



Soil and earthworm-derived enzymatic activities in soil microcosms following addition of a N- and P-enriched polysaccharide-based vs a conventional starch-based mulching film

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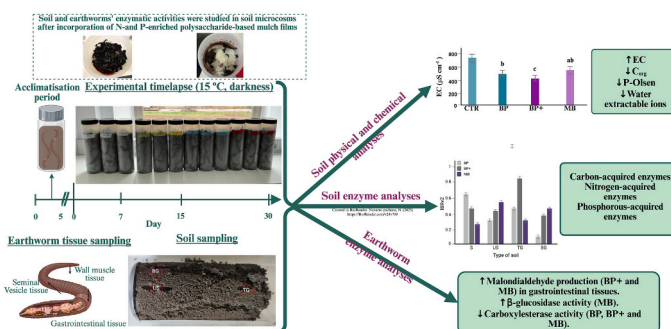
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HIGHLIGHTS

- Bio-based polymers with different C/N selectively stimulated soil C mineralization
- No significant differences in soil enzyme activities at burrow and undisturbed soil.
- Earthworms' exposure to all mulching films inhibited carboxylesterase activity.
- Oxidative stress was induced in earthworms exposed to BP+ and MB mulching films.

GRAPHICAL ABSTRACT



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ABSTRACT

Bio-based mulching films are gaining increasing attention in contemporary agriculture as feasible and environmentally friendly alternatives to polyethylene films whose use causes the undesirable release of plastic debris into the environment, thus contributing to pollution and negatively affecting soil quality. Being a source of readily available C, following soil incorporation bio-based mulches can stimulate the biological activity and benefit soil fertility and health.

This work aims to evaluate short-term impacts of an innovative N- and P-enriched carboxymethylcellulose, sodium alginate and chitosan-based biodegradable mulch film on physical (aggregate stability index), chemical (pH, electrical conductivity, total organic C, total N, P-Olsen, water soluble anions) and enzymatic soil activities using soil microcosms added or not with an epigeic earthworm (*Lumbricus terrestris* L.) over a 30-d incubation period. Mater-Bi was used as reference. Analysis of earthworm casts, tissues, digestive enzymes and oxidative stress biomarkers was also included since earthworms can play a role both as indicators of pollution and as drivers of bio-based mulch degradation with their borrowing lifestyle and release of hydrolytic enzymes. The C/N ratio of incorporated mulches differently primed C mineralization.

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Mulching films addition did not significantly alter soil enzymatic activities, whereas their combination with earthworms markedly those activities involved in nutrient-cycling. Earthworms exposed to all types of films showed oxidative stress, evidenced by increased glutathione oxidation and carboxylesterase inhibition. This work suggests that biodegradable films can replace conventional plastic films in a more environmentally sustainable way, but their environmental effects still disturb soil biological activity and earthworm activity. Moreover, this study showed that carboxylesterase could be an appropriate biomarker to study the effects of mulching films on earthworms.

1. Introduction

Mulching is an essential agricultural practice in crop production, offering benefits such as protection and increase of crop yields, weed control, soil moisture conservation and regulation of soil temperature (Touchaleaume et al., 2016). Polyethylene (PE) mulching films dominate the mulching film market (Briassoulis and Giannoulis, 2018). In Europe in 2018, 1,740,000 tons of plastics were used, of which 450,000 tons for silage and mulching (<https://openknowledge.fao.org/home>, September 2024). Polyethylene films can break down in the field into small, non-recoverable pieces or be intentionally or unintentionally left in the soil at the end of the crop cycle, leading to an accumulation of microplastics in the soil and irreversible and harmful environmental contamination (Zhang et al., 2019). When the rate of annual accumulation overpasses 240 kg ha⁻¹, soil properties are being severely affected in terms of both soil quality and functioning (Qi et al., 2020a). In fact, microplastics can affect soil properties such as bulk density (De Souza Machado et al., 2018), porosity (Zhang and Zhang, 2020), soil aggregation, water-holding capacity (De Souza Machado et al., 2019), pH (Wang et al., 2022), and cation-exchange capacity, thus exerting positive or negative effects (Boots et al., 2019). Microplastics can also affect, directly or indirectly, the soil biota, in particular the soil microbiota in their activity and taxonomic diversity (Bronick and Lal, 2005). Previous research has explored the impact of soil-applied mulching films on microbial community properties such as microbial biomass (Zheng et al., 2018) and enzymatic activities connected to biogeochemical nutrient cycling (Wang et al., 2014). In their study, Lee et al. (2019) showed that plastic mulching exerted a significant negative effect on soil nitrogen and carbon storage, while other authors found no effect (Zhang et al., 2017). At a global scale, the increasing release of plastics and plastic-derived debris from human activities has reached untargeted organisms through the food chain and this is determining undesirable and unprecedented consequences to plant, animal and human health.

Growing awareness of environmental issues has led to the development of environmentally sustainable alternatives, i.e. the use of innovative polymer matrices used for biodegradable plastic films suitable for burial. Among the most innovative commercial film solutions for biodegradable and compostable mulching, made up of blends of natural polymer/synthetic polymers, which have recently appeared on the market, there is the Mater-Bi® film, a starch-based biopolymer developed and produced by Novamont (Italy) (Bastioli, 1998). However, researches from open field trials have not provided consistent results on the dynamics of the full biodegradation (that is biological conversion to CO₂, H₂O and biomass) of this starch-based blend in soil (Huang et al., 2023; Li et al., 2014). This suggests that several factors such as environmental conditions (temperature, moisture, UV radiation), polymer characteristics, soil type and ecology together with management practice can affect its persistence in the environment and the time it takes for Mater-Bi® mulch films to achieve a complete biodegradation in soil systems cannot be generalized (Hejna et al., 2022; Degli-Innocenti et al., 2022).

Most recently, ongoing research is seeking for producing mulching films from natural polymers of renewable origin which have shown the potential to fully biodegrade after burial in soil at the end of their life service (in a time ranging from 100 to 180 days from exposure to soil microorganisms (Hosokawa et al., 1990; Morgado et al., 2011)), with

obvious benefits for soil health and environmental sustainability (Ciaramitaro et al., 2024). These biopolymers are essentially based on natural polymers such as cellulose and chitosan which are widely represented in nature and easily and economically available on a global scale (Tursi, 2019). Cellulose is constituted by D-glucose, linked by β-1,4-glycosidic bonds and shows a linear structure with a high molecular weight. Cellulose is the most abundant organic compound and occurs in plant cell wall (Tursi, 2019). Chitosan is a linear polysaccharide composed of randomly distributed β-(1 → 4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It is widely present in plant (fungi) and animal (insects, shrimp) cells and shows biodegradability, antimicrobial effects, and good film formation (Lertsuthiwong et al., 2012). Needless to say, these fully biodegradable polymeric formulations can represent a carbon (C) source for soil microorganisms at the end of their life cycle, thus promoting soil biological activity and nutrient cycling, processes which involve the interplay between soil biota.

Among the different organisms found in the soil biota, earthworms play an essential role in maintaining soil structure and porosity by promoting air movement, water infiltration, nutrient translocation and plant growth. According to Bouché (1977), there are three types of earthworm ecotypes based on their burrowing activity, food habits, and niches: epigeic earthworms found in the topsoil (e.g., *Eisenia fetida*), endogeic earthworms living in the subsoil and creating horizontal burrows (e.g., *Aporrectodea caliginosa*), and anecic earthworms (e.g., *Lumbricus terrestris*) that move between soil surface and subsoil creating vertical burrows which extend through the profile (Bottinelli et al., 2020). The *L. terrestris* burrows are hotspots of nutrient translocation like nitrogen (N) due to the deposited cast or mucus. For example, N-enriched soils were found at a 0–8 mm distance from *L. terrestris* burrow wall (Andriuzzi et al., 2013). Moreover, the presence of earthworms increases the soil biota heterogeneity, bacterial activity, microbial biomass C and enzymatic activities associated with nutrient cycling and the aggregate stability (Liu et al., 2019). The physical association between soil particles and earthworms' cutaneous mucus creates the aggregates where organic carbon can be stored (Guhra et al., 2022; Vidal et al., 2023). Those aggregates can impact the soil's physical properties, nutrient cycling and plant growth, and the transformation of soil organic matter (SOM), thus improving soil fertility and productivity (Bhadauria and Saxena, 2010).

Furthermore, beside nutrients, earthworms can also translocate microplastics through the soil, even fragments larger in size than 5 mm (Adhikari et al., 2023), but with a different mechanism which involves external adhesion to their body (Rillig et al., 2017). Indeed, it has been hypothesized that with its ecological behaviour *L. terrestris* can shape the development of plastisphere and microplastome differently than in bulk soil microplastics (Sanchez-Hernandez, 2024).

Some studies have investigated the transport of low-density polyethylene (LDPE) plastic mulch and biodegradable plastic mulches and their effects on earthworm biomass and mortality. These studies showed that LDPE caused less harmful impacts on *L. terrestris* biomass and mortality than biodegradable plastics, and the plastic mulch was transported up to 10 cm depth in soil (Qi et al., 2018; Ju et al., 2023).

Moreover, several articles suggested negative effects of PLA on earthworms, such as a reduction of offspring in *Eisenia fetida* (Ding et al., 2021), a reduction of reproduction rate in *Eisenia andrei* (Barbir et al.,

2023), and mortality in *L. terrestris* (Huerta-Lwanga et al., 2021). Nevertheless, this unexpected response to PLA concentrations as low as to 5 % can be explained by the ecological behaviour of earthworms ecotype, as proposed by Sanchez-Hernandez et al. (2023). However, few studies have analysed the ecotoxicological effects (mainly avoidance, growth rate, and mortality) of plastic and biodegradable plastic mulch on *L. terrestris* earthworms (Adhikari et al., 2023; Huerta-Lwanga et al., 2021; Huerta Lwanga et al., 2016; Prendergast-Miller et al., 2019).

Hence, there is a need for a broader understanding of the effects of biodegradable mulching films on soil microorganisms and earthworms. Thus, the present paper aims to investigate short-term responses after the addition of two innovative polysaccharide-based biodegradable mulching films on (i) physical (ISS) and chemical (pH, EC, C_{org} , N_t , P-Olsen, water-soluble anions) soil variables, (ii) soil enzymatic activities, and (iii) enzymatic activities from earthworm's gut, muscle tissue and seminal vesicles by a microcosms approach hosting a soil-earthworm system.

2. Material and methods

2.1. Soil and earthworms

The soil used for filling the microcosms was surface (0–20 cm) collected from the an agricultural biologically managed arable soil located near Toledo (Spain; 39°52'08.5"N, 4°02'35.1"W), and shows the following characteristics: sand 44 %, silt 43 %, clay 13 %, loamy texture (according to USDA), total organic C 4.24 ± 0.24 %, total N 0.34 ± 0.02 %, pH_{H_2O} 7.49 ± 0.04 , $EC_{1:2}$ (25 °C) $243.6 \pm 48.2 \mu S cm^{-1}$, CEC $32.1 \pm 1.7 cmol kg^{-1}$, total $CaCO_3$ $122.1 \pm 3.8 g kg^{-1}$. Soon after sampling, the soil was sieved at <2 mm opening sieve and adjusted to 20 % (w/w) moisture content before starting the trial.

Adult earthworms (*Lumbricus terrestris* L.) were purchased by a local commercial supplier (Decathlon, Toledo, Spain) and left acclimatised in plastic containers (345 × 325 × 150 mm) filled with the collected soil at 15 °C in darkness for 5 days. During the acclimatisation period, earthworms were fed with fresh grass placed on the soil surface once per week. After the acclimatisation period, earthworms were checked, and no health issues, like skin scars, damage, or weight loss, were detected. The measurement of the weight before the experiment was used to detect any health issues.

2.1.1. Mulching films

Tested mulching polymers were two innovative experimental biopolymers produced by researchers from the University of Palermo (Italy) together with a commercially available biodegradable mulch film which was taken as a reference treatment.

The two innovative polysaccharide-based and biodegradable composite mulch films were produced in the laboratory by using the solvent casting technique as reported by Ciaramitaro et al. (2024). In brief, the bioplastic film (BP) was obtained by mixing at a 1:1 mass weight ratio two starting dispersions: a 1.5 % (w/v) chitosan/sodium alginate (CS/SA) dispersion and a 1.5 % (w/v) carboxymethyl cellulose (CMC) dispersion. The CS/SA dispersion was prepared by dissolving 1.5 g chitosan (CS), 1.5 g sodium alginate (AS) and 2.0 g glycerol (added as plasticizer agent) in 100 mL of 2 % (v/v) aqueous acetic acid solution. The CMC dispersion was prepared by dissolving 1.5 g carboxymethyl cellulose (CMC) in 100 mL distilled H_2O . After mixing, the CS/SA:CMC 1:1 dispersion was immediately spread on a flat and clean glass area, left at room temperature until a complete dryness was reached, and then gently peeled off the glass surface. The N- and P-enriched bioplastic film (BP⁺) was produced same as above, but adding 5.5 g of monoammonium phosphate ($NH_4H_2PO_4$, MAP) to the CMC dispersion (25 % salt percentage relative to the 22 g mass weight of the dry polymer). The two polysaccharide-based mulch films showed a thickness, although irregular, of about 50 μm , and the following elemental composition: C 28.46 ± 1.30 %, N 1.63 ± 0.10 % and P 0 % in BP, and C 24.85 ± 0.92 %, N

3.05 ± 0.09 % and P 3.49 ± 0.015 % in BP⁺.

The commercial mulching film used in the trial was an ECOPAC BIO BLACK film, (MB; colour black, thickness 15–18 μm), kindly provided by Guarniflon SpA PATI division (San Zenone degli Ezzelini, TV, I) and it was made of Mater-Bi® grade EF04P converted into a film with the addition of black masterbatch (FDM 324006 BK BIOE MASTERBATCH provided by AVIENT). Mater-Bi® EF04P is constituted by with biodegradable polyesters, starch, and a natural plasticizer. Polyesters are made with monomers that biodegrade in soil. The plasticizer is a bio-based, biodegradable polyol that biodegrades completely within 28 days at room temperature, under aqueous aerobic conditions (Degli-Innocenti et al., 2022). ECOPAC BIO BLACK complies with European (UNI EN 13432:2002 (UNI EN 13432, 2000), DIN EN 17033:2018 (DIN EN 17033, 2018)) and American (ASTM 6400:2012 (ASTM 6400, 2012)) standards for compostability and biodegradation, and has an elemental composition of C% and N% of 58.82 ± 0.21 % and 0.068 ± 0.008 %, respectively.

2.2. Experimental set-up

After a 5-day acclimatisation, earthworms were collected, rinsed with tap water, gently dried, and weighed. Then, they were placed into Petri dishes for 48 h to empty their guts and then transferred into soil microcosms (2 earthworms microcosm⁻¹) 4 days before the addition of the finely cut mulching films at a rate of 0.1 %, w/w, corresponding to 1.8 g microcosm⁻¹). This amount corresponds to the mean annual quantity of bioplastic material incorporated into the soil (100 mg kg⁻¹ soil), and constitutes a recommended dose without reaching strongly impacting loading rates (Mazzon et al., 2022; Paliaga et al., 2025). Soil microcosms consisted of high-density polyethylene round-shaped containers (10 × 26 cm; diameter 8.5 cm, height 11 cm), closed at the bottom and filled with 1800 g of freshly sampled soil brought to 20 % (w/w) moisture content before starting the trial. After the mulch film addition, soil microcosms were placed in a cold chamber (15 ± 1 °C) in darkness for 30 days. During the experiment, earthworms were fed 3 times with fresh grass placed on the soil surface. The experimental design included four treatments: control soil with no film addition (CTR), BP (soil added with the innovative CS/SA:CMC biopolymer), BP⁺ (soil added with the innovative CS/SA:CMC biopolymer enriched with MAP) and MB (soil added with Mater-Bi®). No polyethylene was included in the treatments since the aim of the research was to focus on soil-earthworm responses to addition of biodegradable polymers. The experimental design also included additional microcosms with the same treatments but without earthworms addition. Microcosms were randomly arranged in an aleatory complete block design, with three replications, to compare: 4 bioplastics treatments (CTR, BP, BP⁺, MB) and 2 earthworm effects (with and without earthworms). Soil moisture was kept constant at 20 % gravimetric moisture by adding distilled water periodically (every 2 or 3 days).

After 30 days, all microcosms were carefully opened, and earthworm casts on the soil surface (called "aged cast") were sampled. Earthworm soil gallery samples, that is top gallery (TG) and bottom gallery (BG), undisturbed off drilosphere soil (US), and soil without earthworms (S) were carefully sampled for the soil enzymatic analyses.

Physical (ISS) and chemical (pH, EC, C_{org} , N_t , P-Olsen, water soluble anions) soil variables were measured before (T0) and at the end of the incubation time (T30) in soil samples taken from microcosms with no earthworms and from a mixture of top and bottom drilosphere (Gal). After 30 days, earthworms were cleaned, weighed, and kept in Petri dishes for 72 h to empty their guts. Then, the casts were collected (called "fresh cast"). One of two earthworms for each microcosm was dissected, and seminal vesicle and gastrointestinal (the foregut and midgut, including chloragogenic tissue) and wall muscle (integument) tissues were collected and stored at –80 °C before analysis.

2.3. Soil physical and chemical variables

Soil physical and chemical properties were determined according to the standard methods recommended by the Soil Science Society of America (Klute, 1986; Sparks et al., 1996). Briefly, soil texture was determined by the pipette method; the stability of soil aggregates was determined by using the wet sieving apparatus (Eijkelkamp Agrisearch Equipment, The Netherlands) and expressed as the stability index of soil aggregates (ISS) on dry soil; soil acidity was potentiometrically detected in a 1/2.5 (w/v) soil-to-water mixture (pH_{H2O}); the electrical conductivity was measured in a 1/2 (w/v) soil-to-water ratio slurry (EC_{1:2} referred to 25 °C); the cation exchange capacity (CEC) was measured by using 0.1 M BaCl₂ buffered to pH 8.2 with triethanolamine (2.25 %, v/v); the total carbonate content (CaCO₃ tot) was determined by the gas-volumetric method using the Dietrich-Frühling apparatus; total organic carbon (C_{org}) and total nitrogen (N_t) were determined on a finely (< 0.5 mm) ground aliquot of soil by using the elemental analyser LECO CN628 (LECO Corporation, St. Joseph, MI, USA). Available phosphorus (P-Olsen) was extracted in NaHCO₃ 0.5 M buffered to pH 8.5 and colourimetrically determined according to the ascorbic acid method. Readily available nutrients (nitrate, nitrite, chloride, sulphate) were water-extracted (substrate: water, 1/10, w/v) under horizontal shaking at room temperature for 2 h, centrifuged at 5000g for 5 min and then filtered through Whatman™ n. 42 filter paper. Filtrates were passed through a 0.22 µm Millipore™ filter and stored at -20 °C before chromatographic analysis. Inorganic anions were determined by ion chromatography using a Dionex™ ICS 1100 (Dionex Corp., Sunnyvale, CA, USA) equipped with an isocratic pump, a conductivity detector, and an auto-sampler (Dionex™ AS-DV, ThermoFisher Scientific, Waltham, MA, USA). Anions were determined using a self-generating AERS-500 suppressor (4 mm; ThermoFisher Scientific), an analytical column (4 mm ID × 250 mm) Dionex™ Ion-Pac™ AS22 RFIC™ (Dionex Corp.), and an eluent solution (3.5 mM sodium-carbonate/1.0 mM sodium-bicarbonate) at a flow rate of 1.2 mL min⁻¹. Instrumental control and chromatographic data processing were done by the Thermo Scientific Dionex Chromeleon Chromatography Data System 7.2 (ThermoFisher Scientific).

2.4. Soil sample preparation and earthworm tissue homogenisation

Before the soil enzymatic analyses, the soil extracts were prepared with distilled water in a 1/25 ratio (w/v) for every soil sample (soil without earthworm, S; undisturbed soil, US; top gallery, TG, and bottom gallery, BG) and for the aged and fresh cast by orbital shaking for 30 min.

The tissue homogenates (gastrointestinal, wall muscle and seminal vesicle tissues) were prepared with a cold buffer of 20 mM Tris-HCl (pH 7) containing 1 mM EDTA in a 1/10 (w/v) ratio. Then, the homogenates were centrifuged at 9000g at 4 °C for 20 min. The supernatant was transferred into a new Eppendorf tube and kept at -80 °C until the analyses were performed. The earthworm analyses were expressed and based upon the total protein concentration determined by the Bradford method using a calibration curve with bovine serum albumin (1.0–0.1 mg mL⁻¹). Enzymatic analyses of carboxylesterase, alkaline phosphatase, and β-glucosidase activity were similarly performed for soil extracts and earthworm tissue homogenates.

2.5. Soil enzymatic analyses

2.5.1. Carbon-acquired enzymes

2.5.1.1. Carboxylesterase (EC 3.1.1.1) activity. An aliquot of 100 µL soil extract was mixed with 20 µL of 50 mM 1-naphthyl butyrate and 380 µL 0.1 M Tris-HCl (pH 8). Then, 75 µL of a mixture of 2.5 % SDS, 0.1 % Fast-Red and 2.5 % Triton X-100 (w/v) was added and incubated in darkness

for 30 min and 15 min for aged and fresh cast. After the incubation, the absorbance was measured at 490 nm. The carboxylesterase activity was expressed as µmol 1-naphthol (g dry soil)⁻¹ h⁻¹ using the calibration curve with 1-naphthol.

2.5.1.2. β-Glucosidase (EC 3.2.1.21) activity. An aliquot of 200 µL soil extracts was mixed with 200 µL distilled water and 100 µL 50 mM 4-nitrophenyl-β-D-glucanopyranoside and incubated for 1 h at room temperature (~20 °C). Then, 75 µL 0.5 M NaOH was added for colour development and measured at 405 nm. The β-glucosidase activity was expressed as µmol 4-nitrophenol (g dry soil)⁻¹ h⁻¹ using the calibration curve with 4-nitrophenol (10–0.78 mM).

2.5.2. Nitrogen-acquired enzymes

2.5.2.1. Protease (EC 3.4.21.92) activity. Protease activity was measured according to Sanchez-Hernandez et al., 2017 with slight modifications. The reaction consisted of a mixture of 500 µL soil extracts and 500 µL 2 % casein (w/v) dissolved in 50 mM Tris-HCl buffer. After 2 h incubation in a water bath at 50 °C, the reaction was stopped with 0.92 M trichloroacetic acid. The samples were centrifuged at 10,000g for 10 min, and 80 µL of the supernatants were transferred into a 96-well microplate. Then, 120 µL of working solution (50:1:1, v/v, 5 % Na₂CO₃ with 1 mM NaOH; 0.5 % CuSO₄; 1 % Na-K-tartrate; pH 8) and 80 µL Folin-Ciocalteu's phenol reagent were added. The microplate was kept in darkness for 90 min until stable colour development, and then the absorbance was measured at 690 nm. Protease activity was expressed at µmol tyrosine (g dry soil)⁻¹ h⁻¹ using a calibration curve made with tyrosine.

2.5.2.2. Urease activity (EC 3.5.1.5). Urease activity was measured according to Trasar-Cepeda et al. (1999) with minor modifications. An aliquot of 200 µL soil extract and 200 µL 80 mM urea (ratio 1/1) were incubated for 1 h (except fresh cast that 10 µL soil extract and 390 µL 80 mM urea was incubated for 5 min) in an orbital shaking at room temperature (~20 °C). The reaction was finished by adding 1 mL of the cold stop solution (1 M KCl mixed with 10 mM HCl; ratio 2:3). Then, the Eppendorf tubes were shaken at room temperature (~20 °C) for 30 min and, later, centrifuged at 10,000g for 5 min. An amount of 150 µL of supernatant was transferred into a new Eppendorf tube with 75 µL solution A (mixture of 17 % Na-salicylate and 0.12 % Na-nitroprusside dissolved in NaOH, ratio 1/1, v/v) and 30 µL solution B (0.1 % Na-dichloroisocyanurate). Then, they were left for 30 min in darkness until stable colour development and then the absorbance was measured at 620 nm. Urease activity was expressed as µg NH₄⁺-N (g dry soil)⁻¹ h⁻¹ using the calibration curve made with NH₄Cl (20–0.5 µg mL⁻¹).

2.5.2.3. N-acetyl-β-D-glucosaminidase (glucosaminidase, EC 3.2.1.30; EC 3.2.1.52) activity. The enzyme activity was assessed according to Popova et al. 2010 (Popova and Deng, 2010a) with slight modifications. An aliquot of 200 µL soil extracts was mixed with 200 µL sodium acetate buffer (pH 5.5) and 100 µL of 10 mM p-nitrophenyl-N-β-D-glucosaminide and incubated for 24 h except for aged and fresh cast, which were incubated for 2 h. After the incubation, an aliquot of 150 µL was transferred into a 96-well microplate, and 75 µL 0.5 M NaOH added to stop the reaction, which was measured at 490 nm. N-acetyl-β-D-glucosaminidase activity was expressed as nmol (g dry soil)⁻¹ h⁻¹ using a calibration curve with 4-nitrophenol (10–0.78 mM).

2.5.3. Phosphorous-acquired enzymes

2.5.3.1. Alkaline (EC 3.1.3.1) phosphatase activity. An aliquot of 200 µL soil extracts was mixed with 200 µL distilled water and 100 µL 50 mM 4-nitrophenyl-phosphatase and incubated at room temperature (~20 °C) for 1 h (except for aged and fresh cast, which were incubated for 30

min). Then, 75 μL 0.5 M NaOH was added until stable colour development and the absorbance measured at 405 nm. The alkaline phosphatase activity was expressed as μmol 4-nitrophenol (g dry soil) $^{-1}$ h $^{-1}$ using the calibration curve with 4-nitrophenol (10–0.78 mM).

2.5.4. Soil microbial activity

2.5.4.1. Fluorescein diacetate hydrolysis activity (FDH). Fluorescein diacetate hydrolysis (FDH) activity was based on Margesin and Schinner (2005) and Shawy and Burns (2005) with slight modifications (Margesin and Schinner, 2005; Shawy and Burns, 2005). An aliquot of 275 μL soil extracts was mixed with 275 μL 60 mM Na₂HPO₄ buffer (pH 7.6) and 25 μL fluorescein solution (1 mg mL $^{-1}$) and incubated for 45 and 25 min (undisturbed soil and soil with no earthworms, and top and bottom gallery, respectively). After the incubation, 200 μL acetate was added and centrifuged at 10,000g for 5 min. Then, 250 μL supernatant was transferred to the 96-well microplate, and the absorbance was measured at 490 nm. Fluorescein diacetate hydrolysis activity was expressed as μg fluorescein (g dry soil) $^{-1}$ h $^{-1}$ using a calibration curve made with fluorescein standard (100–3.1 μg mL $^{-1}$).

2.5.4.2. Dehydrogenase (EC 1.1) activity. Dehydrogenase activity was measured according to Sanchez-Hernandez et al., 2017. Soil samples (1 g) and aged and fresh cast (0.5 g) were mixed with the substrate iodinitrotetrazolium chloride (INT; 1 mL and 0.5 mL for aged and fresh cast) and with Tris buffer (2.5 and 1.25 mL, respectively). The incubation was carried out at 40 °C for 1 h (for soil samples) or for 30 min (for aged and fresh cast). Then, the reaction was stopped with 5 mL (for soil samples) or 2.5 mL (for aged and fresh cast) solution (1:1, v/v, ethanol: N,N-dimethylformamide). Slurries were centrifuged at 4500g for 10 min, and the supernatant absorbance was measured at 450 nm. The activity was expressed as μmol iodinitrotetrazolium formazan (ITNF) (g dry soil) $^{-1}$ h $^{-1}$ using a calibration curve with ITNF.

2.5.4.3. Catalase (EC 1.11.1.6) activity. The catalase activity was determined according to Sanchez-Hernandez et al., 2017 with few modifications. 400 μL sample extracts were mixed with 1.2 mL H₂O₂ and incubated for 1 h or 20 min for aged and fresh cast, respectively. The reaction was stopped by adding 200 μL H₂SO₄. Slurries were centrifuged at 4500g at 10 °C for 10 min. An aliquot of 10 μL supernatant was mixed with 290 μL solution containing 110 mM Na-phosphate buffer (pH 7.4), 30 mM 4-aminoantipyrine, 5.3 % phenol and 1.2 U mL $^{-1}$ of peroxidase type-I from horseradish (Sigma-Aldrich). The absorbance was measured at 490 nm, and catalase activity was expressed as μmol H₂O₂ (g dry soil) $^{-1}$ h $^{-1}$ using a calibration curve made with H₂O₂.

2.6. Enzymatic indexes of soil quality

Four numerical indexes were used to assess the effects of BP, BP⁺, and MB on soil enzymatic activities: the geometric mean index (GMean (Hinojosa et al., 2004)), the data was normalised prior to calculate the index, the weighed mean index (WMean (Lessard et al., 2014)) was calculated with normalised data, the treated-soil quality index (T-SQI (Mijangos et al., 2010)), and the integrated biological response index (IBRv2), a modified version by Sanchez et al. 2012 (Sanchez et al., 2013). The indices were calculated as reported by Sanchez-Hernandez et al., 2017.

2.7. Earthworm digestive enzymes and oxidative stress biomarkers

2.7.1. Earthworms digestive enzymes

The acid, alkaline, β -glucosidase and lipase activity were exclusively analysed in gastrointestinal tissues as those enzymes are only found in this type of tissues. The carboxylesterase was selected due to its role in lipid digestion (Ross and Edelmann, 2012; Ríos et al., 2014). The

earthworm analyses were performed similarly to soil extracts with slight changes.

2.7.1.1. Acid (EC 3.1.3.2) phosphatase activity. The acid phosphatase activity was analysed according to Popova and Deng (2010) (Popova and Deng, 2010b) with slight modification. 40 μL of 1/25 dilution (v/v) of homogenates was mixed with 90 μL Tris HCl (pH 6.5) and 20 μL of 50 mM 4-nitrophenyl-phosphatase and incubated at room temperature (~20 °C) for 20 min. Then, 50 μL 0.1 M NaOH was added before the measurement at 405 nm. The acid phosphatase activity was expressed as nmol 4-nitrophenol (mg protein) $^{-1}$ h $^{-1}$ using the calibration curve 4-nitrophenol (10–0.78 mM).

2.7.1.2. Alkaline (EC 3.1.3.2) phosphatase activity. 40 μL of 1/25 dilution (v/v) of homogenates was mixed with 90 μL Tris HCl (pH 9.5) and 20 μL of 50 mM 4-nitrophenyl-phosphatase and incubated for 10 min. Then, 50 μL 0.1 M NaOH was added before the measurement at 405 nm. The alkaline phosphatase activity was expressed as nmol 4-nitrophenol (mg protein) $^{-1}$ h $^{-1}$ using the calibration curve 4-nitrophenol (10–0.78 mM).

2.7.1.3. β -glucosidase (EC 3.2.1.21) activity. The earthworm analyses were performed similarly to β -glucosidase activity in section 2.5.1 Carbon-acquired enzymes with slight changes. An aliquot of 40 μL of 1/25 dilution (v/v) of homogenates was mixed with 90 μL Tris HCl (pH 7) and 20 μL of 50 mM 4-nitrophenyl- β -D-glucanopyranoside and incubated for 40 min. Then, 50 μL 0.1 M NaOH was added before the measurement at 405 nm. The β -glucosidase activity was expressed as nmol 4-nitrophenol (mg protein) $^{-1}$ h $^{-1}$ using the calibration curve 4-nitrophenol (10–0.78 mM).

2.7.1.4. Lipase (EC 3.1.1.3) activity. Lipase activity was performed according to Gupta et al. (2002) with minor modifications (Gupta et al., 2002). 1.6 mM final concentration of the substrate 4-nitrophenyl-palmitate was mixed with 200 μL of 50 mM Tris-HCl (pH 8.00) containing 0.4 % (w/v) Triton X and 0.1 % (w/v) arabic gum. Then 50 μL of gastrointestinal tissues were added and measured for 20 min at room temperature (~20 °C) at 405 nm. The lipase activity was expressed as nmol 4-nitrophenol (mg protein) $^{-1}$ min $^{-1}$ using the calibration curve 4-nitrophenol (10–0.78 mM).

2.7.1.5. Carboxylesterase (EC 3.1.1.1) activity. An aliquot of 4 μL homogenate tissue was mixed with 10 μL of 50 mM 1-naphthyl acetate and 486 μL 0.1 M Tris-HCl (pH 7) and incubated at room temperature (~20 °C) for 10 min. Then, 125 μL of 2.5 % SDS was added and smoothly shaken. Then, another 125 μL of a mixture of 0.1 % Fast-Red and 2.5 % Triton X-100 (w/v) was added and incubated in darkness for 15 min. After the incubation, the absorbance was measured at 490 nm. The carboxylesterase activity was expressed as μmol 1-naphthol (mg protein) $^{-1}$ min $^{-1}$ using the calibration curve with 1-naphthol.

2.7.2. Oxidative stress biomarker

The concentrations of reduced (GSH) and oxidised (GSSG) glutathione, as well as malondialdehyde formation (lipid peroxidation) were chosen as indicators of cellular oxidative stress. An amount of 100 μL homogenate was mixed with 100 μL of 0.6 % cold sulfosalicylic acid to avoid glutathione oxidation. Then, the extract was centrifuged at 10,000g at 4 °C for 5 min. The supernatant was transferred into a clean Eppendorf tube to measure reduced and oxidised glutathione (GSH and GSSG, respectively), according to Rahman et al. (2006). Lipid peroxidation was determined according to Romero-Haro and Alonso-Alvarez (2014) with slight changes. An amount of 50 μL of gastrointestinal tissues (or 100 μL and 150 μL of wall muscle tissue and seminal vesicle, respectively) was added with 450 μL of thiobarbituric acid and kept at 90 °C for 30 min. Then, after cooling for 5 min, 500 μL of 1-butanol was

added, vortexed and centrifuged at 10,000g for 5 min. After transferring the supernatant into the new Eppendorf tube, 250 μL were pipetted into the 96-well microplate and measured at 535 nm. The concentration of malondialdehyde formed was expressed as $\text{nmol mg protein}^{-1}$ using the calibration curve with 1,1,3,3-tetraethoxypropane ($5\text{--}0.039 \mu\text{M}$).

2.8. Statistics

Soil variables were first checked for deviations from normality (Kolmogorov-Smirnov test). Soil-related data were statistically processed by a three-way analysis of variance (mulch type \times earthworm addition \times time). Tukey's post hoc test comparison (at $P < 0.05$) was applied for multiple pairwise comparisons of means. Statistical analyses were run using the Systat 13.0 software (SYSTAT Software Inc., Erkrath, D). Graphs were drawn by using SigmaPlot 10.0 software (SYSTAT Software Inc.). Soil enzymatic and earthworm analyses were checked for normality (Shapiro-Wilk test) and residuals' normality (Kolmogorov-Smirnov and Shapiro-Wilk test). One-way ANOVA and Dunnett's comparison test were applied for the parametric analyses. Kruskal-Wallis was applied for the non-parametric analyses, followed by pairwise Wilcoxon test. Statistical analyses and graphs were run using GraphPad Prism version 9.5.0 (GraphPad Software, LLC.).

3. Results

3.1. Soil physical and chemical variables

The ISS, an indicator of soil physical fertility, was significantly influenced by the occurrence of the earthworms that decreased the initial values to the lowest one of 52.63 % in the drilosphere soil (Gal). No significant effect of mulch addition on ISS was evidenced by the 2-way ANOVA, independently of the mulching film type. Moreover, no statistically significant differences were found among treatments at each sampling time (Fig. 1).

On the contrary, soil pH was significantly affected by the mulching

film addition but not by the earthworm occurrence (Fig. 2). Even though a slight degree of variability was appreciated in soil pH at the initial stage (ranging from 7.52 to 7.44), the treatment with the different biodegradable mulching films influenced the pH, especially in the drilosphere (Gal) where a statistically lower value (7.34) was found in the BP+ treatment.

The EC, a soil chemical variable expressing the total content of soluble salts, was statistically influenced by mulching polymers, earthworms' presence and their interaction (Fig. 2). Moreover, significant increases were appreciated in the drilosphere (Gal) at the end of the observation period, with the highest EC value observed in the CTR soil ($651 \mu\text{S cm}^{-1}$) and the lowest one in the treatment BP+ ($445 \mu\text{S cm}^{-1}$; Fig. 2). The treatment with MB provided a reading of $613 \mu\text{S cm}^{-1}$, whereas that with BP presented a value of $518 \mu\text{S cm}^{-1}$.

C_{org} was influenced by the presence of different mulching films with a statistically significant difference among final readings in soil microcosms with no earthworms (Fig. 3). Precisely, at the end of the observation period, C_{org} ranged from 38.81 g kg^{-1} (BP) to 44.66 g kg^{-1} (MB). On the contrary, N_{total} (Fig. 3) showed significant differences in soil without earthworms, with a maximum value of 3.54 g kg^{-1} in the treatment with the commercial biodegradable mulching film (MB) and a minimum value after the addition of BP and BP+ and in CTR.

Soil incubation significantly influenced the P-Olsen which was found to increase from average $48.92 \mu\text{g P g}^{-1}$ to final $86.13 \mu\text{g P g}^{-1}$ considering all microcosms (Fig. 4). The highest value was observed in CTR (Fig. 4).

Water extractable ions (Cl^- , NO_2^- -N, NO_3^- -N, SO_4^{2-} -S) were markedly influenced by the mulching films, earthworms' addition, and in some cases by their interaction. In brief, an increase in the chloride content was found after 30-day incubation either in the microcosm soil without earthworms where values ranged from 23 (CTR) to $39 \mu\text{g Cl}^- \text{ g}^{-1}$ (MB) or in the drilosphere soil (Gal), where values ranging from 68 (BP) to $78 \mu\text{g Cl}^- \text{ g}^{-1}$ (MB) were observed (Fig. 5). NO_2^- -N markedly increased in the drilosphere soil (Gal) after 30 days of exposure when the highest values were detected in the control and the BP treatments: 2.8 and $3.0 \mu\text{g}$

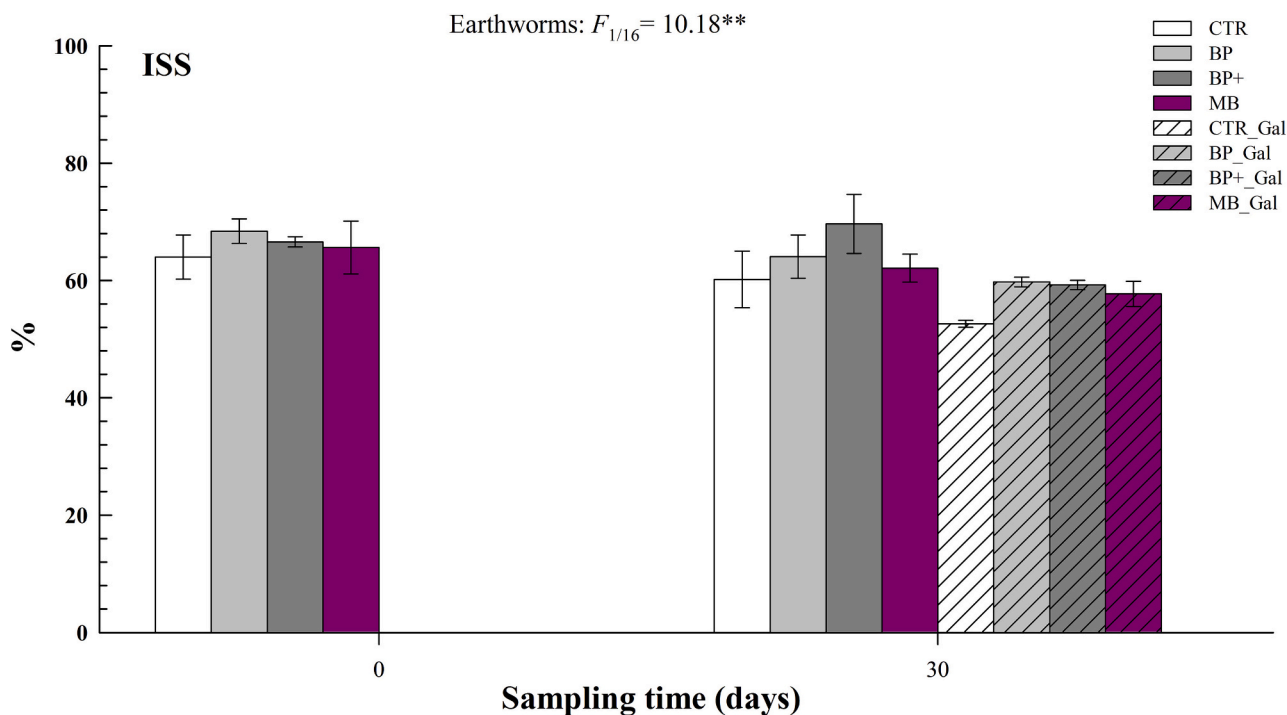


Fig. 1. ISS. Variation of the index of the soil structural stability (ISS) before and after 30 days of incubation with CTR and different mulching biopolymers (BP, BP+ and MB as in M&M) in soil (no earthworms) and in the drilosphere (with *Lumbricus terrestris* L.). Within each sampling period and soil/soil treatment, different letters indicate significant differences among soil treatments (Tukey's HSD test at $P < 0.05$).

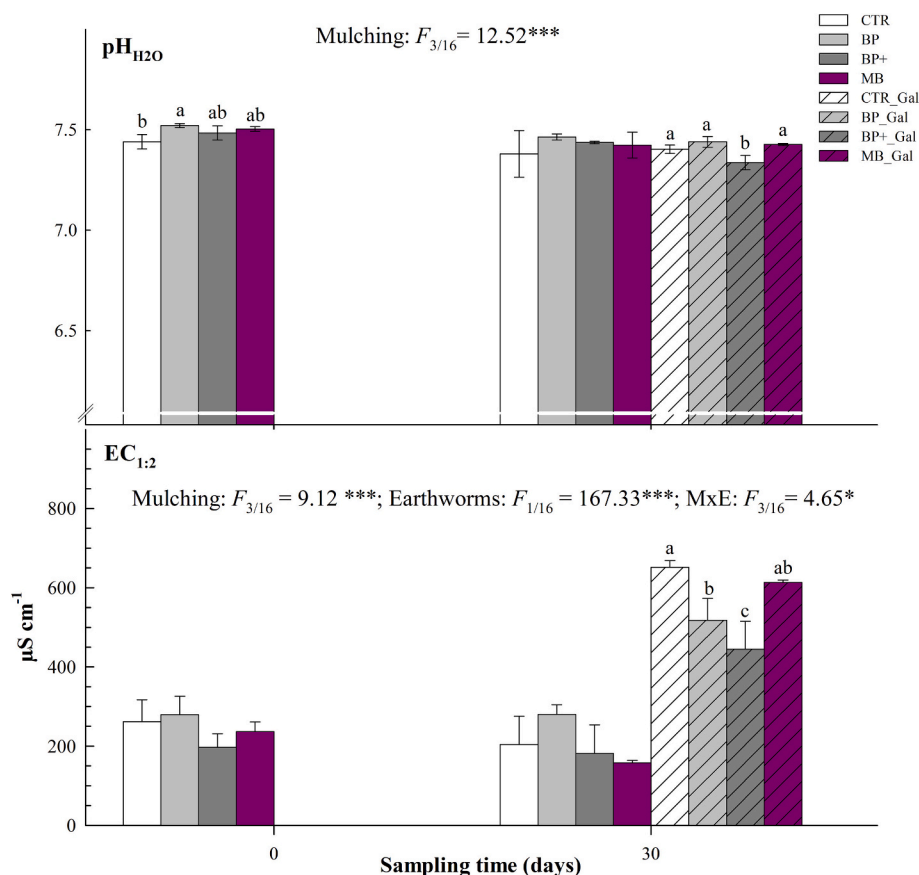


Fig. 2. pH and EC. Variation of soil pH and electrical conductivity (EC) before and after 30 days of incubation with CTR and different mulching biopolymers (BP, BP+ and MB, as in M&M) in soil (no earthworms) and in the drilosphere (with *Lumbricus terrestris* L.). Within each sampling period and soil/soil treatment, different letters indicate significant differences among soil treatments (Tukey's HSD test at $P < 0.05$).

$\text{NO}_2^- \text{N g}^{-1}$, respectively (Fig. 5). The lowest increase was found in the MB treatment ($1.8 \mu\text{g g}^{-1}$, corresponding to a 2.8-fold increase compared to the same treatment with no earthworms). Treatments at the initial stage showed a slight significant difference in the $\text{NO}_3^- \text{N}$ content, with values varying from $8.05 \mu\text{g g}^{-1}$ to $8.90 \mu\text{g g}^{-1}$ (Fig. 5). However, after 30 days of incubation, these values strongly increased in all treatments, especially in the earthworm-treated soil. Notably, in CTR and BP+ treatments a 2.1-fold and 3.8 increase was found, respectively, in the bulk soil compared to initial values; whereas in the drilosphere (Gal) a higher increase was observed: 2.7-fold and 4.3-fold in CTR and BP+ treatments, respectively, compared to initial values (Fig. 5). Same as before, a strong increase in the $\text{SO}_4^{2-} \text{S}$ content was observed after the 30 days, especially in the earthworm-treated soils where it ranged from $31.28 \mu\text{g g}^{-1}$ (BP; Fig. 5) to $26.37 \mu\text{g g}^{-1}$ (MB; Fig. 5).

3.2. Soil moisture and earthworms body mass

Soil moisture was calculated before and after the experiment (30 days). The moisture decreased significantly in the soil without earthworms (Fig. S1A) within CTR ($18.69 \pm 0.4 \%$) and in each mulching film ($17.93 \pm 0.6 \%$ in BP, $18.02 \pm 1.08 \%$ in BP+ and $17.93 \pm 0.5 \%$ in MB) after 30 days of incubation compared to the start of the experiment (20.6%). However, the interaction of the earthworms in the soil did not decrease the soil moisture after 30 days (Fig. S1B).

The analyses of earthworm body mass were performed to observe whether the incubation with the different mulching films would negatively affect their body mass. There was a decrease in body mass in earthworms incubated with BP ($5.5 \pm 0.53 \text{ g}$) and with MB ($6.3 \pm 0.25 \text{ g}$) compared to CTR ($7.3 \pm 0.8 \text{ g}$; Fig. 6). Notably, the BP+ mulching film (7.2 ± 0.48) did not have a negative effect on the earthworm's body

mass (Fig. 6). There was no decrease neither mortality in control earthworms before and after the experiment (Fig. 6).

3.3. Soil enzymatic analyses

3.3.1. Carbon-acquired enzymes

The carboxylesterase activity was inhibited and varied depending on the type of soil where the enzymes were analysed. The most significant inhibition for the US was in the mulching film BP (−6%). However, in the soils where the earthworms were in contact (TG, BG, and aged cast), the inhibition was observed at the MB mulching film (−10%, −12% and −10%, respectively; Table S1). The exception was in BG, where the BP mulching film had the same inhibition as MB (−12%, Table S1). Interestingly, in the fresh cast collected from earthworms after 30 days, the inhibition activity of carboxylesterase changed. The highest inhibition was in BP (−12%), followed by BP+ (−3%), and MB had the lowest % of inhibition (−2%; Table S1).

There were statistical significances for the β -glucosidase activity, where we could observe an enhancement of the activity in BP+ (33%) in S and TG soil, and MB (62%) in the aged cast (Table S1). Like carboxylesterase activity, the activity inhibition was observed in BP+, BP, and then MB (−22%, −18%, and −2%, respectively) in fresh cast. We observed that MB had the lowest inhibition of the activity in the fresh cast.

3.3.2. Nitrogen-acquired enzymes

No statistical differences were observed in protease and urease activity (Table S2). Protease activity was inhibited in BP (−0.13%) in the US soil and in BP and MB in TG soil (−6% and −8%, respectively) compared to CTR. The protease activity was strongly inhibited in fresh

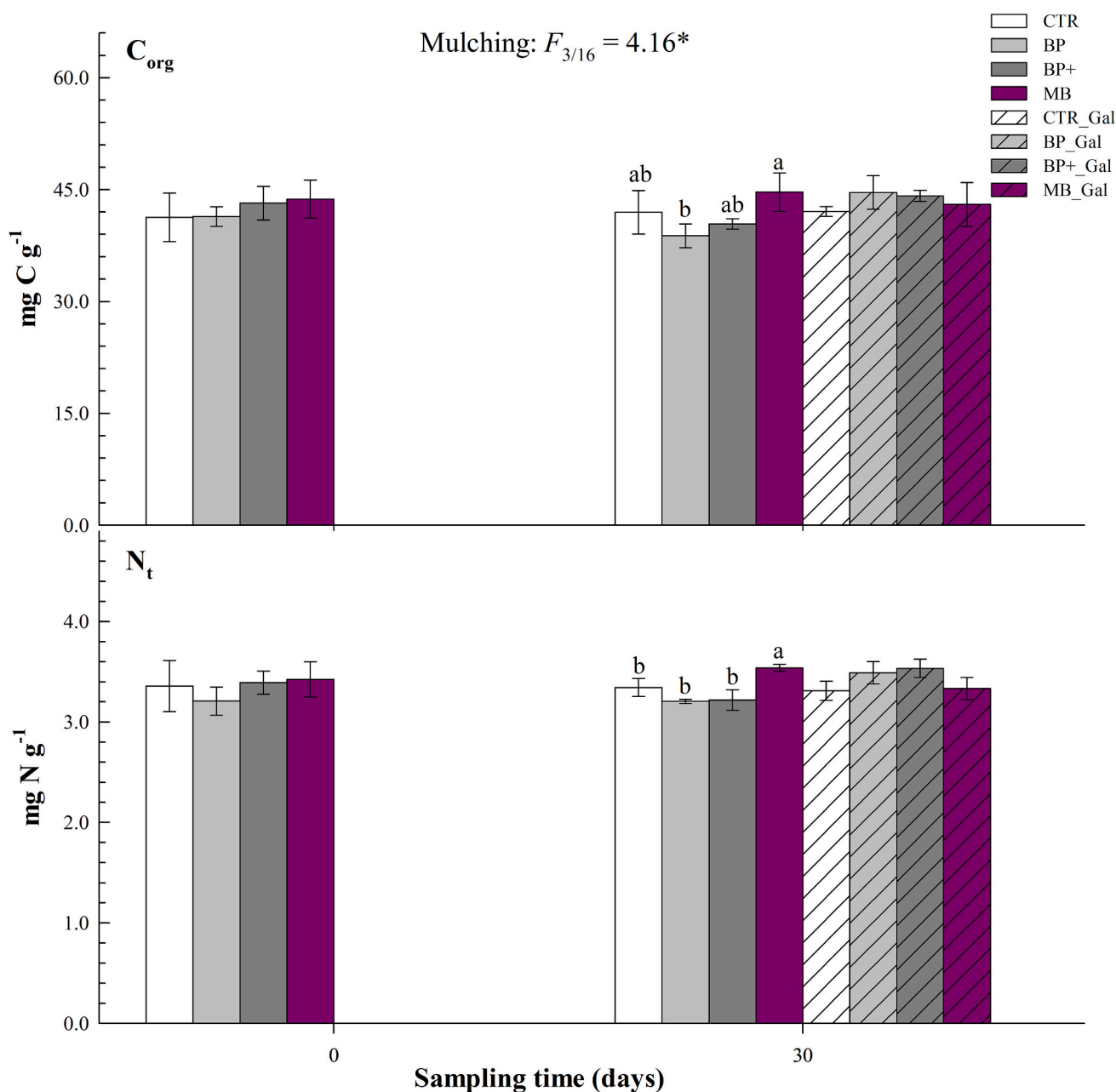


Fig. 3. C_{org} and N_t . Variation of the organic carbon content (C_{org}) and total nitrogen (N_t) before and after 30 days of incubation with CTR and different mulching films (BP, BP+ and MB, as in M&M) in soil (no earthworms) and in the drilosphere (with *Lumbricus terrestris* L.). Within each sampling period and soil/soil treatment, different letters indicate significant differences among soil treatments (Tukey's HSD test at $P < 0.05$).

cast for BP (−13 %) and BP+ (−15 %; Table S2). The urease activity was inhibited in MB in the US (−17 %) and TG (−20 %; Table S2). There was an increase of urease activity in BP and BP+ (20 % in each one) and MB (14 %; Table S2) in aged cast.

3.3.3. Phosphorous-acquired enzymes

There were statistical differences in alkaline phosphatase activity, which was strongly inhibited in MB (−67 %) and increased activity in BP (22 %) and BP+ (19 %; Table 3) in US. There was strong inhibition for all mulching films in BG (−24 % in BP, −15 % in BP+ and −6 % in MB; Table S3). There was a statistical difference in glucosaminidase activity where there was a great increase in BP (56 %; Table S3) compared to CTR. Same as seen for alkaline phosphatase, glucosaminidase activity was inhibited for all mulching films (−36 % in BP, −33 % in BP+ and −3 % in MB; Table S3) in fresh cast.

3.3.4. Soil microbial activity

There were no statistical differences in FDH activity. There was inhibition of the activity in MB in S (−8 %), US (−6 %), TG (−2 %), BG (−6 %) and aged cast (−6 %; Table S4). However, a stronger activity increase was observed in BP+ (66 %) in BG soil and BP+ (56 %) in fresh cast. There were statistical differences in dehydrogenase activity in BP+ (55 %) and MB (116 %, Table S4) in US soil. The highest inhibition was observed in MB (−50 %) in the fresh cast (Table S4), followed by MB in S (−20 %), MB in the aged cast (−7 %) and MB in BG (−3 %; Table S4). In the catalase activity, there was no statistical difference. The inhibition of the activity was observed in BP+ (−11 %) in S, in MB (−8 %) in TG, in BP (−1 %), in BP+ (−5 %) and in MB (−5 %) in BG soil (Table S4). Moreover, there was inhibition in BP+ (−18 %) in the aged cast and MB (−13 %) in the fresh cast (Table S4).

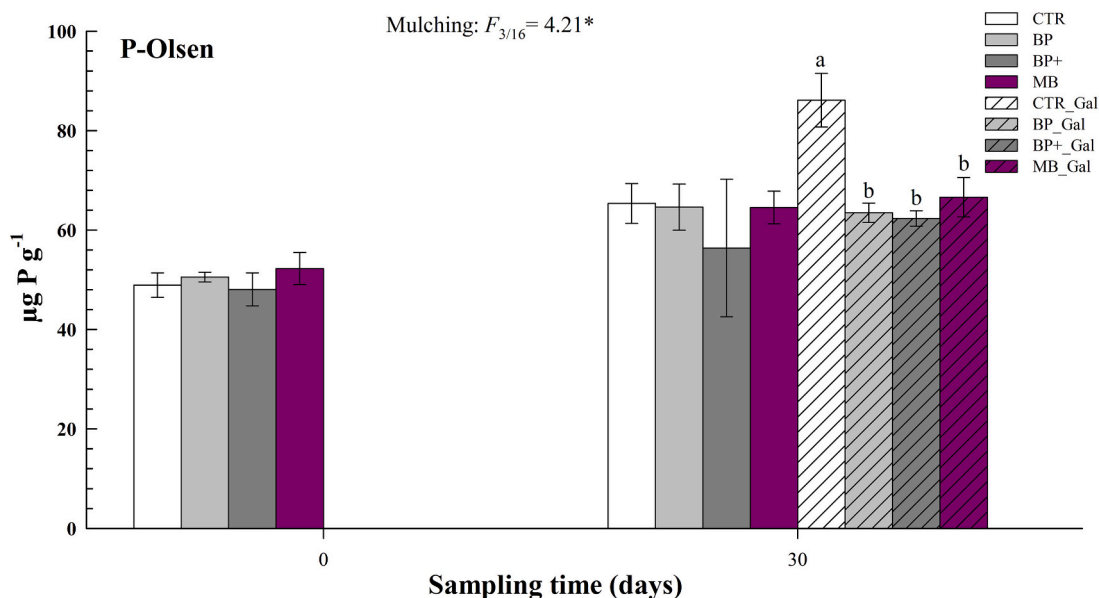


Fig. 4. P-Olsen. Variation of available phosphorus (P-Olsen) in soil before and after 30 days of incubation with CTR and different mulching films (BP, BP+ and MB, as in M&M) in soil (no earthworms) and in the drilosphere (with *Lumbricus terrestris* L.). Within each sampling period and soil/soil treatment, different letters indicate significant differences among soil treatments (Tukey's HSD test at $P < 0.05$).

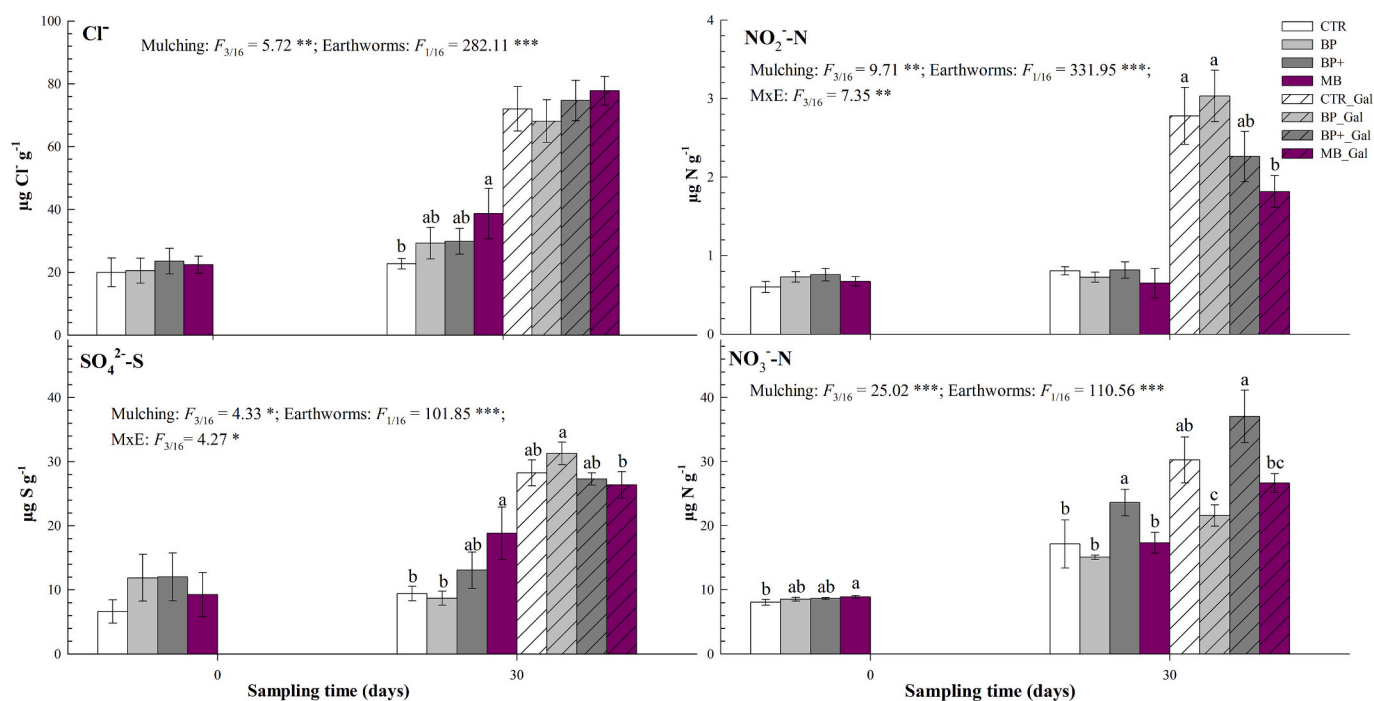


Fig. 5. Water extractable ions. Variation of water extractable ions (Cl^- , NO_2^- -N, NO_3^- -N, SO_4^{2-} -S) before and after 30 days of incubation with CTR and different mulching biopolymers (BP, BP+ and MB, as in M&M) in soil (no earthworms) and in the drilosphere (with *Lumbricus terrestris* L.). Within each sampling period and soil/soil treatment, different letters indicate significant differences among soil treatments (Tukey's HSD test at $P < 0.05$).

3.4. Enzymatic indexes of soil quality

The total response of the soil enzymatic analyses was observed by four enzymatic indexes. The GMean (Fig. 7A) showed the trend of the different types of soils (S, US, TG, BG, aged cast and fresh cast). Remarkably, BP (22.17 ± 1.06) was statistically significant compared to CTR (19.09 ± 0.8) in the type of soil S (Fig. 7A). The WMean calculation included the principal component analyses (PCA) on nine enzyme datasets (Fig. S2). Moreover, the Kaiser-Meyer-Olkin ($P > 0.8$) and

Barlett's test of sphericity ($P < 0.001$) were to ensure that the samples were adequate for the analyses and that the correlation was significant enough for PCA, respectively.

Similarly to GMean, this index did not show any statistical significance or variation between the different soil types and the different mulching films (BP, BP+ and MB) compared to CTR (Fig. 7B). T-SQI showed that the US had strong values compared to other soil types, with BP+ being the highest one (Fig. 7C); the values obtained from the IBRV2 index could be seen in the differences between the soil types and the

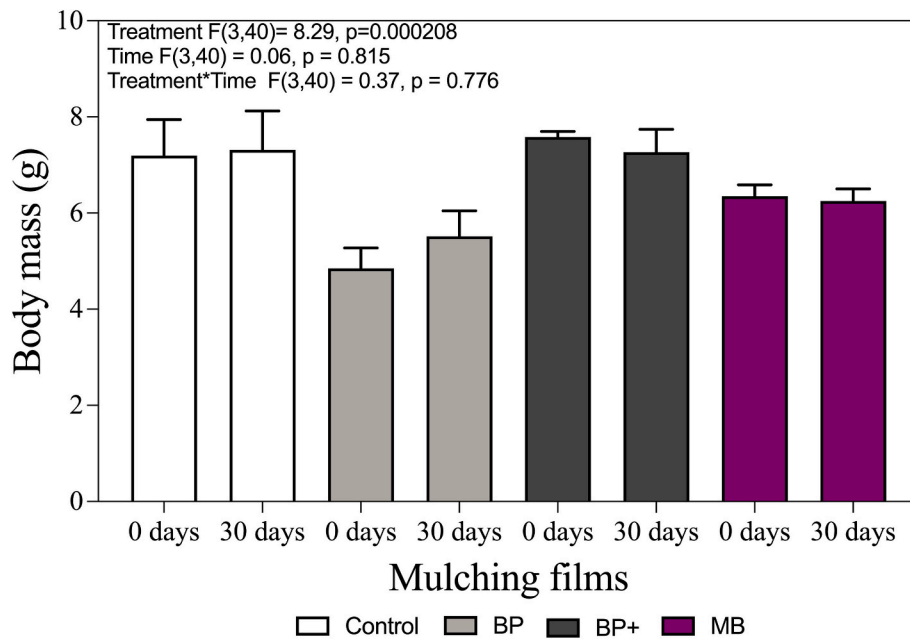


Fig. 6. Earthworm body mass. Earthworm body mass (g) variation from the start of the experiment and after 30 days (30 d) within the CTR and the different mulching films (BP, BP+ and MB). * indicate $P < 0.05$ according to Kruskal-Wallis followed by pairwise Wilcoxon test.

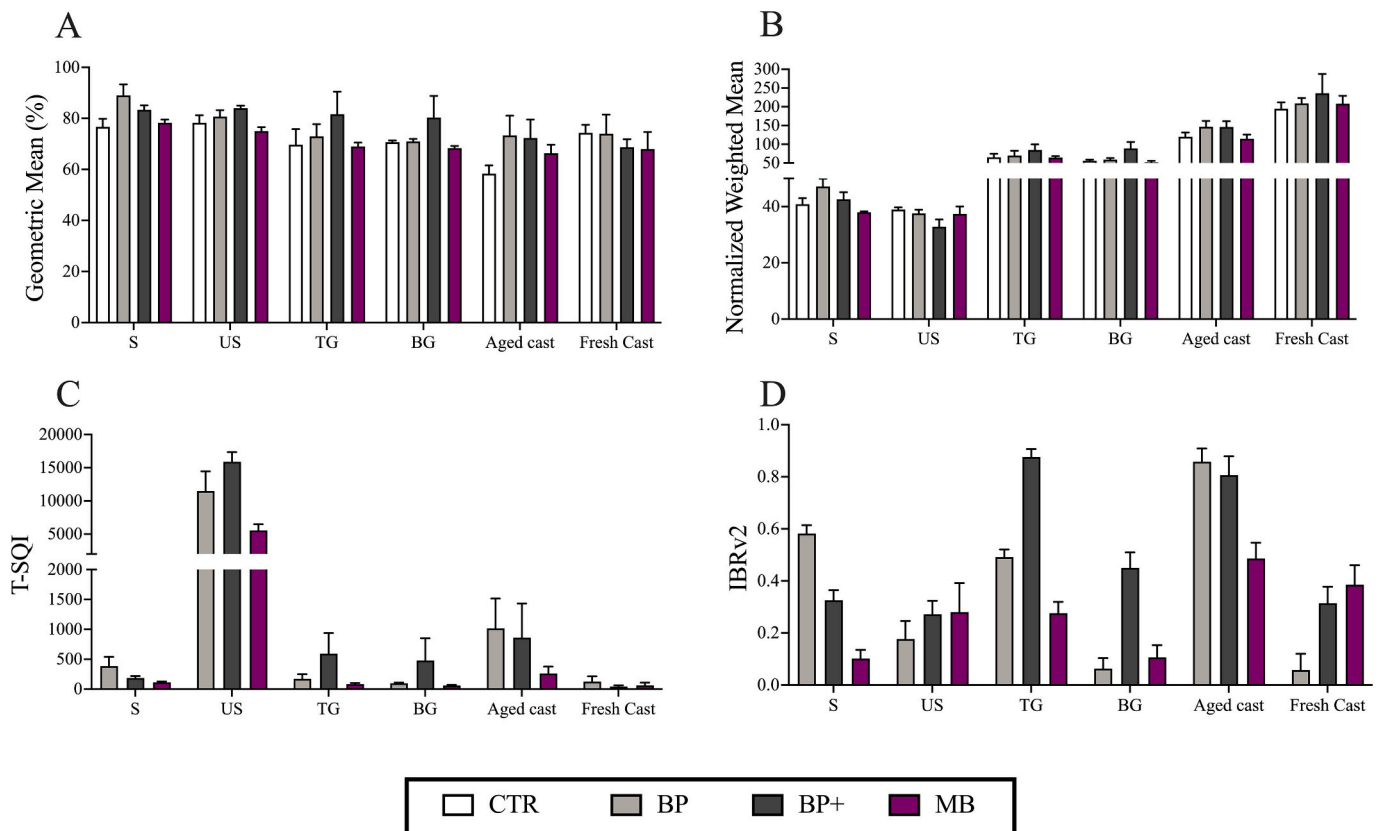


Fig. 7. Soil indexes. The soil indexes were calculated for each type of soil (S, US, TG, BG, aged cast and fresh cast) within the CTR and the different mulching films (BP, BP+ and MB) after 30 days. A) the GMean index, B) the WMean index, C) the T-SQI index and D) the IBRv2 index (abbreviations as in M&M).

different mulching films (BP, BP+ and MB) without showing any statistical differences (Fig. 7D). However, the sunray plots of T-SQI (Fig. S3) and IBRv2 (Fig. S4) showed the effect of the different mulching films with the different soil types. For the T-SQI sunray plots, the S soil type showed slightly inhibited alkaline phosphatase for MB, catalase for

BP+ and dehydrogenase for BP+ and MB compared to CTR (Fig. S3A). However, the US showed inhibition for 3 enzymes (urease, glucosaminidase and alkaline phosphatase) for MB (Fig. S3B). While TG showed an increase of glucosaminidase for all mulching films and FDH for BP and BP+ (Fig. S3C), MB had inhibition of glucosaminidase in BG (Fig. S3D).

The aged and fresh cast showed strong differences between each other. In the aged cast, BP+ inhibited catalase and, similarly to TG, BP, BP+, and MB increased glucosaminidase (Fig. S3E). In the fresh cast, the glucosaminidase was inhibited for BP and BP+ compared to CTR and enhanced FDH for all mulching films (Fig. S3F).

The sunray plots of IBRv2 showed strong inhibition of dehydrogenase, alkaline phosphatase and FDH in MB, while FDH was strongly enhanced for BP and BP+ (Fig. S4A) in S soil type. There was slight inhibition of glucosaminidase for all mulching films in the US soil (Fig. S4B), similarly observed in BG (Fig. S4D). However, in TG (Fig. S4C) the glucosaminidase was enhanced for all mulching films compared to CTR. Within the aged (Fig. S4E) and fresh casts (Fig. S4F), there were differences in the glucosaminidase activity, which was strongly inhibited for the fresh cast and enhanced for the aged cast. While FDH was inhibited for MB in the aged cast (Fig. S4E), FDH was increased for all mulching films in the fresh cast (Fig. S4F).

3.5. Earthworm digestive enzymes and oxidative stress biomarkers

3.5.1. Earthworm digestive enzymes

Regarding the digestive enzymes assessed on earthworms' gastrointestinal tissues, it was observed that there was no statistical significance in alkaline phosphatase activity (Fig. 8B) between the BP, BP+ and MB compared to CTR. At the same time, there was statistical significance in acid phosphatase activity as it was strongly increased in MB mulching film (91.82 ± 18.66 nmol mg protein⁻¹ h⁻¹; Fig. 8A). However, β -glucosidase activity showed an increasing tendency and statistical difference in MB (103.5 ± 12.12 nmol mg protein⁻¹ h⁻¹; Fig. 8C). Moreover, lipase activity showed statistical differences in the

gastrointestinal tissue of earthworms exposed to BP+ (333.4 ± 73.72 nmol mg protein⁻¹ min⁻¹) compared to CTR (107.5 ± 33.9 nmol mg protein⁻¹ min⁻¹; Fig. 8D). The measured carboxylesterase activity decreased strongly between the CTR and the different mulching films (572.3 ± 399.9 μ mol mg protein⁻¹ min⁻¹ in BP, 909.3 ± 836.5 μ mol mg protein⁻¹ min⁻¹ in BP+ and 99.73 ± 35.93 μ mol mg protein⁻¹ min⁻¹ in MB; Fig. 8E).

3.5.2. Oxidative stress biomarker

Those chosen biomarkers indicated that the earthworms' exposure to the different mulching films caused oxidative stress. There were statistical differences in GSSG in the gastrointestinal tissue; the highest increase was observed in MB (Fig. 9A). The most significant increase of GSSG was observed in MB (Fig. 9B) in wall muscle tissue, while there were no statistical differences in the seminal vesicle tissue (Fig. 9C). Similarly to GSSG, the GSH total showed a statistically significant increase in MB in the gastrointestinal tissue (Fig. 9D). However, the highest rise of GSH total was in BP+ in the wall muscle tissue (Fig. 9E). Similarly to GSSG, the seminal vesicle tissues showed no statistical differences compared to CTR in GSH total (Fig. 9F).

Malondialdehyde formation (MDA) is indirectly produced due to the interaction between radical oxygen species (ROS) and lipids. According to the results, the MDA formed from least to greatest production was BP < BP+ < MB. We could observe that independently of the type of tissue (gastrointestinal, wall muscle or seminal vesicle), MB had strong significance (Fig. 10) and was the strongest in gastrointestinal tissue ($23,289 \pm 1394$ nmol MDA mg protein⁻¹, Fig. 10A). BP+ had strong statistical significance in gastrointestinal tissue ($17,104 \pm 2613$ nmol MDA mg protein⁻¹, Fig. 10A) and slight statistical significance in wall

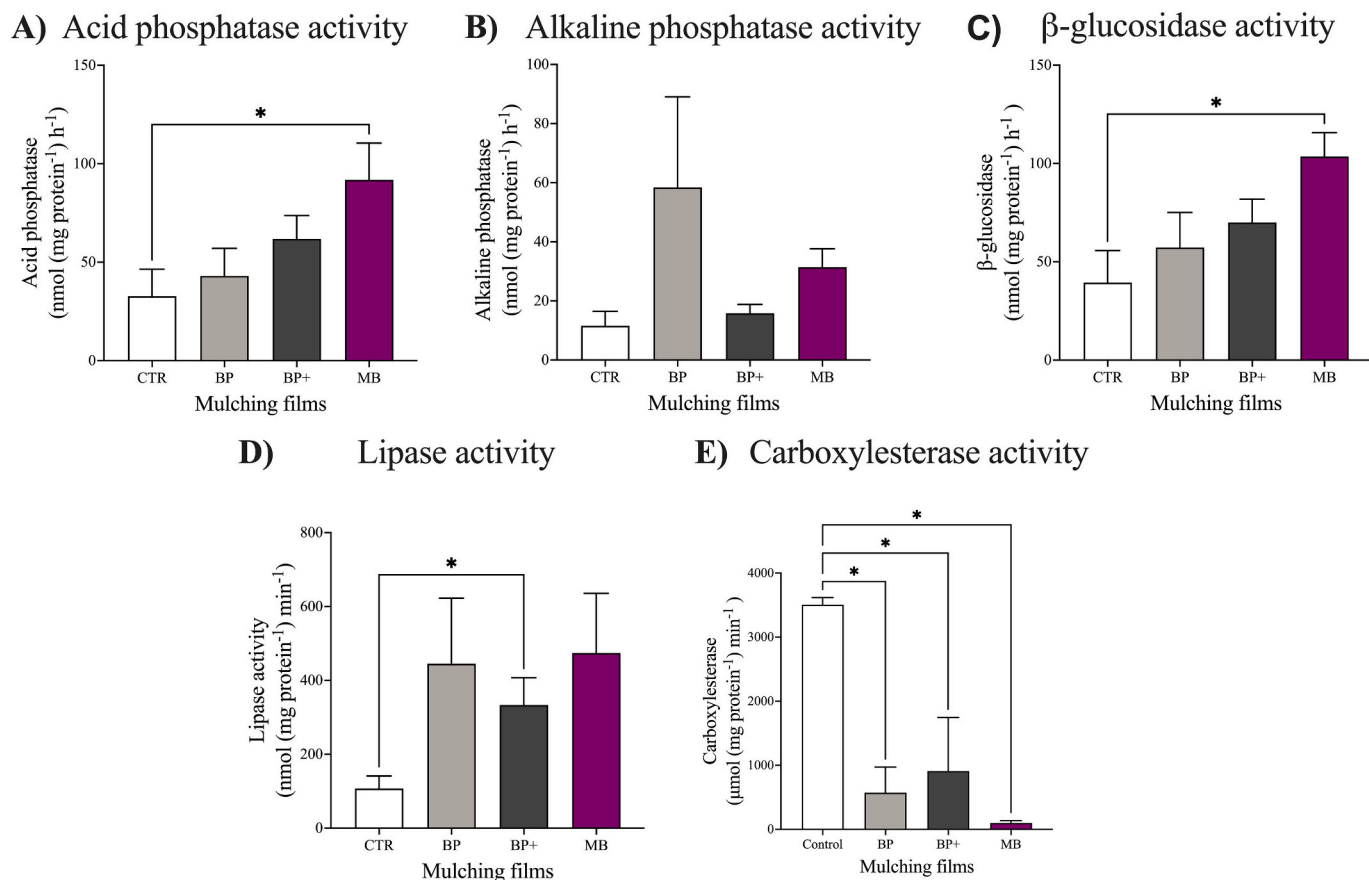


Fig. 8. Digestive enzymes. The different digestive enzymes calculated at the gastrointestinal tissue of earthworm *Lumbricus terrestris* L. incubated in CTR and with the different mulching films (BP, BP+ and MB; abbreviations as described in M&M) after 30 days. A) Acid phosphatase, B) Alkaline phosphatase, C) β -Glucosidase, D) Lipase and E) Carboxylesterase activity. * $P < 0.05$ according to One-way ANOVA and Dunnett's comparison test.

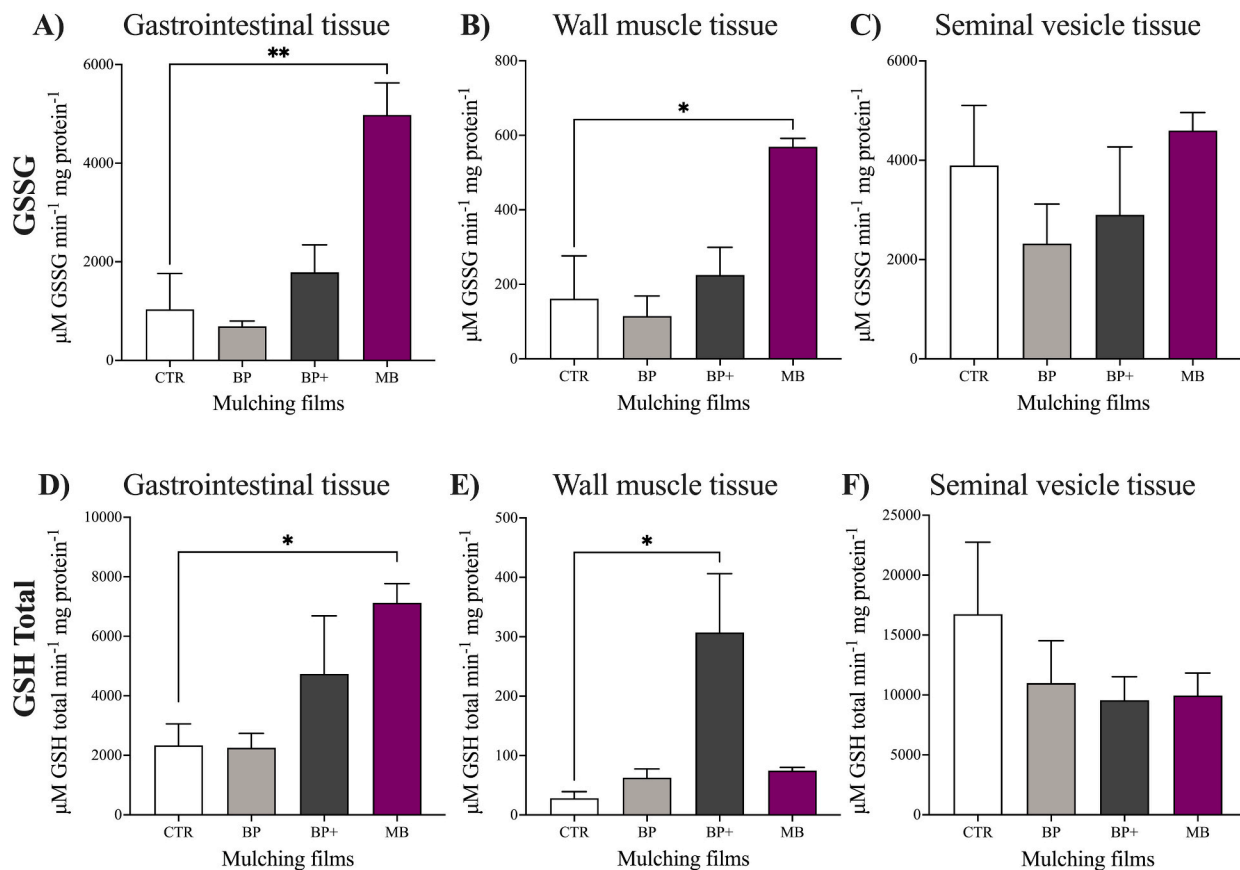


Fig. 9. GSH Total and GSSG. GSSG production in A) gastrointestinal tissue, B) wall muscle tissue and C) seminal vesicle tissue and GSH total formation in D) gastrointestinal tissue, E) wall muscle tissue and F) seminal vesicle tissue after 30 days incubation with CTR and the different mulching films (BP, BP+ and MB). * $P < 0.05$ and ** $P < 0.005$ according to One-way ANOVA and Dunnett's comparison test.

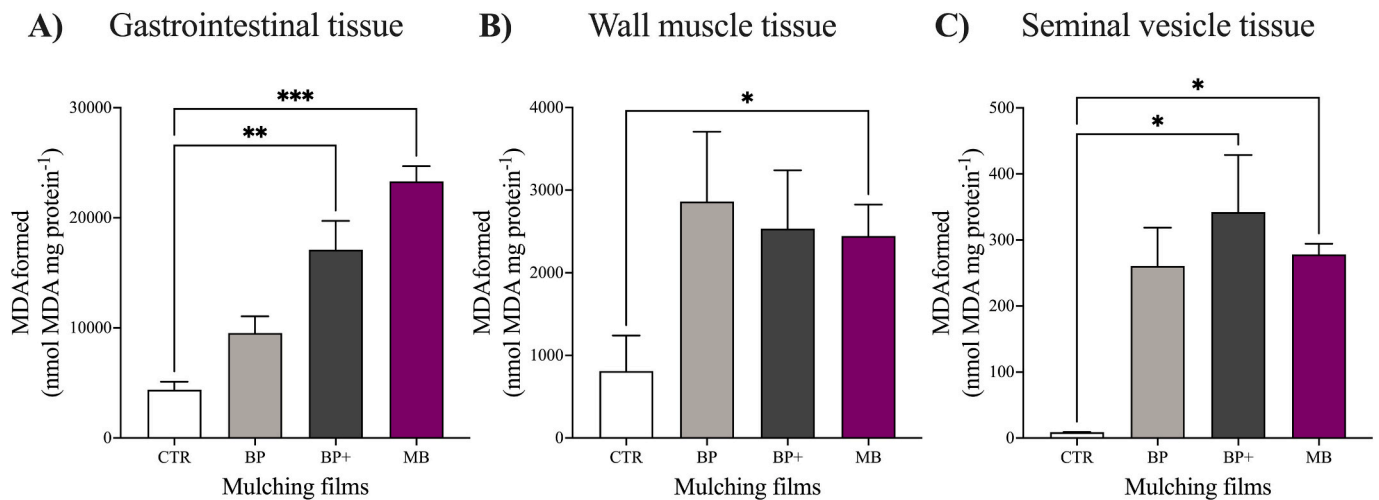


Fig. 10. MDA formed. MDA production in A) gastrointestinal tissue, B) wall muscle tissue and C) seminal vesicle tissue after 30 days of incubation with CTR and the different mulching films (BP, BP+ and MB). * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.0005$ according to One-way ANOVA and Dunnett's comparison test.

muscle tissue ($2533 \pm 708,7 \text{ nmol MDA mg protein}^{-1}$, Fig. 10B). The MDA formed due to the interaction between BP and the earthworms was not statistically significant in gastrointestinal (Fig. 10A), wall muscle tissue (Fig. 10B), or seminal vesicle (Fig. 10C).

4. Discussion

Differently from most studies which report on open field trials, the

present work aims to analyze in detail the soil-bioplactic-earthworms interaction using a microcosm approach and investigate how and at what degree soil incorporation of different kinds of bio-based and biodegradable mulching films can affect soil properties and earthworm-derived enzymatic activities.

It is known that earthworms positively affect the physical properties of soils (Blanchart et al., n.d.; Frouz et al., 2014). However, a similar result was not found in the present work, and the observed decrease in

the stability of soil aggregates could be explained considering the limited time period between the physical disturbance during the microcosm filling and the end of the trial (that was ~30 days). In fact, soil mixing occurring during the set-up of enclosed model units could have altered physical and biological soil properties in a way that a conditioning time depending on soil properties is often required before the tillage effect has become expired (Tortorella and Gelsomino, 2011). Previous studies have shown that soil pH responses to microplastic additions are highly variable, being increased in most cases, reduced or left unaltered. The observed opposite effects on this chemical soil variable were attributed to several influential factors such as soil physicochemical properties, nutrient availability, polymer composition, fragmentation and dose, time of exposure, cultivation and management practice (Qi et al., 2020a; Wang et al., 2022; Qi et al., 2020b). As uncertain as pH changes there could be in response to plastic mulch addition, we hypothesize that the soil pH decline observed in our microcosms, especially in the drilosphere where the whole biological activity was further elevated due to presence of *Lumbricus terrestris*, was likely due to the release of carboxylic acids originating from decaying starch-based or polysaccharide-based polymers that promoted an increase in soil acidity. This process was further magnified when soil incorporation of MAP-enriched mulch provided a source of ammonium that stimulated the nitrification process with consequent release of nitrate (a finding already observed by Paliaga and coworkers (Paliaga et al., 2025), and here corroborated by the increase of nitrate-N) and production of H⁺ ions.

Addition of mulch films can exert a variable impact on soil salinity, due to the direct and indirect effects through multiple mechanisms, varying with polymer type, film shape, doses and size (Wang et al., 2022). The decline in soil EC found at the end of the observation period in all treatments where no earthworms were present, including that with MAP-enrichment, can be justified by the increased microbial uptake of soluble substrates released from decaying biopolymers. On the contrary, a marked increase of soil salinity was found in samples from the drilosphere. Not unexpectedly, the finding is due to burrowing activity of earthworm and the release of their casts, which are known to contain a significant amount of soluble salts and nutrients which can positively affect the fertility status of the soil (Lavelle and Spain, 2001). The organic carbon content in this work increases with the presence of commercial mulching film (MB) and decrease in the soil without earthworms treated with the experimental film (BP), despite an estimated amount of approximately 512.3 mg C kg⁻¹ entered the soil together with the mulch film (BP). Noteworthy, biodegradable mulching films are constituted by readily hydrolysable organic polymers (Hejna et al., 2022) that once incorporated within the soil microcosms could have exerted a priming effect on soil microbiota since they represent a readily available C source for soil microorganisms. This hypothesis would be supported also by the low C to N ratio of both BP (21.9) and BP+ (8.1) which could have contributed to foster the mineralization process during the incubation experiment, thus providing a decreased final C reading. On the contrary, soil incorporation of Mater-Bi®, which is characterized by a C to N ratio as large as 865, lowered the microbial mineralization thus leaving the total C reading unaffected.

Most represented nutrients are inorganic N- and P- containing chemical forms (Chaoui et al., 2003; Van Groenigen et al., 2019). It is known that several concurring pedomorphic conditions regulate P availability in soils, and the concentrations of the chemical species of phosphate ions present is determined by the solution pH (Brady and Weil, 2017). Even though phosphate availability is at its highest when soil pH is maintained within the 6.0–7.0 range (Wang et al., 2022; Huang et al., 2023), the slight decline of pH here observed during the experimental period cannot explain the noticeable increase in P-Olsen content. Nevertheless, an increased mineralization of the soil organic pool, further stimulated by aeration and mixing during the experimental set-up phase by earthworms, could have promoted the release of soluble P. In this work the presence of biodegradable mulching films in soil does not lead to any significant variation despite one of the buried films was

enriched with approximately 62.82 mg kg⁻¹ of P. Phosphatase activity in soil might be associated with the increased P demand from growing microorganisms, as P can become a limiting factor for microbial growth when easily degradable C substrates enter the soil, especially those substrates with a high C/P ratio, as for instance for BP or MB mulching polymers. In these conditions, an increased availability of C substrates induced by incorporating C-rich biodegradable films could have stimulated microbial uptake of inorganic P, thus preventing P-Olsen from accumulating in soil. On the other hand, available P increases in the microcosm without film but with the presence of earthworms, thus confirming what observed by Lopez-Hernandez et al. (1993) and Le Bayon and Binet (2006) on the role of earthworms ability to influence the distribution and release of soluble P in soils.

Using a microcosm system, Parkin and Berry (1999) showed that the drilosphere is characterized by high rates of nitrification and denitrification processes (Parkin and Berry, 1999). In other words, C and N pools released in the drilosphere stimulate the growth and the activity of N-transforming microbial populations, which in turn result in an enrichment of NO₃⁻ in the drilosphere.

It has been shown that the addition of biodegradable mulch fragments can influence the microbial populations involved in the S cycle by reducing its assimilation into the soil (Huang et al., 2023; Sun et al., 2022a; Sun et al., 2022b). In this work, instead, the presence of Mater-Bi® fragments increases SO₄²⁻ ions, especially in the drilosphere, probably because the prokaryotic communities hosted in the casts expressed a biochemical activity involved in the sulfate cycle.

4.1. Effects on soil enzymatic activities

This study also focused on the effects of mulching film on soil enzymes and the different biochemical hotspots occurring in soil fabric (burrows created by *L. terrestris*, undisturbed soil, soil without earthworms and cast produced by earthworms). Due to its geofagous lifestyle, *L. terrestris* accidentally ingests fragmented plastics during the feeding activity, and then translocate them throughout the soil profile when releasing its cast on the burrows and at the soil surface (Huerta Lwanga et al., 2016). During the experiment, we could observe that BP and BP+ mulching films that were deposited upon the soil surface were lately moved down in the burrows by the earthworms. Although the accidental ingestion has been described with microplastic (Adhikari et al., 2023; Huerta Lwanga et al., 2016), we could expect similar behaviour with our polysaccharide-based composite mulching films. Interestingly, the cast amended with microplastics and secreted by earthworms might not alter the soil microbial community when the cast is part of the soil, as found with microplastics like low density polyethylene (LDPE), poly (butylene adipate-co-terephthalate (PBAT) and polylactic acid (PLA) (Adhikari et al., 2023; Meng et al., 2024). Our study showed that the N-acquired enzymes (urease and protease activity) remained unaffected by BP, BP+ and MB addition, a finding consistent with the observation that earthworms do not alter the bacterial community functionally connected with the nitrification and denitrification processes. However, there was an exception with *N*-acetyl-β-D-glucosaminidase activity (NAG), which was significantly induced by BP, BP+ and MB at the burrows and the aged cast of earthworms. The T-SQI sunray plot (Fig. S3) showed that NAG was strongly induced at BP and BP+ exposure compared to MB in the S and BG, while similar induction was observed at TG and aged cast. Nevertheless, the increased NAG activity observed in BP and BP+ -treated soil corroborates what recently reported by Paliaga et al. who observed that chitosan degradation process in soil involves several steps including the hydrolysis of *N*-acetyl bonds (Paliaga et al., 2025). The increased secretion/induction of this enzyme is likely due to several factors. 1) The ecological role of the plastsphere (that is the microbial community residing on the surface of plastics, including biodegradable mulching films (Rillig et al., 2017; Rillig et al., 2024)) in secreting specific enzymes to foster the degradation of chitosan and the release soluble C and N substrates which are made available

for microbial use. 2) The ecological interaction between the plastisphere and the earthworms, as suggested by Rillig and coworkers (Sanchez-Hernandez, 2024), which leads to the formation of burrows cemented with casts containing digestive enzymes, microorganisms, and microplastics, and hosting a specific microbial community (Sanchez-Hernandez, 2024; Helmberger et al., 2020). Moreover, it was observed that earthworms are prone to ingest the plastisphere (Helmberger et al., 2020), and that their microbiome gut can shift towards the dominance of Actinobacteria, Proteobacteria and Firmicutes after exposure to microplastics (Papazlatani et al., 2024). Hence, the earthworm's interaction with the plastisphere could lead to ingestion of BP and BP+, then gut microorganisms could lead to a NAG activity increase, and it is detected in the soil through the cast that is part of the earthworm burrows. 3) Prokaryotic compositional shifts, accompanied by the increase of Actinobacteria, that produce *N*-acetylglucosamine within other polysaccharide compounds that would enhance the attachment to plastic surfaces (Riveros et al., 2022), and Nocardioidaceae, that are capable of degrading chitin and starch, make *N*-acetylglucosamine accessible to microorganism as a C source (Cruz et al., 2024).

Mater-Bi® EF04P contains starch, natural plasticizer, and biodegradable polyesters without any specification for polyester type (Degli-Innocenti et al., 2022; Bernetti et al., 2025). However, we could suggest that the induction of NAG in MB could depend on the kind of polyester it is made of.

On the other hand, introducing earthworms into the soil would change the soil microbiota (Lu et al., 2024), which is in concordance with our results, which provided a slight increase in soil enzyme activities in soils with earthworms (Table 2). Interestingly, dehydrogenase activity (DHA) is another soil enzyme associated with microbial respiration, so a decrease in DHA would worsen the soil quality (Wolinska and Stepniewsk, 2012; Santini et al., 2022). Combined with the fluorescein diacetate (FDH) results, slight changes were observed between mulching films. FDH was higher at the TG and BG and the fresh cast (Fig. S3-S4). The FDH is used to measure total microbial activity, which is indirectly associated with the turnover of the organic matter (Schnürer and Rosswal, 1982). Our results suggest that the earthworms influence the microbial activity, which further increases when earthworms are exposed to BP+, as we could observe at the TG, BG, and fresh cast (Fig. S3). Indeed, this is entirely in concordance with Gallego et al. who suggested that earthworms' presence increases the soil enzymatic activities, unless they are exposed to biologically active chemicals such as valsartan (antihypertensive drug) (Gallego et al., 2021).

4.2. Effects on earthworms

Weight loss is one of the main endpoints used to check the effects of any substance on earthworms. According to our analyses, exposure to BP and MB determined a decrease in earthworm weight, probably caused by some stressing factors like material composition. Some studies have mentioned that some biodegradable mulching films could cause weight loss (Ju et al., 2023). Similar to Sforzini et al. mortality was not observed (Sforzini et al., 2016). However, Huerta Lwanga et al. (2016) observed that mortality of *L. terrestris* occurred after a 60-day exposure (Huerta Lwanga et al., 2016). Thus, weight loss and mortality are dependent on the type of mulching film (plastic like polyethylene, PE, LDPE; biodegradable plastic like PBAT or PLA), time of exposure, and size and shape of the mulching film (Forsell et al., 2024; Weltmeyer and Roß-Nickoll, 2024). Nevertheless, the observed increase in earthworms' weight could be considered a direct response to the addition of readily available N and P entering the soil with the BP+ mulch film. However, an indirect effect can be also considered, as for instance a change in the efficiency of the whole digestive processes and, hence nutrient absorption, due to a compositional shift in the gut microbiota when earthworms are feeding on BP+. Although not experimentally assessed in the present paper, this hypothesis could be supported by Papazlatani et al., 2024, who found a shift in the composition of gut microbiota in anecic earthworms exposed

to microplastics (Papazlatani et al., 2024). Similarly, Swart et al. (2020) showed a shift of epigeic earthworms gut microbiota when worms are exposed to different industrially derived materials (i.e. copper oxide nanoparticles (Swart et al., 2020)). Needless to say, further studies in this field should consider also the earthworms' gut microbiota shifts.

The mulching films and plastics can be ingested by earthworms (Adhikari et al., 2023; Huerta Lwanga et al., 2016; Khaldoon et al., 2022) and either accumulate in their gut, causing tissue damage or a shift in gut microbiota (Li et al., 2022), and secreted through the cast. Some studies showed that *L. terrestris* engulfed LDPE and PBAT, and found them in the gut and the cast (Adhikari et al., 2023; Huerta Lwanga et al., 2016). Notably, Meng et al. (2024) suggested that the digestion and casting process influenced the bacterial communities (Meng et al., 2024). In fact, the digestion process is accompanied by a high enzymatic activity (digestive enzymes assessed in this study; Fig. 8) exerted by both microorganisms symbionts in the earthworm gut and those ingested from the soil (Aira et al., 2019; Drake and Horn, 2007). According to our study, Mater-Bi® induced acid phosphatase and β -glucosidase (Fig. 8A-8C), while carboxylesterase enzyme was inhibited in all the mulching films (Fig. 8E). The fact that the carboxylesterase activity was strongly inhibited in all types of mulch film suggests that carboxylesterase activity could be a suitable biomarker due to its high sensitivity. Similar inhibition was also described by Sanchez-Hernandez et al. (2018) when earthworms were exposed to organophosphorus pesticides like chlorpyrifos (Sanchez-Hernandez et al., 2018). Moreover, the inhibition of carboxylesterase activity was stronger in Mater-Bi® than in the other mulching films, which could also explain the reason for the weight loss of the earthworms. It has been reported that a protein corona could form spontaneously upon nanoplastics interacting with digestive enzymes (Peng et al., 2019). According to Sanchez-Hernandez (2024), the protein corona would interact with the digestive enzymes and block the cells to uptake the nutrients with the consequent effects of weight loss (Sanchez-Hernandez, 2024). If this is true in our study, ingested fragments of BP and MB could have formed surface coatings with the carboxylesterase (a digestive enzyme), thus altering the digestive processes, the cell's uptake of nutrients and then leading to weight loss. Further studies should explore the potential formation of protein corona in earthworms feeding on different mulching films, including BP and BP+. Rodríguez-Seijo et al. (2018) suggested the importance of the earthworm's enzymatic system for their health and preventing harm at the cellular and tissue level (Rodríguez-Seijo et al., 2018). Lipases are enzymes that catalyse the hydrolysis of ester bonds (Barbir et al., 2023; Huo et al., 2024). Lipase activity is another digestive enzyme that was induced by exposure to BP+ (Fig. 8D). Interestingly, Huo et al. (2024) showed that lipase hydrolyzed the ester bonds of PBAT, leading to an increase of lipase in *Artemia persimili nauplii* exposed to BPE-AMF-PLA, a blend of PLA and PBAT (Huo et al., 2024). The digestion of the BP+ probably released some oligomers that could stimulate the lipase activity, as similarly happened with *Artemia persimili nauplii* (Huo et al., 2024). Regarding β -glucosidase activity induced by exposure to MB (Fig. 8C), the induction could have been caused by one of the main components of MB, the starch. It is known that glucosidases hydrolyse starch and other types of carbohydrates into monomers (Inamdar et al., 2022).

Oxidative stress is another endpoint for assessing the effects of substances, in this case, mulching films, on earthworms. Two types of biomarkers were chosen for the oxidative stress in the earthworm tissue. A role of GSH is scavenging reactive oxygen species (ROS), forming glutathione disulfide (GSSG) (Sanchez-Hernandez et al., 2023).

However, MDA is formed when reactive oxygen species (ROS) are not scavenged, or the antioxidative system is not capable of reducing them, which leads to their interaction with lipids, especially polyunsaturated fatty acids (Ayala et al., 2014). MDA can interact with DNA and other biomolecules, causing severe damage to cells and cell death (Navarro Pacheco et al., 2021). Our study showed that MB and BP+ induced the production of antioxidative enzymes and MDA in the tissues (Fig. 10).

Interestingly, the seminal vesicles looked more sensitive to the mulching films than the other tissues (Fig. 10C). However, some studies showed that reproduction in earthworms (*E. fetida* or *L. terrestris*) was not affected by exposure to LDPE, PE, PBAT or PLA (Ju et al., 2023; Huerta Lwanga et al., 2016; Weltmeyer and Roß-Nickoll, 2024; Yu et al., 2022). Sforzini et al. (2016) showed that *Eisenia andrei* reproduction was not affected, although it was exposed to Mater-Bi® EF04P (Sforzini et al., 2016). Similar results in the oxidative stress biomarkers were observed in *E. fetida* earthworms when exposed to the highest concentration (1000 mg kg⁻¹ dry soil) of LDPE pellets (Rodríguez-Seijo et al., 2018). Moreover, a similar increase in MDA production was observed when *E. fetida* was exposed to biodegradable plastic (PLA) for 14 days (Yu et al., 2022). Although the tissue-specific approach is not applied, it broadens the knowledge of what tissues could be damaged by mulching films. Further studies are needed to comprehensively understand the effects of mulching films on *Lumbricus terrestris*, as there have been few studies. Moreover, future studies should be focused on the interaction between soil microbiota-earthworms-mulching films in long-term exposures (> 7 months).

5. Conclusions

Overall, the application of commercial biodegradable mulching film (Mater-Bi®) and the two experimental bio-based and biodegradable mulch films (BP and BP+) did not significantly alter the physicochemical properties of the soil. However, slight variations in soil pH may be attributed to the presence of *Lumbricus terrestris*. The study demonstrated that the introduction of *L. terrestris* into the soil can promote the release of soluble phosphorus and influence microbial activity. While the mulching films did not induce major changes in soil enzyme activities, an increase in *N*-acetylglucosaminidase (NAG) activity was observed in treatments with BP, BP+, and Mater-Bi®. Interestingly, enhanced microbial activity, as indicated by dehydrogenase (DHA) and fluorescein diacetate (FDA) hydrolysis, was only detected in soils treated with BP+. This suggests a potential shift in the soil microbiota. It should be noted, however, that the experimental design did not consider the transient nature of the effects caused by biodegradable materials. Such materials may perturb the system during degradation, followed by a return to a stable state. Few studies have explored the interaction between earthworms and mulching films. This study shows that mulching films can impact the antioxidant system of earthworms and induce oxidative stress, especially Mater-Bi®. Notably, carboxylesterase activity in the earthworm gut emerged as a sensitive biomarker of exposure. Although this experiment was limited to a 30-day period, longer-term studies are needed to detect potential changes in both soil and gut microbiota. Further research is also recommended to investigate the interactions between soil, bioplastics, and earthworms more comprehensively, including tissue-specific effects and longer exposure durations, to better understand the ecological implications of biodegradable mulching films.

CRedit authorship contribution statement

Maria Teresa Rodinò: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Natividad Isabel Navarro Pacheco:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Antonio Gelsomino:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization, Methodology, Visualization.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2025.180013>.

Data availability

Data will be made available on request.

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