Journal of Hazardous Materials 504 (2026) 141339

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Effects of a mixture of mulching film microplastics on soil properties, microbial activities, and plants in terrestrial mesocosms with and without earthworms

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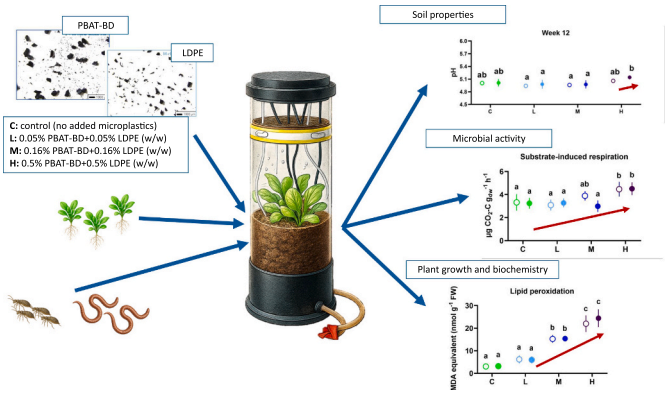
HIGHLIGHTS

- Microplastic (MP) mixture derived from agricultural mulch films was tested.
- Realistic MP concentrations caused adverse effects in a soil mesocosm study.
- MPs increased soil compaction, but earthworms mitigated their impact on soil structure.
- C mineralization increased, while N mineralization decreased due to MP exposure.
- MPs triggered biochemical responses in plants.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Agricultural plastics
Conventional and biodegradable plastics
Soil respiration
Plant growth
Oxidative stress

ABSTRACT

The increasing use of plastics in agriculture, e.g., mulching films, may lead to an accumulation of microplastics (MPs) in soil. This study assessed the effects of a 1:1 mixture of MPs produced from two commonly applied types of mulching film, conventional low-density polyethylene (LDPE) based and biodegradable starch polybutylene adipate terephthalate (PBAT-BD) blend-based plastics, under realistic conditions in terrestrial mesocosms. To assess the potential role of earthworms on the effects of microplastics, the study included treatments with and

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https://doi.org/10.1016/j.jhazmat.2026.141339
Received 14 October 2025; Received in revised form 30 January 2026; Accepted 31 January 2026
Available online 2 February 2026

without earthworms. After 12 weeks of exposure, effects were determined on different endpoints: soil pH, soil compaction, carbon and nitrogen mineralization, and plant growth and biochemistry. MPs increased soil compaction, especially in the absence of earthworms, suggesting that earthworm activity can mitigate their physical impact on soil structure. MPs stimulated soil respiration at high concentrations, while simultaneously reducing nitrogen mineralization. This suggests these MPs acted both as a stimulant and a stressor for soil microbial communities. Although plant growth and chlorophyll content were unaffected by MP exposure, significant biochemical responses in lettuce, such as oxidative stress and activation of defence mechanisms, indicate that MPs can impact plant health at the molecular level, even in the absence of visible symptoms. Nitrogen mineralization was significantly increased in the mesocosms with earthworms, probably due to bioturbation and consequent increased aeration of the soil. However, the presence of earthworms did not have significant effects on other microbial and plant-related endpoints, suggesting that the earthworms affected the toxicity of the MPs only to a limited extent.

1. Introduction

Microplastics (MPs) are now found everywhere in the environment, raising concerns about their potential negative impacts on ecosystem and human health [18,61,62]. Soil has been considered one of the largest global environmental sinks for MPs [8,31]. The total concentrations of MPs in soils vary widely, ranging from hundreds to tens of thousands of particles per kilogram of soil [73]. A crude conversion suggests that mass-based concentrations of microplastics in soil can range from < 0.0001–0.1 % w/w (weight/weight), with higher values possible, for example, in cases of mismanaged mulching films [46]. An extreme case of 6.7 % w/w (67,500 mg per kg soil) has been reported for a heavily contaminated industrial site [12].

The main pathways of soil pollution by MPs commonly comprise atmospheric deposition, wastewater application, and the use of biosolids. However, the use of plastics for crop production has grown rapidly in recent decades, and the soil pollution originating from agricultural plastics (e.g., mulching films, ground cover fabrics, solarization and fumigation films, nets, and irrigation pipes) has garnered increased attention [11,21]. Plastic mulching films are commonly used to reduce water loss, increase soil temperature for seed germination, and control weeds, boosting agricultural productivity [42]. They are usually made of low-density polyethylene (LDPE) and, in the case of biodegradable mulch films, of partially or fully biodegradable polymers (e.g., polybutylene adipate terephthalate (PBAT)/polylactic acid, or PBAT/modified starch blends [11,53]. However, thin films fragment easily due to environmental factors like UV radiation, microbial activity, and mechanical damage [3,41,57]. Repeated use of these films has led to significant accumulation of plastic debris, particularly in the form of microplastics, in soils, especially in the case of very thin films (e.g., 8 μm thick) [27,49,52]. For instance, Li et al. [49] discovered that after 32 years, plastic mulching films represented 33 %–56 % of the total load of microplastics in agricultural soil, with an average of 8885 particles per kilogram in the 0–10 cm topsoil layer. Although the impact of MPs originating from mulching films on soil ecosystems is not fully understood, their presence and accumulation are critical concerns for maintaining sustainable soil health and preventing further environmental damage. The lack of harmonized methodologies and comparable data makes it challenging to assess the distribution and prevalence of MPs in soils, but the current understanding emphasizes the urgent need to address their impact on soil ecosystems [54]. Considering the increased use of different types of mulching films, such understanding is not only needed for the impact of MPs from single plastic types, but even more for mixtures of MPs resulting from different commonly used mulching film plastics, such as conventional (e.g., LDPE) and biodegradable plastics (e.g., PBAT-starch blend based).

MP contamination has the potential of having direct and indirect effects on soil ecosystems. Direct impacts of MPs on crops have been observed, even at environmentally relevant concentrations [76]. For example, Lian et al. [51] showed that lettuce growth, measured as shoot fresh weight and length, was inhibited by PE MPs at concentrations ranging from 0.01 % to 0.1 % w/w. Effects of MPs have also been shown on a broader range of soil characteristics and functions, including microbial activity [16,44,64,78] and soil properties, such as soil pH and bulk density [16,58,71]. For example, Kim et al. [44] reported a significant dose-related increase in soil respiration by biodegradable starch polybutylene adipate terephthalate (PBAT-BD) MPs (concentration range: 0 l – 0.8 % w/w in Lufa 2.2 soil) in a mesocosm study. In the case of plants and microbes, effects may be also be indirectly, due to changes in soil properties.

Earthworms are dominating components of soil ecosystems, often indicated as ecosystem engineers [40]. Their burrowing behaviour may strongly impact conditions of the soil, e.g. by improving aeration and water infiltration in the soil, changing soil properties and improving soil texture. This may, for instance, increase soil microbial activity and enhance nutrient cycling leading to an increased plant growth [65]. As a consequence, the activity of earthworms may however, also influence the response of soil ecosystems to contaminants like microplastics.

Because the effects of MPs on the soil microbial community, plants, and other organisms may be highly dependent on the fluctuating field and anthropogenic conditions, mesocosm experiments are a useful tool to help explaining the effects on processes and interactions in the soil environment under controlled settings. Mesocosm studies may provide insight into these impacts in a holistic way, by assessing the effects of MPs on microorganisms, plants, soil invertebrates, soil physicochemical properties, and their interactions all at once. Previous mesocosm experiments have reported effects of MPs on all these endpoints separately, often at environmentally relevant concentrations [2,9,44]. However, most studies to date have focused on testing individual plastic types and short-term exposures [38,39]. As a result, there is a critical gap in understanding the long-term effects and potential interactions arising from mixtures of different plastic types in one comprehensive study, which better reflect real-world environmental conditions.

This study is part of a larger experiment that mainly focused on the fate of microplastics originating from typical mulching films in a simulated soil ecosystem under the influence of earthworms. Three different concentrations (from realistic to worst case scenario) of a 1:1 (w/w) mixture of conventional low-density polyethylene (LDPE) and biodegradable starch polybutylene adipate terephthalate (PBAT-BD) blend MPs were tested. These represent commonly used polymers for conventional and biodegradable mulching films on the European market [7,60]. The aim of this study was to assess the effects of the MPs on various soil health endpoints in the treated mesocosms, in the presence and absence of earthworms as ecosystem engineers. The selected endpoints illustrate a coherent chain of interactions, starting from the influence of MPs on soil properties (soil pH, soil compaction), followed by soil microbial activity (carbon and nitrogen mineralization) and plant growth and biochemistry (shoot length, shoot fresh weight, number of leaves, chlorophyl *a*, *b*, and total chlorophyl, lipid peroxidation, salicylic acid, salicylic acid glucoside, and total phenolic content).

2. Materials and methods

2.1. MP production, characteristics, and analyses

Two batches of microplastic test materials were used in this study, corresponding with the two polymer types under investigation: conventional non-biodegradable conventional low-density polyethylene (LDPE) and biodegradable starch-polybutylene adipate terephthalate (PBAT) blend (hereafter, PBAT-BD). Both batches were produced from real agricultural mulching films purchased from the European market, representing film fragments. Both films were black in colour, and MPs were produced through a process of shredding and cryomilling. Full details of the production and characterisation of the test materials are described in Hurley et al. [30], where the codes M-PEDE-45-black-A0 and M-BIOEL-15-black-A0 are used for the LDPE and PBAT-BD MPs, respectively. Hurley et al. [30] also give a thorough physical and chemical characterisation of the virgin films and subsequent batches of MPs that were produced. A summary of particle characteristics is provided in Supplementary Figure S1.

MP concentrations were analyzed in soils sampled from the upper spiked layer at the end of the experiment (0–10 cm). Soils were carefully extruded from the columns, transferred to a metal bowl, and homogenized thoroughly with a metal spoon. Subsamples were taken into foil envelopes and transported to the Norwegian Institute for Water Research (NIVA) for MP analysis. The detailed MP analyses procedure is explained in Text S1 and Figure S2 in the Supplementary Material.

2.2. CLIMECS set-up

Lufa 2.2 standard natural soil (Lufa Speyer, Germany) was airdried upon arrival in the laboratory at 40 °C for 48 h to prevent fungal growth. The soil had a sandy loam structure, and, according to the provider, a soil organic carbon content (% C) of 1.77 ± 0.56 %, a cation exchange capacity of 8.5 ± 2.0 $cmol_c\ kg^{-1}$, a pH of 5.6 ± 0.3 (0.01 M $CaCl_2$), and a water holding capacity (WHC) of 43 %.

Soil was mixed with MPs using a concrete mixer (PROMISCHER PM 145 L) at 24–29 rpm. Batches of Lufa 2.2 soil were spiked with low (L; 0.05 % LDPE + 0.05 % PBAT-BD), middle (M; 0.16 % LDPE + 0.16 % PBAT-BD), and high (H; 0.5 % LDPE + 0.5 % PBAT-BD) concentrations (%w/w dry soil) of both MP types. Soils and the appropriate amounts of MPs were mixed dry for five minutes, with a paper cover on the mixer, to prevent dusting. Water was then added in small batches and mixed before the addition of more water to prevent clumps from forming and to prevent conglomeration of the MPs. Enough water was added to bring the soil to 50 % of its water holding capacity. After the addition of all the water, the soil was mixed for another five minutes. Control soil without MPs received the same mixing and water addition treatment.

Columns with a 16.6 cm diameter and a height of 40 cm were filled with soil. Two soil layers were created inside the column: a 10 cm loose simulated plough-layer on top of a 30 cm more compact layer. The bottom 30 cm of each column was first filled with soil without added MPs, after which the top 10 cm was added using the soil spiked with MPs. The bottom 30 cm was compacted by inserting a 15 kg tightly fitting steel cylinder into the column after the addition of enough soil to create a 5 cm layer after compaction and repeating this six times. The top 10 cm was only slightly compacted using a 7.5 kg column. Eight columns for each MP concentration and the control were prepared.

After weighing the constructed soil columns, they were inserted into the CLImatic Manipulation of ECosystem Samples, or CLIMECS system (CLIMECS, Amsterdam, The Netherlands; [23]). Here, they were kept at 15 °C (deeper soil layers) and a topsoil temperature of 18 °C, which was maintained by the heat from the lighting above each column. The systems were set to a day: night cycle of 14: 10 h. These settings were chosen to resemble the conditions during the crop-growing season in Northwestern Europe. Columns were inserted into the system in a randomised fashion, with four rows containing one column of each treatment. Care was taken to prevent two columns with the same treatment from being next to each other in two rows.

Half the columns of each treatment and control received two adult earthworms of (Ew) the species *Lumbricus terrestris* and two of the species *Aporrectodea caliginosa*, after having acclimatized in clean Lufa 2.2 soil for 24 h. *L. terrestris* is an anecic species, digging deep in the soil and coming to the surface for feeding and mating. In this way, this species may enhance the transportation of MPs to deeper soil layers. *A. caliginosa* is an endogeic species, mainly living in the topsoil layers, feeding on soil particles and associated organic matter, and thereby potentially also ingesting MPs. By combining both species, it was tried to simulate a natural earthworm community of agricultural soils (see e.g., [15]). The remaining columns did not receive any earthworms. To increase biological activity and complete the soil invertebrate community, springtails were added to all columns at week 0; each column received 125 adult and 125 subadult individuals of *Cerotophysella denticulata, Heteromurus nitidus*, and *Protaphorura fimata*. Since the main focus of this experiment was on the behaviour of the MPs in soil under the influence of earthworms, at the end of the 12-week incubation period no attempts were made to assess effects on springtails or earthworms. For a detailed description of the soil invertebrates used, see Text S2 in the Supplementary Material.

Lettuce seeds (*Lactuca sativa L.*, Zwart Duits) were sourced from Dutch Garden Seeds (Volendam, the Netherlands). Ten patches of three seeds each were sown into the soil columns to ensure sufficient plant material for sampling across different timepoints. The seeds were allowed to germinate and grow for two weeks, during which the column received 20 mL of water using a mist-forming spray. At week 0, seedlings were thinned to retain ten per column: three for sampling at week 0, three for week 6, and three for week 12, and one additional seedling as a backup.

Every two weeks, each column received 130 mL of demineralized water to maintain soil moisture levels. At week 0 and week 6, the columns were restored to their original weight by the addition of water.

2.3. Soil physicochemical properties

Samples of six grams of moist soil, the approximate equivalent of five grams of dry soil, were taken in duplicate from the columns at weeks −2 (when plant seeds were sown), 0 (when soil invertebrates were introduced), 6, and 12 from the 0–5 cm soil layer. At week 12, samples were taken at different depths: 0–5 cm, 5–10 cm, 10–15 cm, 15–25 cm, 25–35 cm, and 35–40 cm. To each sample, 24 mL of 0.01 M $CaCl_2$ solution was added, after which the suspension was shaken at 200 rpm for two hours. After allowing the suspension to settle overnight, pH was measured using a digital pH meter (WTW pH7710).

At week 12, the force needed to penetrate the soil with a soil penetrometer with a 1.43 cm^2 tip to give insight into potential MP effects on soil structure or crust formation. Penetrability was measured at different depths: the top of the soil, at 5 cm, 10 cm, 15 cm, 25 cm, and 35 cm. Penetration was defined as the force needed for 2 mm penetration of the tip, and all measurements were taken in triplicate.

2.4. Soil microbial activity

Microbial activity, specifically carbon and nitrogen mineralization, was analyzed in all replicates from both the controls and MP treatments. Samples from each mesocosm were measured in triplicate.

Carbon mineralization was determined through basal respiration and substrate-induced respiration (SIR) according to ISO guidelines 16072 [35] and 14240–1 [33], respectively. To stabilize the soil environment, soil samples were preincubated in darkness at 21 ± 2 °C for 14 days prior to measurements [28]. The basal respiration measurement involved distributing the equivalent of 10 g of dry soil weight (g_{dw}) in 150 mL infusion bottles to create a thin layer of soil at the bottom. The soil moisture content was adjusted to 60 % of the water holding capacity by

adding demineralized H_2O onto the soil surface. The soil samples were allowed to stabilize in closed bottles for two days. Then, they were aerated, hermetically sealed, and kept in darkness at 21 ± 2 °C for 24 h. Carbon dioxide (CO_2) levels were measured using an Agilent GC 6850 (manual injection, GasPRO column, helium mobile phase, thermal conductivity detector, SW Clarity). The data are presented as μg CO_2-C g_{dw}^{-1} h^{-1}. For the SIR measurement, 5 mg glucose per gram of dry weight (g_{dw}^{-1}) was added to each aerated infusion bottle at the beginning. After that, the subsamples were sealed hermetically and underwent a process similar to basal respiration. CO_2 measurements were taken after 0, 3, and 6 h. SIR was calculated using linear regression and expressed as μg CO_2-C g_{dw}^{-1} h^{-1}.

Nitrogen mineralization was assessed by two methods of microbial activity measurement: potential ammonium oxidation (PAO) and potential ammonification (PAMO). PAO was estimated following ISO 15685 [34]. Ten grams of dry soil weight were placed in jars in three sub-replicates. As a substrate, 20 mL of diammonium sulfate at a concentration of 1.5 mmol L^{-1} was added. The jars were then sealed with perforated lids and placed on a horizontal shaker. The activity was measured after 0, 3, and 6 h. The final analyses were conducted in FIAlab-2500 (with automatic injection, mobile phase of 1 M KCl, and SW FIASoft). The spectrophotometric measurement determined the amount of ammonium ions produced. PAO was calculated using linear regression, with results expressed as μg NO_2-N g_{dw}^{-1} h^{-1}. PAMO was observed in four sample replicates, each containing the equivalent of 2 g_{dw} in a 15 mL plastic tube. At the beginning of the experiment, 1 mL of L-arginine (L-arg) at a concentration of 1 mg L-arg mL^{-1} in dH_2O was added to each tube. The tubes were then sealed and placed on a tube roller in an incubator set at 30 °C for 4 h. Activity was measured spectrophotometrically by determining the production of NH_4-N ions in comparison to controls (without L-arginine). The analysis was conducted using a FIAlab-2500 (similar to PAO, with a 2 M KCl mobile phase). Results are reported as μg NH_4-N g_{dw}^{-1} h^{-1}.

2.5. Plant sampling and biochemical analyses

Sampling of lettuce was conducted at weeks 0, 6, and 12 to assess shoot length, shoot fresh weight, number of leaves, chlorophyll content (*a*, *b*, and total chlorophyll), and biomarker responses (lipid peroxidation, salicylic acid, salicylic acid glucoside, and total phenolic content). These biochemical endpoints were selected as representative markers of plant stress responses and have been shown to be sensitive indicators of both biotic and abiotic stress [1,55]. At each timepoint, three replicate plants were collected from each column. Shoot length, fresh weight, and leaf count were measured for the three seedlings, and at week 12, two of the sampled plants were stored at −80 °C for subsequent biochemical analysis. Prior to analysis, the frozen leaves were ground in liquid nitrogen, and the plant material was divided for specific biomarker assays.

Chlorophyll a (Chl *a*) and chlorophyll *b* (Chl *b*) concentrations were measured spectrophotometrically according to the protocol by Warren [72]. To 0.1 g of grounded lettuce leaves, 1 mL of 100 % methanol was added, and following centrifugation (5 min, 10 000 g), the supernatant was collected, and the pellet was re-extracted. Combined supernatants were used to measure the absorbance at 663 nm and 645 nm with a microplate reader (BMG Labtech, ClarioStar). Chlorophyll concentrations were calculated as proposed by Warren [72].

Lipid peroxidation was measured through a lipid peroxidation marker, malondialdehyde (MDA) [26]. Grounded lettuce leaves (0.25 g) were mixed with 1 mL 0.1 % trichloroacetic acid (TCA). Following centrifugation, 0.5 mL of supernatant was mixed with 0.5 mL 20 % TCA with 0.5 % thiobarbituric acid (TBA). Controls did not have TBA. Samples were incubated at 95 °C for 0.5 h and immediately cooled in an ice bath. The absorbances were measured in a microplate reader (BMG Labtech, ClarioStar) at 532 nm and at 600 nm to subtract non-specific absorption of sucrose at 440 nm. The results were expressed as MDA equivalent (nmol g^{-1} FW).

The measurement of total phenolic content (TPC) followed the protocol of Herald et al. [25]. Grounded plant material (0.3 g) was mixed with 3 mL of 80 % methanol and incubated at room temperature for 1 h. Following centrifugation (5 min, 10000 g), 0.025 mL of supernatant was mixed with 0.075 mL of water and 0.025 mL of Folin-Ciocalteu reagent and incubated at room temperature for 6 min. Then, 0.1 mL 7.5 % Na_2CO_3 was added, mixed, and the samples were incubated at room temperature in the dark for 1.5 h. The absorbance at 765 nm was measured with a microplate reader (BMG Labtech, ClarioStar). Gallic acid was used as a standard. TPC was expressed as μg gallic acid (GA) equivalent g^{-1} FW.

Free salicylic acid (SA) and SA-glucoside concentrations were measured according to Allasia et al. [4]. Grounded plant material (0.25 g) was mixed with 1 mL 70 % ethanol with 0.032 mL anisic acid (15.25 ng μL^{-1}). After centrifugation, the pellet was re-extracted with 1 mL of 90 % methanol. Methanol and ethanol from combined supernatants were evaporated in a vacuum concentrator (Speed Vac, 173 2–18 Cdplus, Thermo Fisher) and the pellet was treated with 0.065 mL 20 % of TCA, and 0.650 mL of ethyl acetate: cyclohexane (1:1). After centrifugation, the upper phase was collected, and the water phase was re-extracted. The combined upper phase was evaporated to dryness in a vacuum concentrator (Speed Vac). The pellet was dissolved in 0.100 mL of 10 % methanol containing 0.1 % trifluoroacetic acid (TFA). This extraction provided free SA. The water phase was later mixed with 0.3 mL 12 M HCl and incubated at 80 °C for 1 h. After cooling down, 0.018 mL of anisic acid (15.25 ng μL^{-1}) was added, and samples were extracted twice with 0.9 mL of ethyl acetate: cyclohexane (1:1). Combined upper phases were evaporated in a vacuum concentrator, and the dry residue was dissolved in 0.100 mL 10 % methanol with 0.1 % TFA. This fraction represented SA-glucosides. Free and hydrolyzed SA were measured with HPLC (Arc HPLC Waters, US), with a C18 column (Phenomenex, 250 × 4.6 mm, 5 μm) eluted with a methanol gradient from 10 % to 82 % at a flow rate of 1 mL min^{-1} at a temperature of 30 °C. Eluent contained 0.1 % TFA. Detection of SA and glucoside of SA (thus hydrolyzed SA, HSA) was done with a fluorescence detector (excitation at 305 nm and emission at 407 nm). SA and HSA concentrations were expressed as μg SA g^{-1} FW.

2.6. Statistical analyses and data storage

The statistical significance of differences between the control group (0 % MPs), treatments with different MP concentrations, and the presence or absence of earthworms was tested using analysis of variance (two-way ANOVA). A Tukey's post hoc test was performed to evaluate the differences between treatments. All analyses were conducted using Statistica 14 (StatSoft, Inc. 2024).

3. Results

3.1. Soil MP analyses

No LDPE and PBAT-BD MPs were found in the controls (Table 1). Increasing numbers of MPs were detected for the low, middle, and high concentration treatments, however, the target concentrations low (L; 0.05 % + 0.05 %), middle (M; 0.16 % + 0.16 %), and high (H; 0.5 % + 0.5 %) differed from those actually measured and were a bit higher for LDPE and a bit lower for PBAT-BD MPs than expected (Table 1). Nevertheless, there was a clear concentration gradient, and the treatments with and without earthworms revealed comparable MP concentrations, so these treatments could be compared directly.

3.2. 3.2. Soil properties

The addition of MPs to the Lufa 2.2 soil caused a dose-related increase in soil pH, which was significant ($p < 0.05$) at the highest dose of 0.5 % LDPE + 0.5 % PBAT-BD MPs at the start of the 12-week mesocosm

Table 1
Microplastic (MP) concentrations measured in the top 10 cm soil layer at the end of the mesocosm experiment (week 12). The values represent mean (± standard deviation, n = 4) concentrations of the controls (C), and of columns dosed with a 1:1 mixture of low-density polyethylene (LDPE) and starch-based polybutylene adipate terephthalate biodegradable (PBAT-BD) MPs at low (L; 0.05 % + 0.05 %), middle (M; 0.16 % + 0.16 %), and high concentrations (H; 0.5 % + 0.5 %) in the top 10 cm Lufa 2.2 soil layer (on top of 30 cm clean soil). Shown are results for treatments without and with earthworms (Ew). Letters show significant differences between treatments, separately for LDPE and for PBAT-BD ($p < 0.05$).

MP type	C	L	M	H
LDPE	0.00 ± 0.00 % a	0.068 ± 0.010 % ab	0.206 ± 0.00016 % c	0.778 ± 0.063 % d
LDPE Ew	0.00 ± 0.00 % a	0.056 ± 0.010 % a	0.185 ± 0.025 % bc	0.735 ± 0.127 % d
PBAT-BD	0.00 ± 0.00 % a	0.019 ± 0.004 % ab	0.105 ± 0.034 % c	0.253 ± 0.026 % d
PBAT-BD Ew	0.00 ± 0.00 % a	0.027 ± 0.016 % ab	0.078 ± 0.038 % bc	0.191 ± 0.055 % d

test (Fig. 1; Supplementary Tables S1-S9). After 6 and 12 weeks, in all treatments and the control, soil pH was lower than at the start and was no longer significantly higher in the MP-spiked soil. Only exception was pH after 12 weeks in the high treatment with earthworms, which was significantly higher than in the low and medium treatment but did not differ from the control (Supplementary Table S4). At the start of the exposures, soil pH was significantly ($p < 0.05$) lower in the columns without earthworms, both in the control and all MP treatments, but this difference disappeared at later sampling times. No significant effects of the MPs on soil pH at different depths in the mesocosms were seen after 12 weeks of incubation (Supplementary Figure S3).

At the end of the 12-week exposure period, the Lufa 2.2 soil in the mesocosms without earthworms showed a non-significant but dose-related increase in compaction, which was most pronounced in the top 5 cm layer (Supplementary Figure S4; Tables S10-S15). In the mesocosms with earthworms, soil compaction was slightly but not significantly higher than the control only at the highest MP concentration (0.5 % + 0.5 %), at all soil depths (Supplementary Figure S4).

3.3. Soil microbial activity

Carbon mineralization measured as basal respiration did not significantly differ between the controls (0 %) and the MP spiked soils in both the presence and absence of earthworms ($p > 0.05$, Fig. 2A, Supplementary Table S16). In contrast, SIR increased in the high concentrations of MPs compared to the controls and L concentrations in both variants (with and without earthworms) ($p < 0.05$) and also compared to M concentration with earthworms ($p = 0.010$, Fig. 2B; Supplementary Table S17). PAO decreased significantly compared to the controls at the low and middle MP concentrations without earthworms ($p = 0.001$ and 0.041, Fig. 2C; Supplementary Table S18), but the decrease was not statistically significant in the highest MP concentration ($p = 0.281$). The presence of earthworms significantly increased PAO in the controls and all MP treatments ($p < 0.05$). A significant increase of PAMO was found in the control without earthworms compared to the control with earthworms, compared to the L concentration without earthworms, and M concentration with earthworms ($p = < 0.05$, Fig. 2D; Supplementary Table S19). For all other treatments (controls vs MP treatments, MP treatments with and without earthworms), the differences were not statistically significant ($p < 0.05$).

3.4. Plant growth and biochemical analyses

No effects were recorded on the physical growth parameters of lettuce seedlings except shoot fresh weight in week 0 (Fig. 3; Supplementary Tables S20–28). The shoot length was not affected by MP

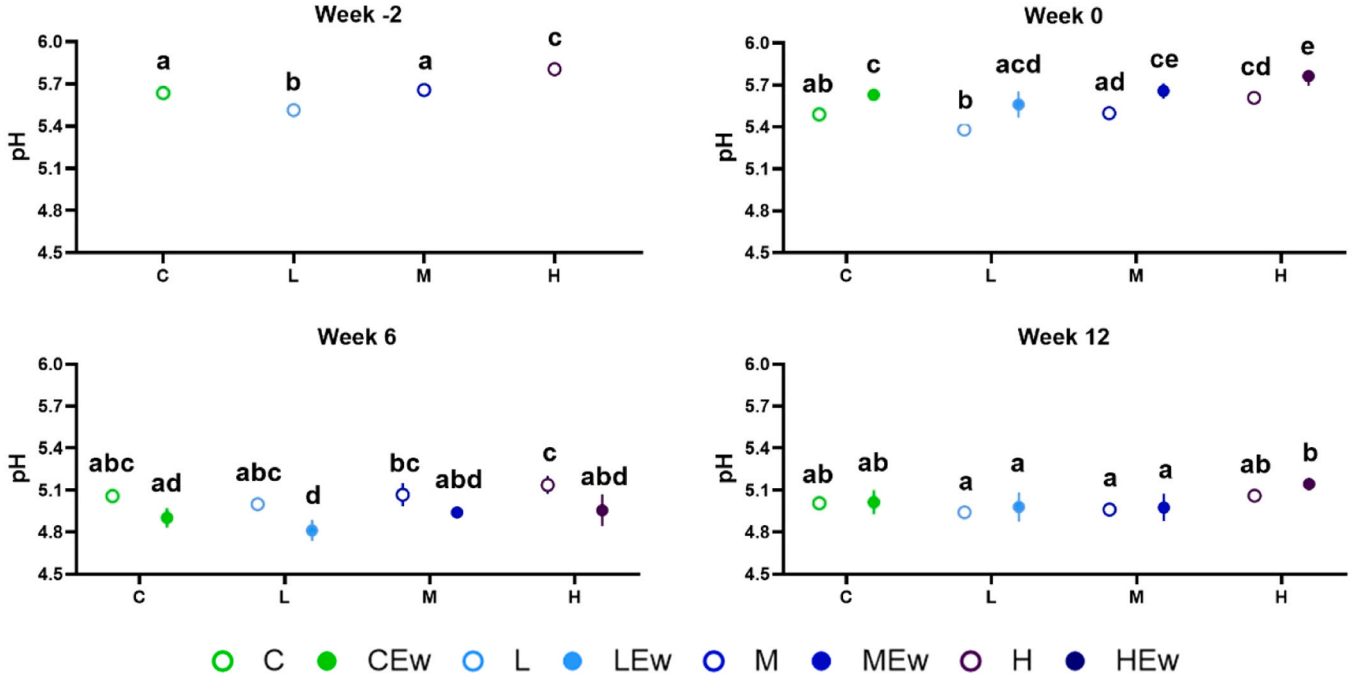

Fig. 1. Effect of microplastics (MPs) on pH (0.01 M $CaCl_2$) of 0–5 cm soil layer at different timepoints of the mesocosm experiment (-2, 0, 6, and 12 weeks). The values represent means (± standard error). Treatments from left to right include control (C), and a 1:1 mixture of low-density polyethylene (LDPE) and starch-based polybutylene adipate terephthalate biodegradable (PBAT-BD) MPs at low (L; 0.05 % + 0.05 %), middle (M; 0.16 % + 0.16 %), and high concentrations (H; 0.5 % + 0.5 %) in the top 10 cm Lufa 2.2 soil layer (on top of 30 cm clean soil). Empty symbols represent treatments without earthworms; full symbols represent treatments with earthworms (Ew). Letters show significant differences between treatments ($p < 0.05$). Note that the Y axis covers only the pH range between 4.5 and 6.

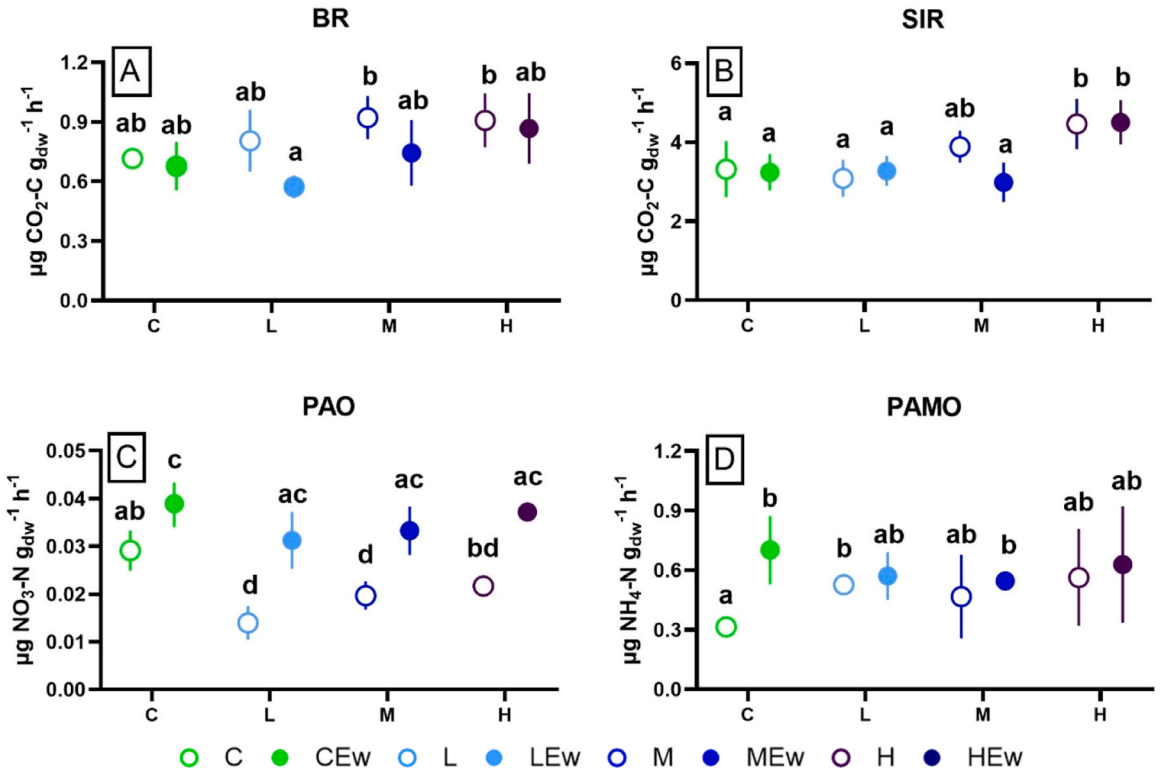

Fig. 2. Effects of microplastics (MPs) on basal respiration (BR), substrate-induced respiration (SIR), potential ammonium oxidation (PAO), and potential ammonification (PAMO) in Lufa 2.2 soil in CLIMECS mesocosms. The values represent means (± standard deviation, n = 4). Treatments from left to right include control (C), and a 1:1 mixture of low-density polyethylene (LDPE) and starch-based polybutylene adipate terephthalate biodegradable (PBAT-BD) MPs at low (L, 0.05 % + 0.05 %), middle (M, 0.16 % + 0.16 %), and high concentrations (H, 0.5 % + 0.5 %) in the top 10 cm Lufa 2.2 soil layer (on top of 30 cm clean soil). Empty circles represent treatments without earthworms; full circles represent treatments with earthworms (Ew). Letters show significant differences between treatments ($p < 0.05$). Note that the Y axes have different units and different ranges.

treatments in weeks 0, 6 and 12, which corresponds with 2, 8 and 14 weeks after planting the seeds in the mesocosms, respectively ($p > 0.05$, Supplementary Tables 20–22). Shoot fresh weight was also not affected by MP treatments ($p > 0.05$, Supplementary Tables 23–25), nevertheless, the presence of earthworms had a negative effect on shoot fresh weight in week 0 ($p = 0.020$, two-way ANOVA) in the control (Fig. 3). Even though a significant effect of MP treatments was found ($p = 0.044$, two-way ANOVA) on the number of leaves, no difference between treatments was observed in week 0. Also, no difference between treatments was observed in week 6 and week 12 ($p > 0.05$, Supplementary Tables 26–28) for the number of leaves of lettuce seedlings.

Total chlorophyll concentrations were highest at time 0, decreased at the second timepoint, and at the same low level at the last timepoint (Supplementary Figure S5 and Tables S29-S37). The concentrations of Chl *a*, Chl *b*, and total chlorophyll did not differ between treatments (addition of MPs vs control, Supplementary Figure S5, $p > 0.05$). The presence or absence of earthworms did not affect the chlorophyll concentration in the lettuce plants (Supplementary Figure S5, $p > 0.05$).

At the end of the 12-week exposure time, the concentration of MDA (a marker of lipid peroxidation) in the lettuce plants did not differ between treatments with and without earthworms (Fig. 4A, $p = 0.444$) but showed a dose-related increase in plants grown on MP-spiked soils. More precisely, medium and high MP concentrations significantly elevated MDA concentration compared to the control (Supplementary Table S38; $p < 0.001$).

The addition of low and medium amounts of MPs resulted in an increase of SA concentrations in the plants compared to the control (Fig. 4B, $p < 0.001$). However, the highest MP concentration did not elevate plant SA concentration (Supplementary Table S39; $p = 0.999$). Similarly, only low and medium MP doses caused an increase in HSA concentration in the plants compared to the control ($p < 0.001$ and $p = 0.026$, respectively, Fig. 4C). There was no effect of earthworms on SA and HSA concentrations in the plants after 12 weeks of exposure (Supplementary Table S40; $p = 0.239$).

The concentration of total phenolics in the lettuce plants was significantly elevated by low and medium MP concentrations compared to the control ($p < 0.001$), but not by the highest MP concentration ($p = 0.061$, Fig. 4D). The treatments with and without earthworms did not differ in total plant phenolics concentrations (Supplementary Table S41; $p = 0.352$).

4. Discussion

This study investigated the effects of a 1:1 mixture of conventional (LDPE) and biodegradable (PBAT-BD) microplastics originating from commonly used mulching films on various endpoints under simulated realistic environmental conditions using mesocosms. Studies taking into account the increasing usage of different types of mulching films in agriculture are rare but needed for understanding the impact of MP mixtures on soil fertility, nutrient cycles, and preventing further environmental damage. The results show the effects of the MPs on soil properties (pH, compaction), soil microbial activity (carbon and nitrogen mineralization), and plant biochemistry (lipid peroxidation, salicylic acid, salicylic acid glucoside, and total phenolic content), already at realistic environmental exposure levels.

4.1. Soil properties

At the start of the experiment (week 0), so 2 weeks after spiking the soils, soil pH was significantly increased by the high concentration (0.5 % + 0.5 %) of the 1:1 LDPE + PBAT-BD MPs (Fig. 1). After 6 and 12 weeks, this MP effect on soil pH was no longer significant, perhaps due

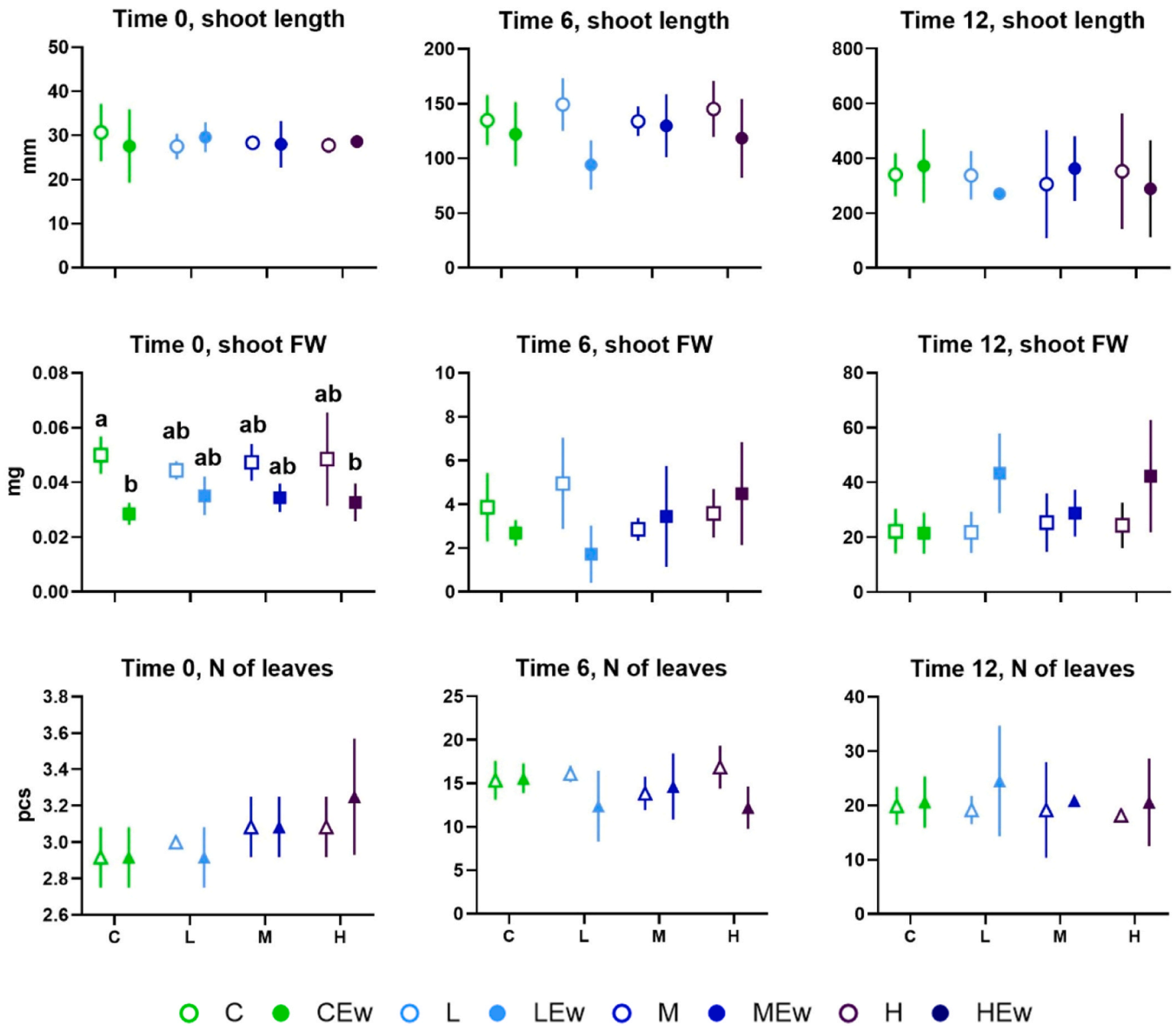

Fig. 3. Effects of microplastics (MPs) on the shoot length (circles, in mm), shoot fresh weight (squares, shoot fresh weight (FW), in mg), and number of leaves (triangles, pieces) of lettuce seedlings harvested at three timepoints of a CLIMECS mesocosm experiment (0, 6, and 12 weeks after set-up, corresponding with 2, 8 and 14 weeks after planting the seeds). The values represent means (± standard deviation, n = 4). Treatments from left to right include control (C), and a 1:1 mixture of low-density polyethylene (LDPE) and starch-based polybutylene adipate terephthalate biodegradable (PBAT-BD) MPs at low (L, 0.05 % + 0.05 %), middle (M, 0.16 % + 0.16 %), and high concentrations (H, 0.5 % + 0.5 %) in the top 10 cm Lufa 2.2 soil layer (on top of 30 cm clean soil). Empty symbols represent treatments without earthworms; full symbols represent treatments with earthworms (Ew). Letters show significant differences between treatments $p < 0.05$); if no letters are given, treatments did not differ. Note that the Y axes have different units and different ranges.

to the buffering capacity of the soil. The fact that soil pH was not affected in the deeper, non-spiked, soil layers confirms this effect was indeed due to the MPs introduced into the soil. An effect of MPs on soil pH was also observed in earlier studies, both in single species toxicity tests and in mesocosm tests with both PBAT-BD and LDPE MPs (see e.g., [44,63,67,68]. As soil pH was affected directly after the addition of the MPs, this is a strong indication that the effects observed are chemical, not biological. Whether the effect of MPs on soil pH is due to interactions with chemical groups on the surface of the particles, or due to leaching of one or more chemicals from the particles is, however, unclear.

The addition of MPs to the soil led to a non-significant but dose-related increasing trend in compaction, especially in the top 5 cm soil layer of columns without earthworms and the 5–10 cm layer in all treatments. The activity of the earthworms appeared to counteract this compacting effect of the MPs, as no such effect was seen in the presence of earthworms. The burrowing activity of the earthworms no doubt helped counteracting the compacting effect of the MPs. Earthworms are known to increase soil porosity by their burrowing behaviour, which may lead to a lower level of soil compaction [10]. This explains why the soils in the mesocosms without earthworms showed a (non-significantly) higher compaction. It remains however, unclear how the compacting effect of the MPs could be explained. It may be an effect caused by lowered soil aggregation due to the MPs [9,16]. As aggregate size is lowered by the addition of MPs, the soil may become more compact. However, the addition of MPs to soil is also known to increase moisture retention and bulk density, which may be assumed to lead to a lower level of compaction [16,37,67]. On the other hand, perhaps the way we measured compaction, using a penetrometer, may have caused this outcome. We did not measure bulk density, but did see that it became harder to penetrate the soil at a higher dose of MPs.

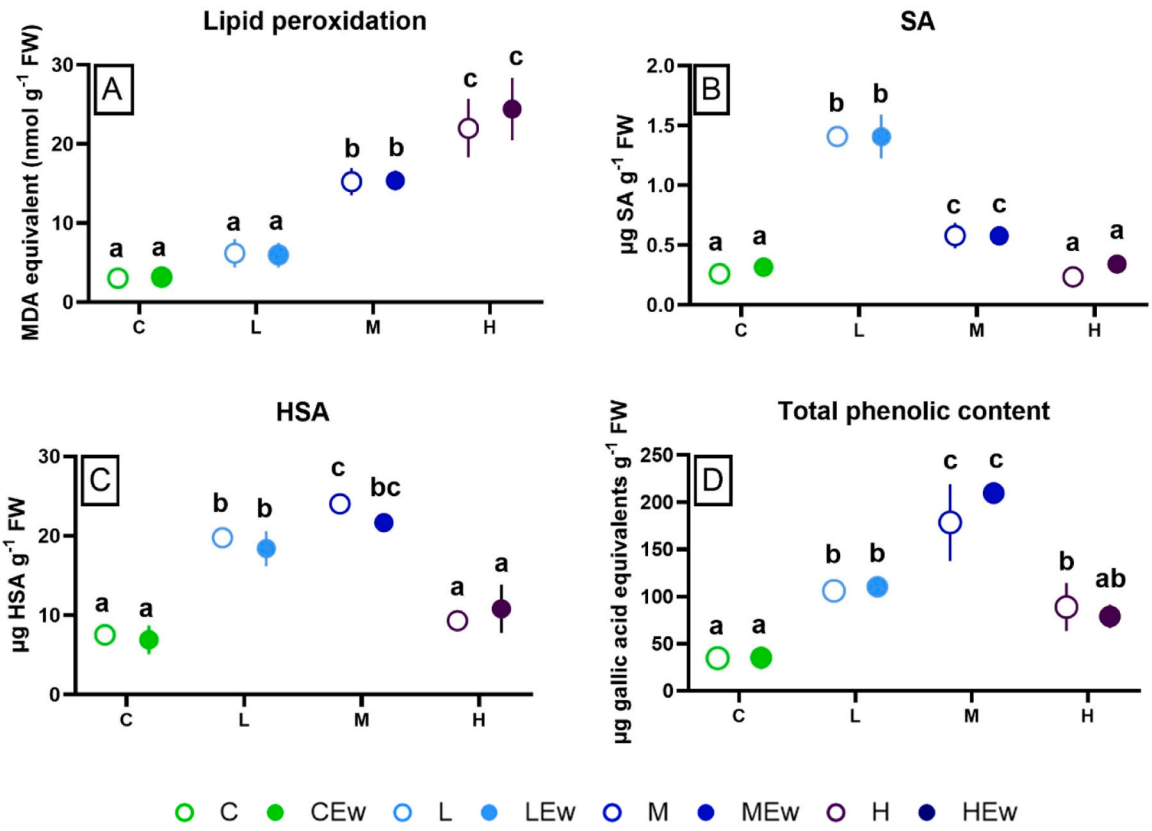

Fig. 4. Effects of microplastics (MPs) on biochemical properties of lettuce plants grown for 12 weeks in Lufa 2.2 soil in a CLIMECS mesocosm experiment: lipid peroxidation (measured as malondialdehyde (MDA) equivalent in nmol g^{-1} FW), salicylic acid (SA; µg SA g^{-1} FW), salicylic acid glucoside (HSA; µg HSA g^{-1} FW), and total phenolic content (in µg gallic acid equivalents g^{-1} FW). The values represent means (± standard deviation, n = 4). Treatments from left to right include control (C), and a 1:1 mixture of low-density polyethylene (LDPE) and starch-based polybutylene adipate terephthalate biodegradable (PBAT-BD) MPs at low (L, 0.05 % + 0.05 %), middle (M, 0.16 % + 0.16 %), and high concentrations (H, 0.5 % + 0.5 %) in the top 10 cm Lufa 2.2 soil layer (on top of 30 cm clean soil). Empty symbols represent treatments without earthworms; full symbols represent treatments with earthworms (Ew). Letters show significant differences between treatments ($p < 0.05$). Note that the Y axes have different units and different ranges.

4.2. Soil microbial activity

As a result of stress (e.g., lack of substrate, decline of temperature, change of soil properties, droughts, or pollution), microorganisms apply a variety of strategies, including dormancy, inactivity, massive cell death, cell size reduction, increase of carbon reserves, and cell wall modification [5,24,48]. On the other hand, the addition of biodegradable microplastics could serve as a carbon source, and an increase in microbial activity might be recorded [32,45,79].

Our results showed a significant increase in SIR at high concentrations of MPs but no effects at the low and middle MP concentrations, which is in accordance with previous studies, where PBAT-BD-MP contamination induced CO_2 emission at comparable MP concentrations [44,59]. At higher concentrations, the positive effect of using PBAT-BD MPs as a carbon source probably outweighed the negative effects (MPs as an additional stressor), and thus, the microbial activity increased. Another possible mechanism contributing to the increase in CO_2 production could be the priming effect of MPs on soil organic matter (SOM). Throughout this effect, MPs alter SOM, and the evolved CO_2 can also stem or partially stem from the SOM [29,77]. A third mechanism could be the change in soil properties, namely pH and soil compaction. In acidic soils like the Lufa 2.2 soil, used in this study, pH elevation can stimulate microbial growth and activity, including CO_2 emissions [56]. On the other hand, higher soil compaction may negatively influence microbial biomass and activity through the decrease of macropore functions and reduced nutrient availability [6,50]. However, pH in our study increased only by 0.05 and 0.13 units in the highest MP treatments (with and without earthworms, respectively) compared to the control, and soil compaction in the 0–5 cm layer, where the microbial activity was also measured, also was not significantly affected by the MP treatments. Therefore, we considered that the changes in pH and soil compaction within the used concentrations had a minor effect on CO_2 production. Although Dijkstra et al. [17] stated that soil community respiration is largely driven by the small minority of actively growing cells, in our study it still seemed to be a sensitive tool for microplastic pollution assessment.

Contrary to the carbon mineralization, nitrogen mineralization decreased with MP contamination for PAO in the treatments without earthworms. This is in accordance with our previous study with the same agricultural MPs, where PAO was reduced at the low MP concentration [64]. In this case, MP contamination probably had an effect as an additional stressor, as was discussed previously for the carbon mineralization, but the direct mechanism remains unclear.

The presence of earthworms had effects only on nitrogen mineralization. In all treatments for PAO, and also in the controls for PAMO, the presence of earthworms increased the microbial activity. PAO and PAMO are aerobic processes, and bioturbation by earthworms might possibly enhance the accessibility of oxygen. The bioturbation activity of earthworms and consequent increased aeration of the soil may explain the increased oxidation of the already available ammonium, which was measured as an increased PAO level in the presence compared to the absence of earthworms (Fig. 2C). It does, however, not explain why the increased ammonification did not go along with an increased soil respiration in the presence of earthworms. Perhaps nitrogen availability is only enhanced in earthworm casts and not in the bulk soil. It should, however, be noted that the PAMO level in the controls without

earthworms showed a rather low variation (Fig. 2D); as a consequence, the small difference in PAMO between the mesocosms with and without earthworms was already statistically significant while its biological significance remains unclear. Xu et al. [74] demonstrated by correlation analysis that PAO may be promoted by earthworms through the increase of soil pH. This was not the case in our study because pH was comparable in the treatments with and without earthworms for all MP concentrations after 12 weeks.

4.3. Lettuce biochemistry affected by a mixture of plastics

Our results indicate that plant growth and chlorophyll content remained unaffected by exposure to the MP mixture, a finding that contrasts with numerous previous studies, which frequently report adverse impacts of MPs on plant development [2,70]. This discrepancy could be related to differences in experimental design, exposure concentrations, plant species, or the physicochemical properties of the plastics used [76]. Interestingly, when examining growth dynamics over the course of the experiment, no significant differences emerged between the control and treatment groups at any time point. This consistency suggests that the plastic mixture did not induce delayed or progressive effects but rather elicited a stable lack of impact on plant growth throughout the exposure period.

In contrast to the absence of growth effects, we observed pronounced impacts at the molecular level in lettuce, primarily through the activation of the plant's defence mechanisms. This aligns with findings from other studies [2,76], which consistently report that lettuce biochemistry is sensitive to MP exposure. The observed increase in lipid peroxidation indicates that the plants were experiencing oxidative stress, which has the potential to damage essential biomolecules, such as lipids and proteins [19,20]. Conversely, the elevated levels of stress-related hormones, such as salicylic acid, suggest that the plants were actively mounting defence responses due to MP exposure [1]. Such activation of defence mechanisms could be beneficial for response to potential threats, including pathogens [47,69] in field conditions. At the highest MP concentration, salicylic acid, salicylic acid glucoside and phenolic content were no longer increased compared to the control. The reason for this non-monotonic dose-response pattern remains unclear but could be related to a mechanistic relation between these parameters. Salicylic acid is involved in the biosynthesis of phenolic compounds [36], so the induction of this enzyme at the lowest exposure level may explain for the higher production of the latter. It remains unclear, however, why the activity of salicylic acid was lower at the medium and higher MP concentrations than at the low MP concentration. Given that the lipid peroxidation was higher for the medium and high concentrations, we hypothesize that oxidative stress levels exceeded the ability of the plant's defence mechanisms to act at the medium and higher MP concentrations [2]. Another factor could be time as shown for plant responses to MPs in another mesocosm study [2]. The induction of stress responses requires energy and therefore may decrease again with time and perhaps also start decreasing earlier at higher levels of stress. The production of phenolic compounds apparently is slower in responding to the stress caused by the microplastics compared to the response of salicylic acid.

One more point to mention is that previous studies on soil-plant-microbe interactions have shown that changes in soil nutrient availability or pH can directly affect plant physiology, while shifts in microbial community composition can indirectly influence plant growth and stress responses through mechanisms such as nutrient cycling, hormone production, or pathogen suppression [13]. Taken together, these results highlight that even in the absence of visible growth impairments, MP exposure can trigger significant biochemical and physiological changes that may compromise plant health over the longer term.

4.4. Effect of earthworm activity on soil ecosystem response to MPs

In this study only a few endpoints showed a different response to the MPs in mesocosms with and without earthworms. Soil compaction was the parameter where earthworm impact, although not significant, was most consistent, with the absence of earthworms resulting in a higher degree of soil compaction (Supplementary Figure S4). As discussed above, earthworm burrowing may have enhanced soil porosity leading to a lower degree of compaction. This slightly higher degree of compaction did not lead to a consistent lower or higher MP effect on other endpoints. Soil pH was significantly higher in mesocosms without earthworms compared to those with earthworms, but only at the lowest and highest MP dose after 6 weeks and not at other time points (Fig. 1). The absence of earthworm burrowing activity may explain the significantly lower PAO activity in the mesocosms without earthworms (Fig. 2). But this does not explain why the MP effects on other microbial endpoints, like basal and substrate-induced respiration or PAMO, did not differ between mesocosms with and without earthworms. Also the response of the plant-related endpoints did not differ between the mesocosms with and without earthworms (Figs. 3 and 4). Overall, these findings suggest that the earthworms did not affect the toxicity of the MPs to a great extent. Perhaps duration of the mesocosm study was still too short to allow for a clear difference in response to earthworm activity.

4.5. Limitations of this study

This study focused on the influence of earthworms on the effects of a mixture of two MP types on a simulated soil ecosystem, at realistic exposure levels. This makes this study unique, as so far the effects of MPs in soil have mainly been determined in single species tests in the laboratory. Only few data is available on MP effects on soil ecosystems under controlled conditions (see e.g. [2,44,67]). Our study, however, also has some limitations. As mentioned earlier, this mesocosm study was especially aimed at assessing the fate of microplastics in soil under environmentally relevant conditions, in the absence and presence of earthworms. Because of that aim, we unfortunately paid less attention to the effects on earthworms and springtails that were introduced in the mesocosms to increase environmental realism. That is why neither mortality, health data, nor reproduction were recorded. Our previous studies have shown only limited effects of similar MP types on both earthworms [22] and springtails [66,68] in single species toxicity tests within the range of concentrations used in this mesocosms study. Van Loon et al. [67], performing a mesocosm in the same CLIMECS system with LUFA 2.2 dosed at similar exposure concentrations, found some effects of PBAT-BD MPs on earthworm reproduction, but not on survival. We therefore did not expect any direct effects of the tested MPs on earthworm (and springtail) survival within the 12-week exposure period. Therefore we assumed that any differences in response of the different parameters studied in this study can be attributed to the burrowing activity of the earthworms in the mesocosm soil.

Earthworm activity may also have affected the exposure to MPs of other soil organisms. Due to their burrowing activity, they may have stimulated microbial activity and also directly and indirectly degradation of the PBAT-BD MPs [43]. It however, remains unclear whether this degradation may have led to a further decrease of the particle size of the MPs or also to loss of particles. Most likely, the 12-week exposure period was far too short to allow for the complete degradation of the PBAT MP material [14]. Microbial activity may also have stimulated the formation of a so-called biocorona on the surface of the MPs [75], which may have affected the possible impact on other organisms. Since measurement of MP degradation or the formation of a biocorona was beyond the scope of this study, no conclusions can be drawn related to these issues.

5. Conclusions

Overall, our results illustrate a coherent chain of interactions, starting from the presence of MPs, through their influence on soil properties, and ultimately extending to microbial activity and plant biochemistry. Overall, the earthworms did not seem to affect the toxicity of the MPs to a great extent. The changes observed in soil pH, however, highlight that MPs can alter fundamental soil characteristics, but such shifts alone are unlikely to account for the broader biological responses. Microbial activity revealed different response mechanisms for MP contamination. Whereas carbon mineralisation may benefit from using MPs as a carbon source, for nitrogen mineralisation, MP contamination could act as an additional stressor and lead to activity decline. In contrast, the plant responses may be more complex. They could arise either directly from altered soil properties or indirectly through microbially mediated processes. This interplay of direct and indirect effects underscores the difficulty of attributing plant responses to a single driver and highlights the importance of considering multiple, interacting mechanisms when evaluating plant responses to environmental stressors like MPs.

In contrast, this study investigated the effects of a MP mixture from frequently used agricultural mulching films at environmentally relevant concentrations with the presence or absence of earthworms. Mesocosm studies help explain the effects on processes and interactions between soil physicochemical properties, microorganisms, soil invertebrates, and plants all at once, without the variability of the natural environment. Though the changes in plant growth were negligible, MPs caused more profound changes in plant biochemistry, pointing to the triggering of plant response mechanisms against oxidative stress. Therefore, our study emphasises the need of studies using more complex systems dosed with MP mixtures. The next step would be the field plot experiments because, in natural field conditions, the inherent variability of soil and the effects of growing plants can buffer subtle changes caused by microplastics. On the other hand, MPs in combination with other stressors such as contaminants, drought, or waterlogging, may increase the risk of reduced soil ecosystem functioning, and even low MP concentrations could contribute to declining soil health under these multiple stress conditions.

Environmental Implications

The use of plastics in agriculture is expanding both in quantity and in the diversity of applications. Although plastics as soil pollutants may potentially threaten food safety, human health, and environment quality, there is a lack of data on microplastic mixtures originating from materials currently used in agriculture. Here a mixture of microplastics produced from real mulching films was tested to resemble particles found in agricultural fields and using soil mesocosms to simulate realistic environmental conditions. Effects on different biological endpoints were observed at environmentally relevant concentrations, emphasizing that microplastic mixtures may be harmful to soil quality in agricultural fields.

CRediT authorship contribution statement

Luca Nizzetto: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Thijs Bosker:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Sarmite Kernchen:** Writing – original draft, Investigation, Formal analysis. **Chiara Consolaro:** Investigation. **Lotte de Jeu:** Investigation, Formal analysis. **Rachel Hurley:** Writing – original draft, Project administration, Investigation, Funding acquisition, Formal analysis. **Cornelis A.M. van Gestel:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Laura J. Zantis:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Sylwia Adamczyk:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Klára Šmídová:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Salla Selonen:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Sam van Loon:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Jakub Hofman:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization. **Bartosz Adamczyk:** Writing – original draft, Methodology, Investigation, Formal analysis.

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

This study received funding under grant agreement No 101000210 (project PAPILLONS) and was supported under grant agreement No 857560 (CETOCOEN Excellence) from the European Union's Horizon 2020 research and innovation program. This research was supported by the Johannes Amos Comenius Programme (P JAC), project No. CZ.02.01.01/00/22_008/0004605, Natural and anthropogenic georisks financed by MEYS (co-funded by the European Union). This publication reflects only the author's view, and the European Commission is not responsible for any use that may be made of the information it contains. The authors thank the RECETOX Research Infrastructure (No LM2023069), financed by MEYS for the supportive background. Moreover, the authors thank Linda Mutanen (Natural Resources Institute Finland) for help in laboratory work and financial support from the Research Council of Finland (No 360978).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2026.141339.

Data Availability

The data from this study are available on the open-access research data repository Zenodo and can be accessed at: https://zenodo.org/records/15690334.

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