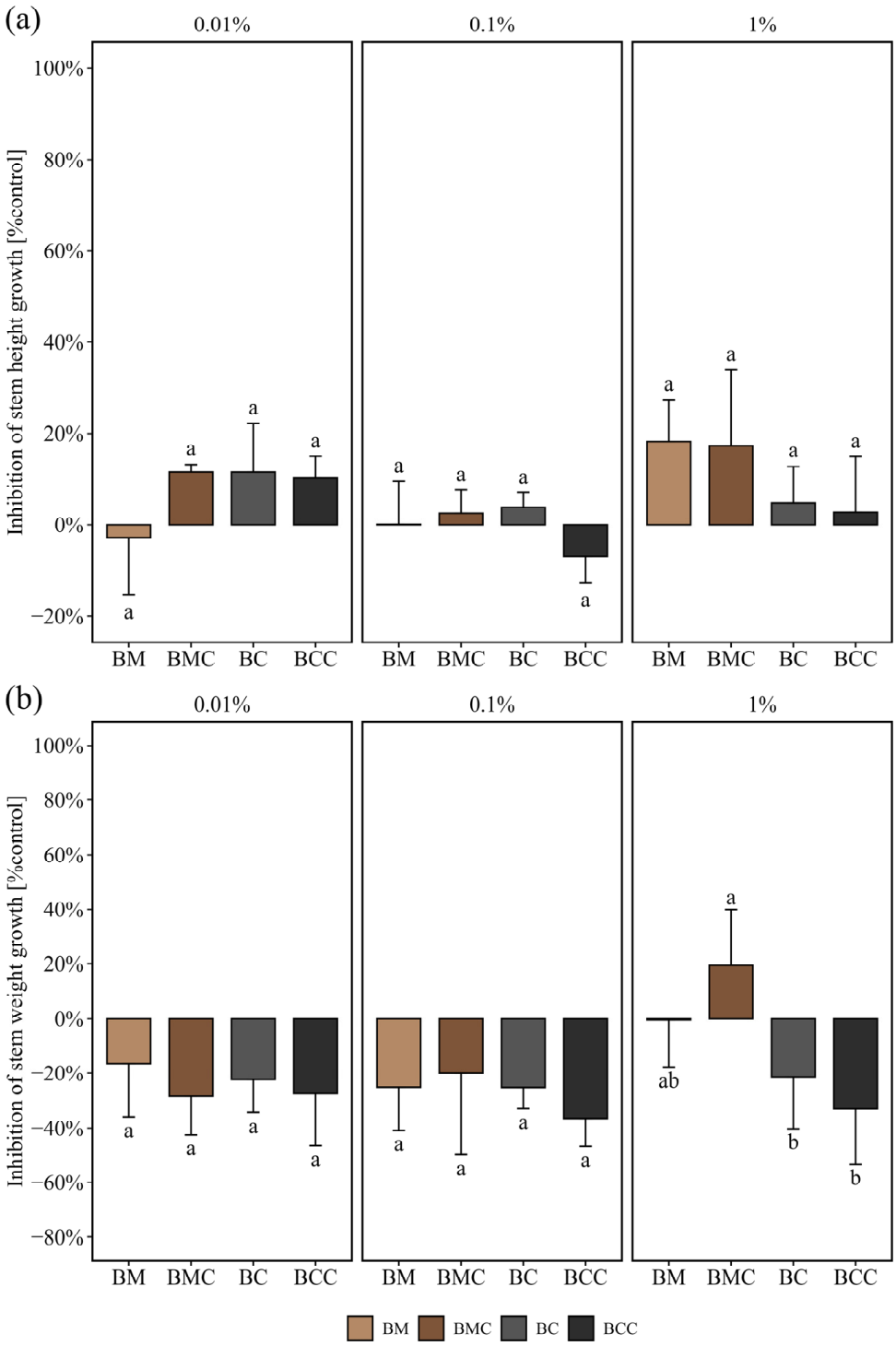

Figure 3. Toxicity of biomass samples and biochars to *C. sativus*: (**a**) stem height growth inhibition and (**b**) biomass growth inhibition. Different lowercase letters indicate statistically significant differences between sample variants within each concentration and endpoint. Bars sharing at least one letter are not significantly different. For stem growth inhibition, differences were assessed using the Kruskal–Wallis test followed by multiple comparisons of mean ranks; for biomass growth inhibition, one-way ANOVA followed by the LSD test was used. Differences were considered significant at $\alpha = 0.05$.

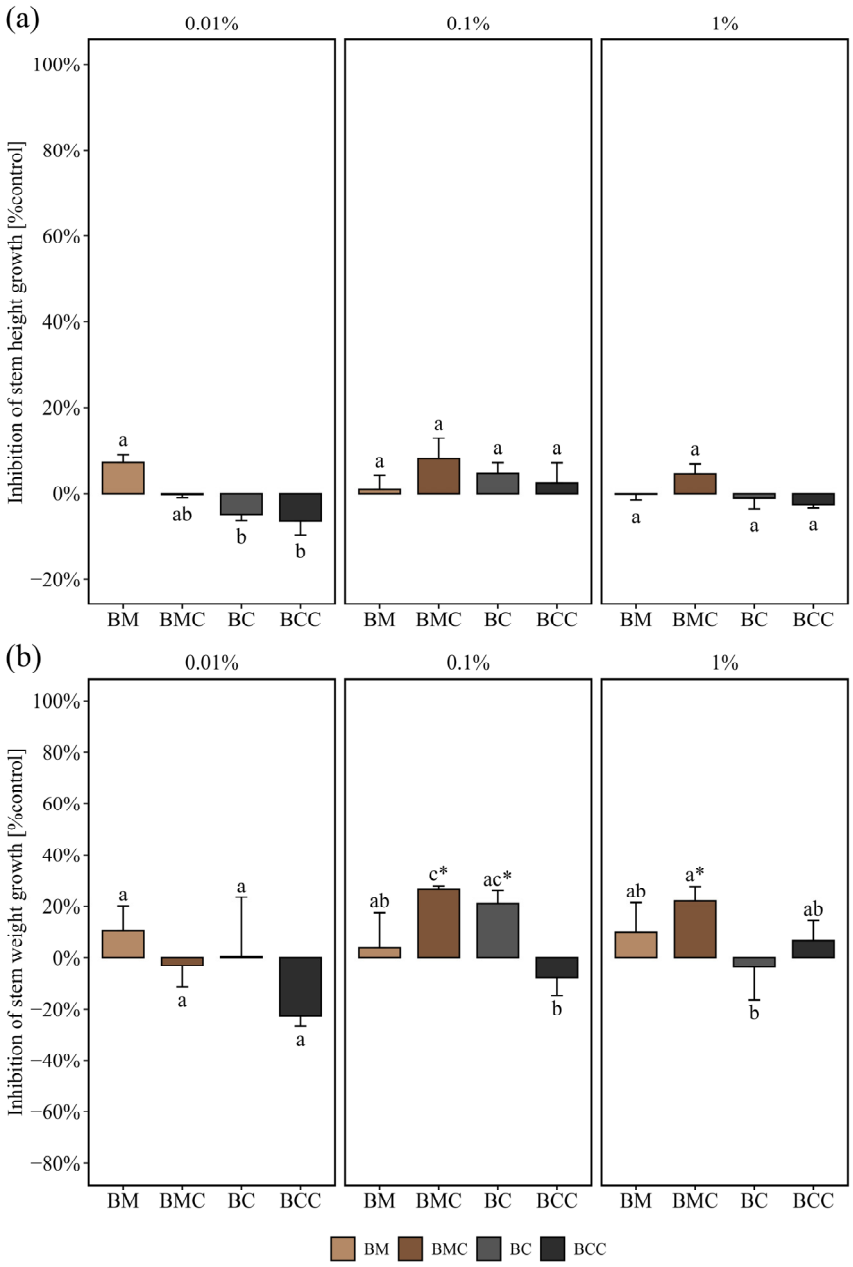

Figure 4. Toxicity of biomass samples and biochars to *T. aestivum*: (**a**) stem height growth inhibition and (**b**) biomass growth inhibition. Different lowercase letters indicate statistically significant differences between sample variants within each concentration and endpoint. Bars sharing at least one letter are not significantly different. Asterisks indicate statistically significant differences relative to the control. For stem growth inhibition, differences were assessed using the Kruskal–Wallis test followed by multiple comparisons of mean ranks; for biomass growth inhibition, one-way ANOVA followed by the LSD test was used. Differences were considered significant at $\alpha = 0.05$.

3.4. Earthworm Toxicity and Avoidance Behavior (Direct Exposure)

No acute mortality or significant behavioral changes were recorded for *Eisenia fetida* exposed to soils amended with biomass or biochar (Figures 5 and 6). In contrast to aquatic assays, where responses were expressed as inhibition relative to controls, earthworm responses are presented as direct biological endpoints; therefore, control values are included for reference and assessment of test validity. Mean mortality remained below 7% after 7 and 14 days across all treatments, and avoided soils were within the range of random distribution (−23.7% for BMC to 53% for BCC). None of the differences were statistically significant relative to the control ($p > 0.05$).

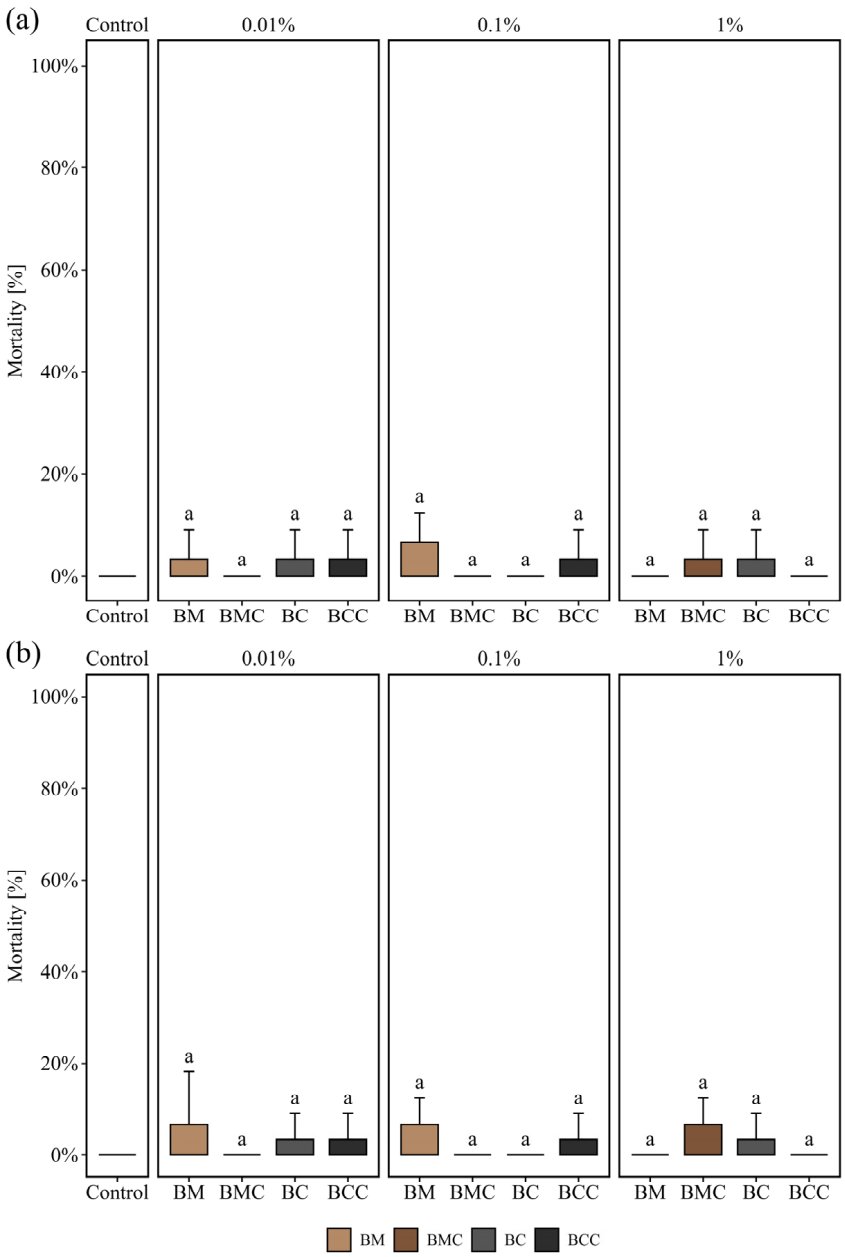

Figure 5. Effect of biomass and biochar amendments on *E. fetida* mortality: (**a**) after 7 days and (**b**) after 14 days. Different lowercase letters indicate statistically significant differences between sample variants within each exposure time. Bars sharing at least one letter are not significantly different. Differences were assessed using one-way ANOVA followed by the LSD test at $\alpha = 0.05$. The control treatment is presented because mortality values represent direct biological responses and additionally serve as a test validity criterion for the earthworm assay.

These results classify all materials as non-toxic for earthworms under OECD 207 criteria [40]. Maintaining the soil at 85% of its water-holding capacity effectively prevented artefactual effects related to biochar porosity and moisture stress, which have been previously shown to cause false-positive avoidance responses [43]. At the tested application rates ($\leq$1%), both biomass and biochar displayed full ecotoxicological safety for soil macrofauna.

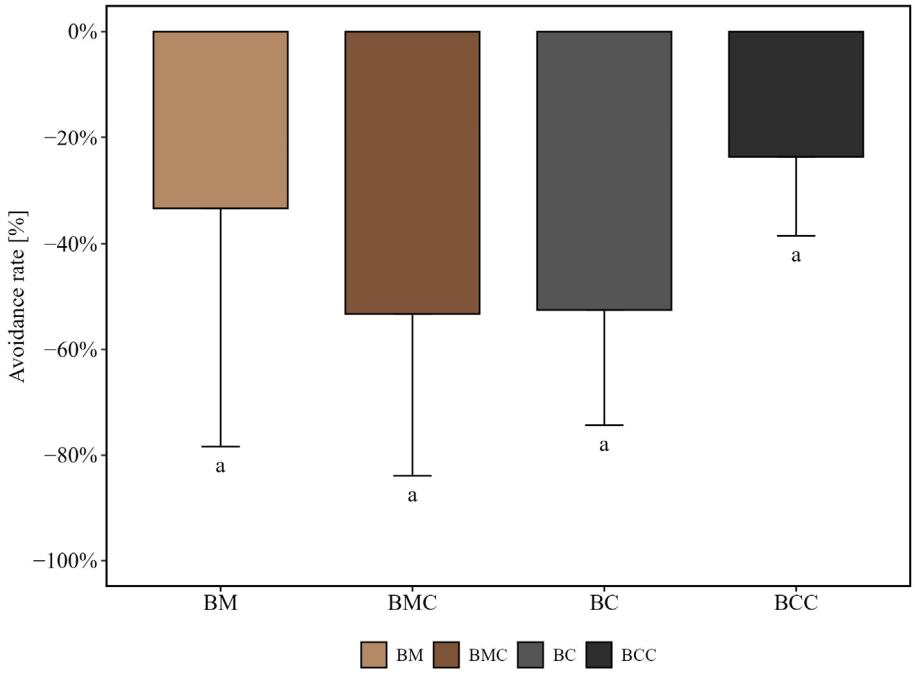

Figure 6. Avoidance response of *E. fetida* exposed to soils amended with biomass and biochar samples. Different lowercase letters indicate statistically significant differences between sample variants. Bars sharing at least one letter are not significantly different. Differences were assessed using one-way ANOVA followed by the LSD test at $\alpha = 0.05$.

3.5. Soil Microbial Community Profiles

Functional diversity analysis demonstrated measurable but moderate changes in microbial activity (Figure 7). Across treatments, response patterns followed the typical sigmoidal AWCD curve, with maximum metabolic activity (A_{max}) reached within 96–120 h.

In general, soils amended with BM showed the highest A_{max} values and shorter times to half-maximum activity (t_{50}), indicating moderate stimulation of microbial metabolism due to the addition of readily decomposable organic matter (Figure 7). In contrast, soils amended with the contaminated biomass (BMC) responded variably depending on the plant species present. In wheat soil, A_{max} was slightly lower and t_{50} was extended, while in cress soil the maximum activity was achieved faster, suggesting a balance between metal-induced stress and organic carbon availability.

Biochars (BC and BCC) had only minor impacts on A_{max} and diversity, reflecting their low content of readily available carbon. Values of the Shannon index (H′), Gini–Simpson (GS), and McIntosh (U) remained close to controls in all treatments, indicating no disturbance to overall community structure or function. Slight increases in metabolic richness (RS) and evenness indexes (J′, EGS, EMc) were observed for BM and BMC amendments, possibly due to short-term substrate enrichment. None of the biochar treatments resulted with significant decreases in microbial diversity ($p > 0.05$).

Distinct plant-specific patterns were noted: soils planted with cress and cucumber showed higher A_{max} values than those with wheat, confirming that root exudates strongly shape microbial metabolic profiles. Overall, the functional diversity data indicate that microbial communities remained relatively resilient to low-rate amendments of both biomass and biochar, with only limited differences observed among treatments. Detailed numerical values of Amax, t_{50}, and functional diversity indexes (RS, H′, GS, U, J′, EGS, EMc, G) are provided in Supplementary Information (Table S2).

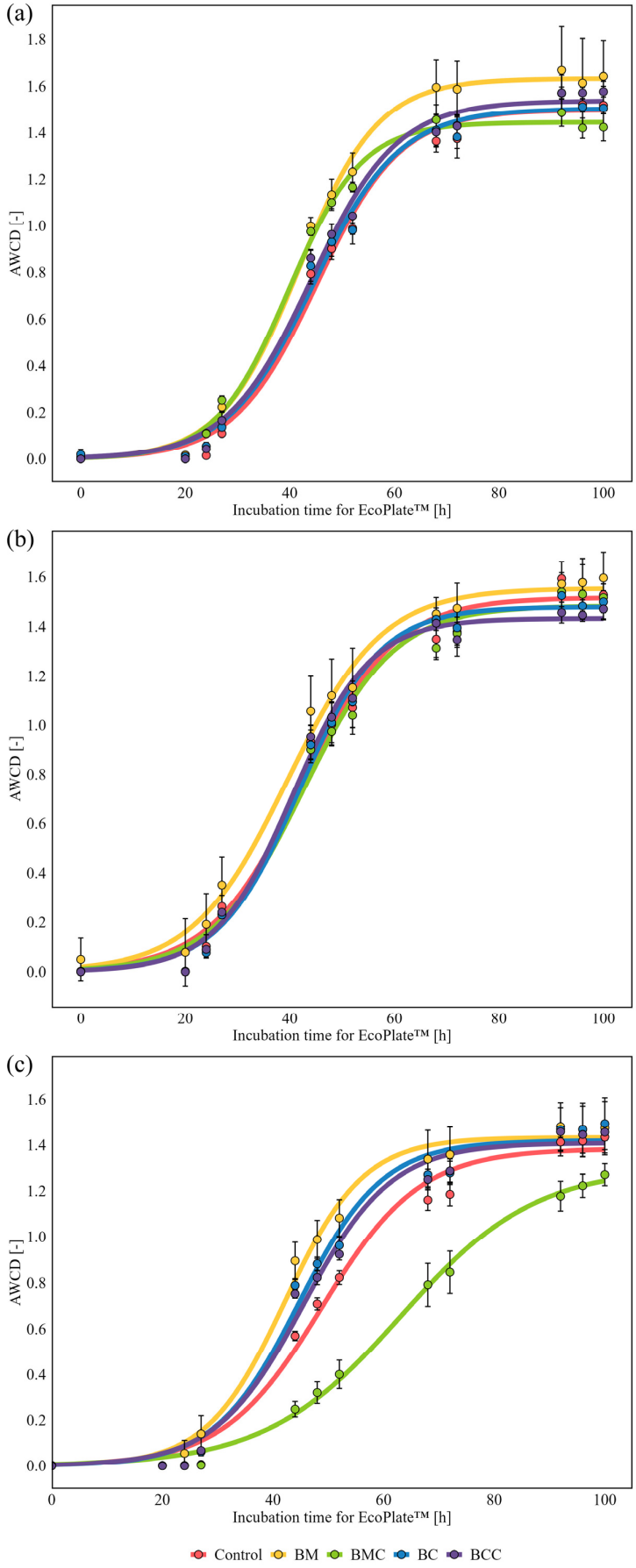

Figure 7. Changes in the overall metabolic activity (as AWCD values) in soil planted with: (**a**) *L. sativum*, (**b**) *C. sativus*, (**c**) *T. aestivum* under different treatments.

4. Discussion

4.1. Transformation of Chemical Composition During Pyrolysis

Pyrolysis substantially modified the physicochemical characteristics of the studied biomass samples, confirming its potential to transform phytoremediation residues into more stable materials. As expected, the total concentrations of Zn and Pb increased in biochars compared with the corresponding feedstocks, reflecting the loss of volatile organics during carbonization and the transfer of metals into the non-volatile solid fraction [53]. Similar enrichments have been widely reported for biomass treated at temperatures above 600 °C [24,25]. The higher retention observed for BMC-derived biochar compared with BM (83% Zn, 100% Pb versus 50% Zn, 46% Pb) may be related to the stronger binding of metals in contaminated biomass, including metal–organic complexes and plant detoxification compartments, which can become trapped in the aromatic or mineral matrix during pyrolysis [54,55].

A pronounced shift from acidic to alkaline reaction (pH 4.6 to 10) was observed after pyrolysis which is commonly attributed to the decomposition of acidic carboxyl and phenolic groups and the concentration of alkaline ash components [56–58]. At the same time, the substantial reduction in TOC and phenolic compounds indicates extensive degradation of labile organic matter. Nevertheless, trace amounts of thermally generated organic compounds, such as polycyclic aromatic hydrocarbons (PAHs) and volatile organic compounds (VOCs), may still be present [26–28].

4.2. Aquatic Toxicity: Balance Between Metal Immobilization and New Organics Formation

The aquatic tests revealed high contrasts between feedstocks and their biochar-derived leachates. Both biomass leachates caused strong inhibitory effects across all aquatic bioindicators, while pyrolysis markedly reduced or eliminated the toxicity of biochar leachates.

D. magna and *A. fischeri* showed a similar trend with BMC leachate classified as more toxic than BM. This was most likely related to the higher concentrations of Zn and Pb, which were the main parameters differentiating both biomass leachates. Both organisms are sensitive to zinc and lead, and synergistic effects of these metals have been reported for *A. fischeri* [9,59]. Reported EC50 values fall within the concentration ranges detected in the leachates, supporting the interpretation that metal mobility was a key driver of toxicity [59,60].

Organic constituents probably acted as additional stressors in biomass leachates. High levels of dissolved organic carbon and phenolic compounds, which are known to inhibit aquatic organisms even at relatively low concentrations [61,62], may affect *D. magna*, *L. minor*, and *A. fischeri* by disrupting metabolic and physiological processes [63–65]. Thus, the extreme toxicity of BMC leachates likely reflected the combined presence of metals and organic toxicants.

Correlation analysis between *TU* values and selected physicochemical parameters (Table 5) further supported this interpretation. *TU* values for *D. magna* and *A. fischeri* were very strongly correlated with Zn and Pb concentrations ($r \approx 0.99$), whereas *L. minor* showed stronger associations with TOC ($r \approx 0.999$) and phenolic compounds ($r \approx 0.965$). The negative correlation between *TU* and pH for all organisms ($r \approx -0.63$ to -0.99) also suggests that increasing alkalinity contributed to the reduction in toxicity in biochar leachates. These results confirm a shift in toxicity drivers from mobile metals and soluble organics in biomass leachates to less acute physicochemical effects in biochar leachates [61–65].

After pyrolysis, leachable metal concentrations decreased by more than an order of magnitude—Pb dropped from 6.9 mg L^{-1} to 0.6 mg L^{-1}, Zn to below the detection limit. This explains the reduced toxicity of BCC leachates compared with BMC leachates and is consistent with previous reports showing immobilization of metals in high-temperature

biochars [24]. The substantial reduction in metal concentrations observed in biochar leachates indicates that pyrolysis effectively promoted heavy metal immobilization. During thermal treatment, metals become incorporated into stable mineral and carbonaceous structures formed during carbonization, which substantially reduces their mobility [24,25,53,54]. Increased alkalinity of biochars additionally favors precipitation of less soluble metal species [56–58], while the porous aromatic structure of biochar enhances metal sorption through surface interactions [17,18]. Consequently, despite increased total metal concentrations caused by mass reduction during pyrolysis [53], the environmentally available and leachable fractions were markedly reduced, explaining the lower ecotoxicological responses observed in the present study.

The residual toxicity of biochar leachates was therefore unlikely to result from direct heavy-metal effects alone. Strong alkalinity (pH ≈ 9.7–10.0) may have induced physiological stress, especially in *A. fischeri*, by disturbing membrane permeability and ion gradients [66–68]. In addition, thermal conversion of lignocellulosic biomass may generate water-extractable VOCs, oxygenated aromatic compounds, and PAHs [26–28,69–72]. Although phenolic compounds decreased below the detection limit, measurable TOC persisted in biochar leachates, indicating residual dissolved organic constituents that may affect organisms through membrane disruption, oxidative stress, or metabolic interference [61–65,69–72]. Similar residual toxicity has been reported for biochars with low metal content, whereas additional thermal treatment or aging may reduce toxicity by limiting volatile constituent release [69–72].

A comparison of bioassay responses revealed clear differences in organism sensitivity. Among aquatic organisms, *A. fischeri* was the most sensitive, particularly in contaminated biomass leachates, which is consistent with the broad susceptibility of luminescent bacteria to metals and organic compounds [73,74]. *L. minor* also showed strong responses, often exceeding those of *D. magna*, especially in biomass-derived leachates rich in phenolic compounds.

Based on the overall dataset, a general sensitivity pattern can be proposed: *A. fischeri* > *L. minor* > *D. magna* > terrestrial plants > *E. fetida*. This gradient reflects differences in exposure pathways and physiological susceptibility, confirming the importance of a multitrophic testing strategy.

4.3. Terrestrial Phytotoxicity and Soil Fauna

In terrestrial systems, the toxicity of raw biomass was primarily associated with soluble organic compounds, particularly phenolics derived from plant decomposition [75,76]. This interpretation remains indirect because individual organic compounds were not identified analytically; it is based on measured phenol concentrations, TOC values, observed toxicity patterns, and published evidence on the phytotoxicity of dissolved organic substances [61–65,75,76].

Among the tested species, *Lepidium sativum* showed the highest sensitivity, consistent with its known susceptibility to allelopathic compounds [77]. Moderate effects observed in *Triticum aestivum* may result from combined exposure to organic compounds and trace metals, whereas *Cucumis sativus* exhibited greater tolerance [78,79].

Pyrolysis eliminated measurable phytotoxic effects, and none of the biochar treatments significantly inhibited plant growth. This pattern agrees with findings by Stefaniuk et al. [80] and Joseph et al. [81], who described stimulatory or neutral impacts at low biochar doses depending on feedstock and production temperature. The absence of adverse effects can also be linked to controlled moisture conditions, which prevented artifacts related to the water retention properties of biochar [43]. Minor inhibition observed in isolated cases may reflect nutrient imbalance rather than toxic stress. Plant responses

observed in the present study should be interpreted as ecotoxicological indicators rather than predictors of agricultural suitability.

Earthworm assays further confirmed the low ecotoxicological risk of both biomass and biochar under the tested conditions. Mortality never exceeded 7%, and avoidance behavior was not significant. This is consistent with previous reports showing that correctly moistened and sieved biochar causes no measurable stress to *Eisenia fetida* [43,82]. Notably, biochar particle size influences worm behavior—smaller fractions may be ingested more readily and even act as carbon supplements [82]. In our 4 mm-sieved material, direct physical exposure appeared negligible.

Aging of biochar can, however, alter its ecotoxicological profile. Shi et al. [83] showed that both abiotic and biotic weathering may increase biochar toxicity through renewed release of water-soluble organics. Therefore, long-term assessments remain necessary.

4.4. Soil Microbial Community Responses

The microbial community analysis revealed that the soil biological compartment was generally resilient to both biomass and biochar amendments. Soils enriched with the uncontaminated biomass displayed slightly enhanced microbial activity (higher A_{max}, lower t_{50}) and increased functional diversity indices (H', GS, U). This reflects stimulation by labile organic carbon inputs, as previously observed in soils amended with organic wastes or lignocellulosic residues [84,85].

For BMC, the effect was variable: stimulation in some plant systems and mild inhibition in others. The dual effect is consistent with the recognized interaction between carbon addition and heavy-metal pressure: at lower metal levels, organic matter and root exudates may stimulate microbial activity, whereas at higher loads toxicity may prevail [86,87].

Biochar amendments did not significantly alter community-level physiological profiles and functional diversity indices remained comparable to control soil, confirming that low application rates (1% w/w) are safe for microbial consortia. This agrees with large meta-analyses highlighting that biochar often increases microbial biomass but has variable effects on diversity [88]. Minor shifts observed here may be related to physical structuring including microhabitats provided by biochar pores, rather than direct chemical pressure [17,18].

Differences among host plants emphasize the strong influence of the rhizosphere in shaping functional microbial profiles [89,90]. Plants-specific exudates, including amino acids, sugars, and phenolics, modify microbial carbon-substrate preferences [91], and may therefore have overridden the moderate effects of soil amendments.

Overall, the CLPP analysis indicates that soil microorganisms adapted rapidly to the low-intensity perturbations introduced by biomass or biochar, and that the activity increases observed for BM were likely transient and substrate-driven.

4.5. Integrated Interpretation and Environmental Implications

The combined results of aquatic and terrestrial bioassays suggest that pyrolysis acts primarily as an ecotoxicological risk transformation rather than simple elimination. It markedly reduces the mobility and toxicity of heavy metals and soluble organic compounds in phytoremediation-derived biomass but may generate or expose new stressors including alkaline ash components or trace aromatic condensates, responsible for residual aquatic toxicity.

Despite these side-effects, biochar produced under controlled conditions and applied at realistic soil doses appears environmentally safe for soil organisms and plants. The observed divergence between aquatic and terrestrial responses underscores the importance of a multitrophic assessment approach. Standardized leachate assays reflect short-term

release and immediate contaminant bioavailability under controlled extraction conditions, whereas real soil systems may differ due to slower release kinetics, sorption, precipitation, aging, and soil buffering capacity [18,24,25]. Conversely, long-term weathering may alter contaminant speciation and increase the release of previously immobilized fractions [83]. Therefore, leachate-based assays should be interpreted as complementary early-warning tools rather than direct predictors of long-term soil ecotoxicity.

Overall, pyrolysis represents a promising strategy for integrating phytoremediation with circular economy principles by enabling the safe valorization of contaminated biomass [10,15]. However, a final environmental qualification should include solid-phase and leachate-based ecotoxicity assays and, where relevant, chemical fingerprinting of PAHs and VOCs to ensure safe reuse.

The extent of risk mitigation achieved through pyrolysis was further quantified using the risk reduction factor (*RRF*) and leachate hazard index-based leaching reduction factor (*LRF*) (Table 6). A substantial decrease in ecotoxicological risk was observed for both materials. For contaminated biomass, *RRF* reached 10.35, indicating an order-of-magnitude reduction in toxicity after pyrolysis (from *TU* = 145.28 to 14.03). A lower but still evident effect was observed for uncontaminated biomass, with *RRF* = 3.95. These results confirm that pyrolysis is particularly effective in mitigating high initial risk levels associated with contaminated feedstocks.

Table 6. Risk reduction and leaching reduction factors.

Material	Max *TU*	*RRF*	*LHI*	*LRF*
BM	45.40	-	0.57	-
BMC	145.28	-	0.95	-
BC	11.48	3.95	0.009	63.3
BCC	14.03	10.35	0.027	35.2

Even stronger changes were observed for the leachate hazard index (*LHI*), which decreased from 0.95 to 0.027 for contaminated biomass and from 0.57 to 0.009 for uncontaminated biomass. This corresponded to high leaching reduction factors (*LRF* = 35.2 and 63.3, respectively), indicating a substantial reduction in hazardous-component mobility. The higher *LRF* observed for BC suggests that pyrolysis effectively stabilizes dissolved constituents across biomass types, even when initial contamination is relatively low.

Together, the *RRF* and *LRF* results show that pyrolysis reduces both ecotoxicity and contaminant release into the aqueous phase. However, the persistence of moderate *TU* values in biochar leachates indicates that risk is transformed rather than completely eliminated.

Figure 8 provides a conceptual synthesis of the obtained results, highlighting that pyrolysis does not simply reduce toxicity but fundamentally transforms the nature of environmental risk—from metal- and phenol-driven toxicity in raw biomass to pH- and trace organic-driven effects in biochar.

Future research should focus on long-term aging and metal speciation in biochars derived from phytoremediation, identification of organics responsible for the residual aquatic toxicity, and life-cycle assessments quantifying environmental and economic trade-offs between biochar storage, land application, and energy recovery routes.

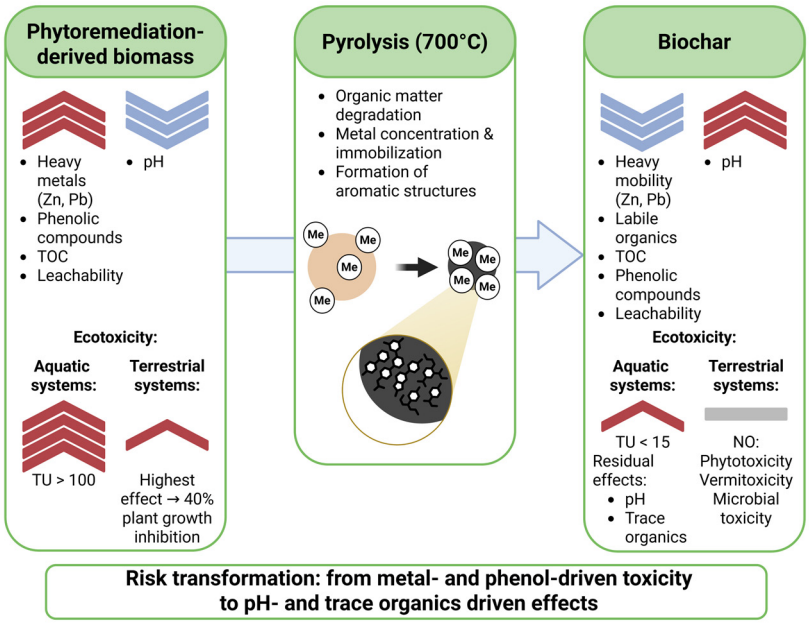

Figure 8. Conceptual model of ecotoxicological risk transformation during pyrolysis of phytoremediation-derived biomass.

5. Conclusions

This study demonstrates that biomass generated during phytoremediation constitutes a highly reactive and potentially hazardous secondary waste stream, particularly due to the mobility of contaminants and the toxicity of its leachable fraction. The applied multi-trophic bioassay battery revealed that untreated biomass exhibited pronounced toxicity in aquatic systems, reaching extreme levels ($TU > 100$), and measurable phytotoxic effects in terrestrial environments.

Pyrolysis at 700 °C substantially altered the environmental risk profile of the material. The process led to a significant reduction in the mobility of both inorganic and organic contaminants, reflected in a decrease in toxic unit values by one to two orders of magnitude in aquatic assays and the complete elimination of phytotoxic effects in all tested plant species. At the same time, no adverse effects were observed for soil macrofauna or microbial functional diversity at environmentally relevant application rates ($\leq 1\%$ w/w), indicating a high level of ecological compatibility in terrestrial systems.

However, the results also indicate that risk reduction following pyrolysis should not be interpreted as complete risk elimination. Residual toxicity persisted in selected aquatic bioassays and was more strongly associated with physicochemical properties of biochar-derived leachates, particularly elevated pH and the potential presence of trace thermally generated organic compounds, rather than with direct heavy metal effects. These findings demonstrate that environmental safety assessment should consider not only contaminant concentrations but also changes in contaminant mobility and material properties resulting from thermal treatment.

From a practical perspective, the environmental applicability of phytoremediation-derived biochars should be evaluated under specific environmental boundary conditions. Factors such as feedstock composition, contamination level, pyrolysis parameters, application rate, soil characteristics, pH conditions, and long-term weathering processes may substantially influence the environmental behavior of the resulting material. Consequently, biochars originating from contaminated biomass should not be considered universally safe for environmental application without prior site-specific evaluation.

The proposed integrated assessment framework combining leaching analysis with multitrophic ecotoxicological testing provides a practical tool for supporting decision-making within circular economy strategies involving waste-derived materials. The combined use of direct exposure and leachate-based approaches may improve the reliability of environmental safety evaluation and reduce the risk of underestimating adverse effects across different environmental compartments.

Future research should focus on long-term aging processes of biochar under field conditions, metal speciation changes during environmental exposure, detailed identification of organic compounds responsible for residual aquatic toxicity, and life cycle assessment of alternative biomass management pathways. Such efforts are essential to ensure that the environmental benefits of phytoremediation are not offset by unintended impacts associated with the handling of its by-products.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app16126104/s1, S1: *Aliivibrio fischeri* luminescent bacteria inhibition test—IC_{50} calculation; S2: Soil microbial functional diversity—detailed methodology and calculations Table S1. Summary of carbon substrates present on the BIOLOG®EcoPlate™ Table S2. Functional diversity indices (mean ± SD) of soil microbial communities under different plant and amendment treatments. S3 Statistical analysis and pairwise comparisons for ecotoxicological endpoints, including Tables S3–S11, which present ANOVA and Kruskal–Wallis test results, effect sizes, and pairwise *p*-value matrices for toxicity units, plant growth inhibition, earthworm mortality and avoidance, and soil microbial functional diversity indices.

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Abbreviations

The following abbreviations are used in this manuscript:

A_{max}	Maximum metabolic activity
AWCD	Average well color development
BC	Biochar
BCC	Contaminated biochar
BM	Biomass
BMC	Contaminated biomass
GS	Gini–Simpson index
H′	Shannon index
HMs	Heavy metals
IC/EC_{50}	Inhibition/effect concentration
PAHs	Polycyclic aromatic hydrocarbons
RS	Metabolic richness

SDG	Sustainable development goal
t_{50}	Time to half maximum activity
TOC	Total organic carbon
TU	Toxic units
U	McIntosh index
VOCs	Volatile organic compounds
WHC	Water-holding capacity

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