Environment International 163 (2022) 107190

Contents lists available at ScienceDirect

Environment International

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

Two types of microplastics (polystyrene-HBCD and car tire abrasion) affect oxidative stress-related biomarkers in earthworm *Eisenia andrei* in a time-dependent manner

Carina Lackmann [a], Mirna Velki [b], Antonio Šimić [b], Axel Müller [a,c], Ulrike Braun [c,d], Sandra Ečimović [b], Henner Hollert [a,e,*]

[a] Department of Evolutionary Ecology and Environmental Toxicology, Goethe University Frankfurt, Max-von-Laue-Str. 13, 60438 Frankfurt/Main, Germany
[b] Department of Biology, Josip Juraj Strossmayer University of Osijek, Cara Hadrijana 8/A, 31000 Osijek, Croatia
[c] Bundesanstalt für Materialforschung und -prüfung (BAM), Fachbereich 6.6: Physik und chemische Analytik der Polymere, Unter den Eichen 87, 12205 Berlin, Germany
[d] Umweltbundesamt (UBA), Fachgebiet III 2.5 - Überwachungsverfahren, Abwasserentsorgung, Schichauweg 58, 12307 Berlin, Germany
[e] LOEWE Centre for Translational Biodiversity Genomics (LOEWE-TBG), 60325 Frankfurt am Main, Germany

ARTICLE INFO

Handling Editor: Adrian Covaci

Keywords:
Microplastics
Earthworms
Toxicity
Biomarker
Oxidative stress

ABSTRACT

Microplastics are small plastic fragments that are widely distributed in marine and terrestrial environments. While the soil ecosystem represents a large reservoir for plastic, research so far has focused mainly on the impact on aquatic ecosystems and there is a lack of information on the potentially adverse effects of microplastics on soil biota. Earthworms are key organisms of the soil ecosystem and are due to their crucial role in soil quality and fertility a suitable and popular model organism in soil ecotoxicology.

Therefore, the aim of this study was to gain insight into the effects of environmentally relevant concentrations of microplastics on the earthworm *Eisenia andrei* on multiple levels of biological organization after different exposure periods. Earthworms were exposed to two types of microplastics: (1) polystyrene-HBCD and (2) car tire abrasion in natural soil for 2, 7, 14 and 28 d. Acute and chronic toxicity and all subcellular investigations were conducted for all exposure times, avoidance behavior assessed after 48 h and reproduction after 28 d. Subcellular endpoints included enzymatic biomarker responses, namely, carboxylesterase, glutathione peroxidase, acetylcholinesterase, glutathione reductase, glutathione S-transferase and catalase activities, as well as fluorescence-based measurements of oxidative stress-related markers and multixenobiotic resistance activity. Multiple biomarkers showed significant changes in activity, but a recovery of most enzymatic activities could be observed after 28 d. Overall, only minor effects could be observed on a subcellular level, showing that in this exposure scenario with environmentally relevant concentrations based on German pollution levels the threat to soil biota is minimal. However, in areas with higher concentrations of microplastics in the environment, these results can be interpreted as an early warning signal for more adverse effects. In conclusion, these findings provide new insights regarding the ecotoxicological effects of environmentally relevant concentrations of microplastics on soil organisms.

1. Introduction

The rapid growth of plastic production since the 1950s and the insufficient disposal of plastic waste has resulted in plastic being considered an emerging contaminant (Geyer et al., 2017; Stubbins et al., 2021). Due to its versatile applications besides its usage as packaging material, its low production costs, and overall superior chemical properties such as lightness, durability and hydrophobicity, the production of plastic products has increased constantly in the past decades (Ganesh Kumar et al., 2020). (Borrelle et al., 2020) categorize plastic pollution as a planetary threat and estimate the growth of plastic waste exceeding any current efforts to mitigate plastic pollution. This consideration of plastic pollution as a planetary threat can be explained not only by their highly uncertain half-lives (Chamas et al., 2020; Duan

* Corresponding author at: Goethe University Frankfurt Department of Evolutionary Ecology and Environmental Toxicology, Max-von-Laue-Str. 13, 60438 Frankfurt am Main, Germany.
E-mail address: hollert@bio.uni-frankfurt.de (H. Hollert).

https://doi.org/10.1016/j.envint.2022.107190
Received 15 December 2021; Received in revised form 18 February 2022; Accepted 14 March 2022
Available online 19 March 2022

et al., 2021; MacLeod et al., 2021) but also the potential impact on carbon and nutrient cycles, habitat changes within soils, sediments and aquatic ecosystems with co-occurring biological impacts on endangered or keystone species due to its ecotoxicity (MacLeod et al., 2021). While recently biodegradable plastics have been developed, none of the most commonly used plastics are completely biodegradable and rather need destructive thermal treatment for complete degradation, and thus so far accumulate in landfills and the environment (Geyer et al., 2017). Plastic debris can however break down into smaller micro- (0.1 – 1000 μm) or nano-sized (≤0.1 μm) particles, from herein referred to as micro- and nanoplastics, respectively, through weathering processes, e.g., biological, chemical or physical agents or physical abrasion or fragmentation (Duan et al., 2021; Gigault et al., 2018; Defu He et al., 2018; Helmberger et al., 2020; Hidalgo-Ruz et al., 2012),.

Microplastic accumulation and its fate on different ecosystems has been reported to be potentially hazardous to marine, freshwater and soil organisms (Chae & An, 2018; Defu He et al., 2018; MacLeod et al., 2021; Ng et al., 2018). Due to their easy distribution by wind or water to many remote urban, riparian and agricultural sites, microplastics' presence in the environment has become ubiquitous and pervasive (Helmberger et al., 2020; Rochman & Hoellein, 2020). Microplastics have been detected in various environmental compartments and scientific research has overall increased in the last 25 years (de Souza Machado et al., 2018), especifically in terrestrial ecosystem (Donghui He et al., 2020). However, research on the occurrence and impact of microplastics in soil have only accounted for 3.8% of publications between 2004 and 2018 (Defu He et al., 2018). This lack of knowledge regarding microplastics in soil is alarming, as (Hurley & Nizzetto, 2018) believe soil to be a significant and possibly dominant environmental reservoir of micro and nanoplastic and thus research should not neglect the potential impact on the soil ecosystem. As in general research on microplastics has been very limited compared to other environmental contaminants adequate data on environmental concentrations in soil is missing (Büks & Kaupenjohann, 2020). What is however known is that there are various potential entry routes of microplastics in soil: such as degradation of plastic debris, industry wastewater, fertilizers, irrigation by waste water or tire abrasion (Chae & An, 2018). The annual emission of tire dust from car tire abrasions (containing rubber, a mostly synthetic polymer) is estimated at up to 110,000 tons in Germany alone, which enters the roadside environment via dust or wash-off (Bläsing & Amelung, 2018). Once microplastics such as tire dust reach the soil ecosystem, they can potentially accumulate, aggregate, persist and affect biota and thus, the overall soil quality.

Earthworms have long been used as indicators for soil quality due to their important role in terrestrial ecosystems (Fründ et al., 2010). Furthermore, they represent a majority of soil biomass of moderate terrestrial areas and are considered to be major ecosystem engineers due to their influence on both the structural integrity and the fertility of soil (Blouin et al., 2013; Jones et al., 1994). By definition, ecosystem engineers influence characteristics of their surroundings and thus affect other living components in their ecosystem. Earthworms specifically influence the chemical and physical properties of soil through bioturbation, i.e. burrowing, mixing of soil layers and litter, transfer organic matter and soil particles and thus improve the structural integrity, stability, aeration and irrigation of the soil (Edwards & Bohlen, 1996; Eisenhauer, 2010; Lavelle et al., 1998). Due to their crucial role for most soil ecosystems earthworms have been established as model organisms in terrestrial ecotoxicology. While there is a clear lack of studies on the effects of microplastics, recent studies have shown the adverse effects microplastics can have on earthworms. (Jiang et al., 2020) observed polystyrene microplastic to induce oxidative stress, histopathological changes and DNA damage in earthworms, while (Kwak & An, 2021) showed an inhibition of spermatogenesis and coelomocyte viability after exposure of earthworms to polyethylene microplastics. However, considering the wide range of plastics and their highly varying chemical properties, more research is needed to fill this immense gap of knowledge surrounding microplastics.

As part of the project "Plastik in Böden- Vorkommen, Quellen, Wirkungen" (commissioned by the Umweltbundesamt (German Environmental Agency)) which aimed to establish methods for sampling of microplastics in soil and determined environmentally relevant concentrations of car tire abrasion and polystyrene in German soils (Braun et al., 2021; Müller et al., 2022), the present study investigated the ecotoxicological effects of microplastic concentrations that were based on these findings.

Therefore, the aim of the present study was to thoroughly investigate the effects of two types of secondary microplastics on the earthworm *Eisenia andrei* in a time-dependant manner and on various levels of biological organization. More specifically, earthworms were exposed to environmentally relevant concentrations of car tire abrasion and polystyrene particles (containing 1% of the flame retardant HBCD). Exposures of various lengths (2, 7, 14 and 28 d) were conducted in natural soil and multiple apical and mechanistic endpoints investigated. Namely, mortality, avoidance behavior, subcellular markers and reproduction were assessed. Enzymatic biomarkers measurements included carboxylesterase (CES), glutathione peroxidase (GPx), acetylcholinesterase (AChE), glutathione reductase (GR), glutathione S-transferase (GST) and catalase (CAT) activities. Furthermore, fluorescence-based measurements of oxidative stress-related markers, namely reactive oxygen species (ROS) and glutathione (GSH), and multixenobiotic resistance (MXR) activity were assessed. Based on the measured responses, this study will help elucidate the potential effects of environmentally relevant microplastic concentrations and help gain insight into the potential risks associated with global microplastic pollution.

2. Material and methods

2.1. Chemicals

The following chemicals were used: acetonitrile (C_2H_3N, CAS 75-05-8), β-nicotinamide adenine dinucleotide 2 -phosphate reduced tetrasodium salt hydrate (β-NADPH) ($C_{12}H_{26}N_7Na_4O_{17}P_3$ xH_2O, CAS 2646-71-1 (anhydrous)), 9-(2-carboxyphenyl)-6-diethylamino-3-xanthenylidene-diethylammonium chloride (rhodamine B) ($C_{28}H_{31}ClN_2O_3$, CAS 81-88-9), CellTracker^{TM} Green CMFDA Dye ($C_{25}H_{17}ClO_7$, CAS 136832-63-8) (ThermoFisher Scientific), 1-chloro-2,4-dinitrobenzene (CDNB) ($C_6H_3ClN_2O_4$, CAS 97-00-7), CM-H_2DCFDA ($C_{27}H_{19}Cl_3O_8$, CAS 1219794-09-8) (ThermoFisher Scientific), hydrogen peroxide (H_2O_2, CAS 7722-84-1), (2-mercaptoethyl)trimethylammonium iodide acetate (acetylthiocholine iodide) ($CH_3COSCH_2CH_2N(CH_3)_3I$, CAS 1866-15-5), disodium hydrogen phosphate (Na_2HPO_4, CAS 7558-79-4), 5,5 -dithiobis-(2-nitrobenzoic acid) (DTNB) ($[-SC_6H_3(NO_2)CO_2H]_2$, CAS 69-78-3), glutathione disulfide (GSSG) ($C_{20}H_{32}N_6O_{12}S_2$, CAS 27025-41-8), 4-nitrophenyl acetate ($C_8H_7NO_4$, CAS 830-03-5), (2*S*)-2-amino-4-{[(1*R*)-1-[(carboxymethyl)carbamoyl]-2-sulfanylethyl]carbamoyl}butanoic acid (glutathione (GSH)) ($C_{10}H_{17}N_3O_6S$, CAS 70-18-8), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$, CAS 13472-35-0), ethylenediaminetetraacetic acid disodium salt hydrated ($C_{10}H_{16}N_2O_8$, CAS 6381-92-6), dimethyl sulfoxide (DMSO) ($(CH_3)_2SO$, CAS 67-68-5), glutathione reductase from baker's yeast (*Saccharomyces cerevisiae*) (ammonium sulfate suspension) EC 1.6.4.2. (CAS 9001-48-3), sodium azide (NaN_3, CAS 26628-22-8. Protein concentrations were measured using the Pierce^{TM} BCA Protein Assay Kit.

2.2. Microplastics

The following microplastics were investigated: (1) car tire abrasion (abbreviated as MP1) (particle size < 600 μm, black in color) from a bulk product of recycled old tires and (2) Polystyrene-HBCD (abbreviated as MP2) (particle size < 500 μm, white in color). Based on a study by (Rodriguez-Seijo et al., 2017), which performed exposures of microplastic particles of up to 1000 μm sizes in *E. andrei*, we assumed that the microplastics in the present study may be ingested by *E. andrei*.

The environmentally relevant concentrations and particle sizes for the present study were chosen based on the results of the project "Plastik in Böden- Vorkommen, Quellen, Wirkungen" commissioned by the Umweltbundesamt (German Environmental Agency) where different test sites (e.g., construction sites and road sites) were sampled (Braun et al., 2021).

Additional information on car tire abrasion composition can be found in (Müller et al., 2022). Polystyrene-HBCD was obtained through cryogenic grinding (Retsch ZM200) of 1x1 cm parts of extruded polystyrene containing 2% of the stabilizer calcium stearate and 1% of the flame retardant hexabromocyclododecane (HBCD) according to the manufacturer. It can be assumed that the particles were not round as the polystyrene-HBCD was ground at temperatures below its glass transition temperature and then behaves brittle and breaks accordingly.

2.3. Test organism

Exposures were conducted using adult earthworms (*Eisenia andrei*) showing a well-developed clitellum (average weight: 0.3 g) supplied from a local earthworm farm and acclimatized at 20 °C prior to all experiments. After selecting adult earthworm, they were thoroughly washed with distilled water and placed on damp filter paper in petri dishes. The petri dishes were then covered with aluminum foil containing aeration holes for at least 12 h to empty the earthworms' gut contents before the start of the experiments.

2.4. Soil matrix

The used soil originated from a former military test site with an area of 12 km^2. For 30 years the site has been used for field trials by the Federal Institute for Materials Research and Testing (BAM). The closest road is kilometers away, and the area is mostly covered by pine forest. The dominant soil is classified as podzolic regosol. A total of 150 kg soil was collected using stainless steel shovels, mixed, and transported in tinplate containers. The sampled soil was contained roots and plant residues and was characterized as fine sand with a silt and clay content of 4.5%. The organic content of 2.5% was determined by ashing in a muffle furnace according to DIN 18128. Thermoanalytical investigations by TED-GC/MS showed no car tire abrasions, polystyrene, or other microplastic contaminations in the soil matrix (Müller et al., 2022).

2.5. Acute toxicity test

Toxicity was determined according to OECD Guideline 207 (OECD, 1984) with changes as described below. All exposures were conducted in glass containers, with 10 earthworms per container in 400 g soil thoroughly mixed with the respective microplastic amount and 40 mL distilled water. To determine the mortality, limit tests were conducted using environmentally relevant concentrations (Braun et al., 2021), namely 1 and 1000 mg/kg MP1 and 0.1 and 100 mg/kg MP2. After the respective exposures of 2, 7, 14 and 28 d (during which the soil was watered to avoid drying out), mortality was checked and recorded. All experiments were conducted in at least three independent replicates with 10 earthworms per replicate ($n = 30$) with negative controls (soil + distilled water) performed in parallel. The glass containers were placed in constant light at 20 °C.

2.6. Assessment of avoidance behavior

Avoidance behavior (ISO 17512-1:2008) was assessed as described in detail in (Lackmann et al., 2018). Shortly, two separate chambers were created in glass containers by using a tray that was placed in the middle of the glass container. One side of the container was filled with the 200 g control soil and the other side with 200 g of treated soil. After removing the separation tray, 10 earthworms per container were placed on the middle line and the glass containers then incubated for in a climate chamber 48 h at 20° C under constant light conditions. At least three replicates each were performed together with the appropriate controls. At the end of the exposure period the number of earthworms on each side was determined by hand sorting. The earthworms that were on the separating line were sorted depending on the location of their heads.

2.7. Subcellular markers

2.7.1. General measurement preparations

2.7.1.1. Exposures. As no mortality was recorded for the tested concentrations during the toxicity tests, all following exposures were performed using the same concentrations, exposure periods and test conditions. Experiments were conducted in two independent replicates with 10 earthworms per replicate with appropriate controls performed in parallel. The glass containers were placed in constant light in a climate chamber at 20 °C for the respective exposure times of 2, 7, 14 and 28 d.

2.8. Sample preparation

After the end of the respective exposure period earthworms were removed from the soil, thoroughly cleaned with distilled water, dried and the weight of each earthworm was determined. Earthworms were then individually placed in 2 mL tubes and homogenized on ice in cold sodium phosphate buffer (0.1 M, pH 7.2, in ratio 1:5 w:v) with an Ultra-Turrax T18 homogenizer. After homogenization, the samples were centrifuged for 30 min at 9000 *g* at 4 °C. Then the supernatant (post-mitochondrial fraction, S9) was transferred to a set of fresh tubes in 3 aliquots per sample and the pellets discarded. The aliquots of the S9 samples were snap frozen and stored at −80 °C until further usage.

2.9. Protein content determination

Measurements of protein concentration were conducted in 96-well plates using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). For each measurement 1.5 μL of the sample were added to 23.5 μL sodium phosphate buffer (0.1 M, pH 7.2) and 200 μL of the working solution. Absorbance was recorded after a 2 h incubation period at 20 °C using a Tecan Spark microplate reader at 562 nm. The calibration curve was constructed using the supplied bovine serum albumin as a standard to calculate protein concentrations accordingly.

2.9.1. Enzymatic biomarker measurements

2.9.1.1. Glutathione S-transferase (GST) measurements. GST activity (Habig & Jakoby, 1981) was measured as described in detail in (Lackmann et al., 2021). Kinetic measurements of absorbance were performed using a Tecan Spark microplate reader at 340 nm for 2 min at room temperature. Protein content was determined and the specific enzyme activity was calculated and given in nmol of conjugated GSH in one min per mg of proteins.

2.9.1.2. Catalase (CAT) measurements. CAT activity (Claiborne, 1985) was measured in triplicates in 96-well UV plates. The assay mixture contained 100 μL sodium phosphate buffer (0.1 M, pH 7.2), 100 μL H_2O_2 (0.019 M) and 3 μL of the sample (S9). Kinetic absorbance measurements were conducted using a Tecan Spark microplate reader at 240 nm for 3 min (measuring every 15 s) at 20 °C. After protein content determination, the specific enzyme activity was calculated and given in μmol of degraded H_2O_2 in one min per mg of proteins.

2.9.1.3. Glutathione reductase (GR) measurements. GR activity (Habig & Jakoby, 1981) was measured as described in detail in (Lackmann et al., 2021). Kinetic measurements of absorbance were performed using a Tecan Spark microplate reader at 340 nm for 5 min at room temperature. After the amount of protein was determined for each sample, the specific enzyme activity was calculated and given in nmol of reduced GSSG in one min per mg of proteins.

2.9.1.4. Acetylcholine esterase (AChE) measurements. Measurements of *AChE* activity (Ellman et al., 1961) were performed as described in detail in (Lackmann et al., 2021). Kinetic measurements of absorbance were performed using a Tecan Spark microplate reader at 412 nm for 2 min (measuring every 15 s) at 20 °C. After protein content measurements, the specific enzyme activity was calculated and given in nmol of acetylthiocholine iodide hydrolyzed in one min per mg of proteins.

2.9.1.5. Carboxylesterase (CES) measurements. CES activity (Hosokawa & Satoh, 2001) was measured as described in detail in (Lackmann et al., 2021). Kinetic measurements of absorbance were performed using a Tecan Spark microplate reader at 405 nm for 2 min (measuring every 15 s) at 20 °C. After protein content measurements, the specific enzyme activity was calculated and given in nmol of 4-nitrophenol produced per one min per mg of protein.

2.9.1.6. Glutathione peroxidase (GPx) measurements. GPx activity (Wendel, 1980) was measured in triplicates in 96-well plates. The reaction cocktail contained 50 mM phosphate buffer with 0.4 mM EDTA (pH = 7.0), 1 mM sodium azide, NADPH (stock solution), glutathione reductase (100 U/mL) and GSH (200 mM). For measurements, 3 μL sample were added to 200 μL reaction cocktail and the mixture left at room temperature for 5 min before 3.3 μL hydrogen peroxide (0.042%) were added and measurements started. Kinetic measurements of absorbance were performed using a Tecan Spark microplate reader at 340 nm for 10 min (every 30 s) at 20 °C. After determination of protein contents in samples, the specific enzymatic activity was calculated and expressed as nmol of oxidized NADPH per mg of proteins.

2.9.2. Fluorescence-based assessment of microplastic effects on oxidative stress markers

Fluorescence-based measurements of oxidative stress-related markers were measured according to (Lackmann et al., 2021). Two fluorescent probes are used, namely, CellTracker Green CMFDA for the detection of thiols and CM-H2DCFDA for the detection of general reactive oxygen species. The samples were diluted in 1:10 ratio and added in triplicate to 96-well plates and blanks were performed in parallel. After a 30 min incubation period at 25 °C, fluorescence was measured using a Tecan Spark microplate reader at 485 nm (ex.) and 530 nm (em.) with the gain set to 50.

2.9.3. Assessment of multixenobiotic resistance (MXR) activity

Measurements of MXR activity were performed based on a protocol by (Hackenberger et al., 2012). Namely, the same exposure conditions were used, but a separate set of exposures was performed. To each glass container 400 g soil were added, the respective microplastic thoroughly mixed in with the soil, 40 mL of a 0.5 mg/ml rhodamine stock solution (prepared in distilled water) added. Ten earthworms were then placed in each container and covered with aluminum foil to keep the exposure in the dark due to the light sensitivity of the fluorescent RB solution. The exposures were conducted at 20 °C for the respective exposure times of 2, 7, 14 and 28 d. After the end of the exposures, samples were prepared and protein content measured as described in *2.5.1.*, but in the dark. Fluorescence of the S9 samples was measured with a Tecan Spark microplate reader in triplicates in 96-well plates at 553 nm (ex.) and 578 nm (em.) with gain set to 55. The calibration curve was constructed using RB (stock solutions were prepared in a 1:2 dilution row starting with 0.25 μM RB) and used for calculation of RB content in each. MXR activity was expressed as nmol RB per mg proteins. The whole experiment was conducted twice to determine the repeatability of the results.

2.10. Reproduction

Assessment of reproduction success was conducted according to OECD Guideline 222 (OECD, 2016) with slight changes described below. The exposures were performed similar to the mortality assessment with the addition of weekly feeding of 3.5 g of cooked potatoes. Ten earthworms were placed in each glass container and kept in a climate chamber at 20 °C under constant light conditions. The exposure was concluded after 28 d when adult earthworms were removed from the soil and weighed. The soil was manually searched for cocoons and juveniles and additionally wet sieved. After the cocoons and juveniles were taken out, they were counted, and the cocoons thoroughly cleaned with distilled water and placed on damp filter paper in petri dishes for another 28 d. Hatching of cocoons was observed and recorded daily and the filter paper regularly moistened to avoid drying out. Exposures were conducted in 5 independent replicates with 10 adult earthworms per replicate with an appropriate negative control group run in parallel to each exposure.

2.11. Data analysis

Prior to further analysis procedures, data was prepared in Microsoft Office Excel 2016. Data of the subcellular markers and reproduction assessment were analyzed using GraphPad Prism 9 (GraphPad Software, Inc., California, USA). Firstly, data were checked for equality of variances (Bartlett test) and normality (Shapiro-Wilk test). Whenever a normal distribution of data was shown, one-way ANOVA was applied followed by the Dunnett's multiple comparison test to determine the significance levels reached in comparison to the control. If data was not normally distributed, Kruskal-Wallis test was performed. Avoidance behavior data was analysed for significance using students' *t*-test. The level of significance was set to $p < 0.05$ throughout the study.

3. Results

3.1. Toxicity of polystyrene-HBCD and car tire abrasions

After 2, 7, 14 and 28 d exposures of earthworms *E. andrei* polystyrene-HBCD and car tire abrasions in soil, no mortality was recorded for any of the tested concentrations. Thus, no dose–response curves and lethal concentrations could be established (data not shown).

3.2. Avoidance behavior

The results of the assessment of avoidance behavior of *E. andrei* after exposures to polystyrene-HBCD and car tire abrasion are shown in Table 1. None of the tested microplastic concentrations significantly

Table 1
Effect of two types of microplastics on the avoidance behavior of *E. andrei* in soil 48 h exposures to the respective microplastics (MP1 – car tire abrasion, MP2 – polystyrene-HBCD, NC-– negative control) in soil for the determination of avoidance behavior. NRHF: no reduced habitat function is considered when > 20% of earthworms in treated soil, n = 3.

Microplastic	Concentration (mg/kg)	Distribution (%)		Net response (%)	Toxicity evaluation
		Control	Treated		
NC	*0*	*43*	*57*	*13.33*	*NRHF*
MP1	*1*	*60*	*40*	*10*	*NRHF*
	1000	*55*	*45*	*20*	*NRHF*
MP2	*0.1*	*53*	*47*	*6.67*	*NRHF*
	100	*53*	*47*	*6.67*	*NRHF*

affected the avoidance behavior of the earthworms.

3.3. Biomarker responses

The results of the 2 d exposures showed no significant changes in enzymatic biomarker activities, multixenobiotic resistance activity or oxidative stress- related markers (Fig. 1).

After the 7 d exposures, significant changes were observed for AChE activity and fluorescence related to ROS levels (Fig. 2). The higher concentration of polystyrene-HBCD, namely, 100 mg/kg polystyrene-HBCD, significantly inhibited the AChE activity compared to the negative control. Fluorescence related to ROS levels was significantly decreased compared to the negative control after exposures to 1 mg/kg car tire abrasion.

The results of the 14 d exposures (Fig. 3) show a change in affected biomarkers, with significant changes observed for both ROS and GSH levels. Relative fluorescence for ROS level determination was decreased for both lower concentrations of the investigated microplastics. The concentration of 1 mg/kg car tire abrasion also significantly decreased relative fluorescence related to GSH levels, while 0.1 mg/kg polystyrene-HBCD only caused a slight non-significant decrease.

After 28 d, significant changes could only be observed for CAT

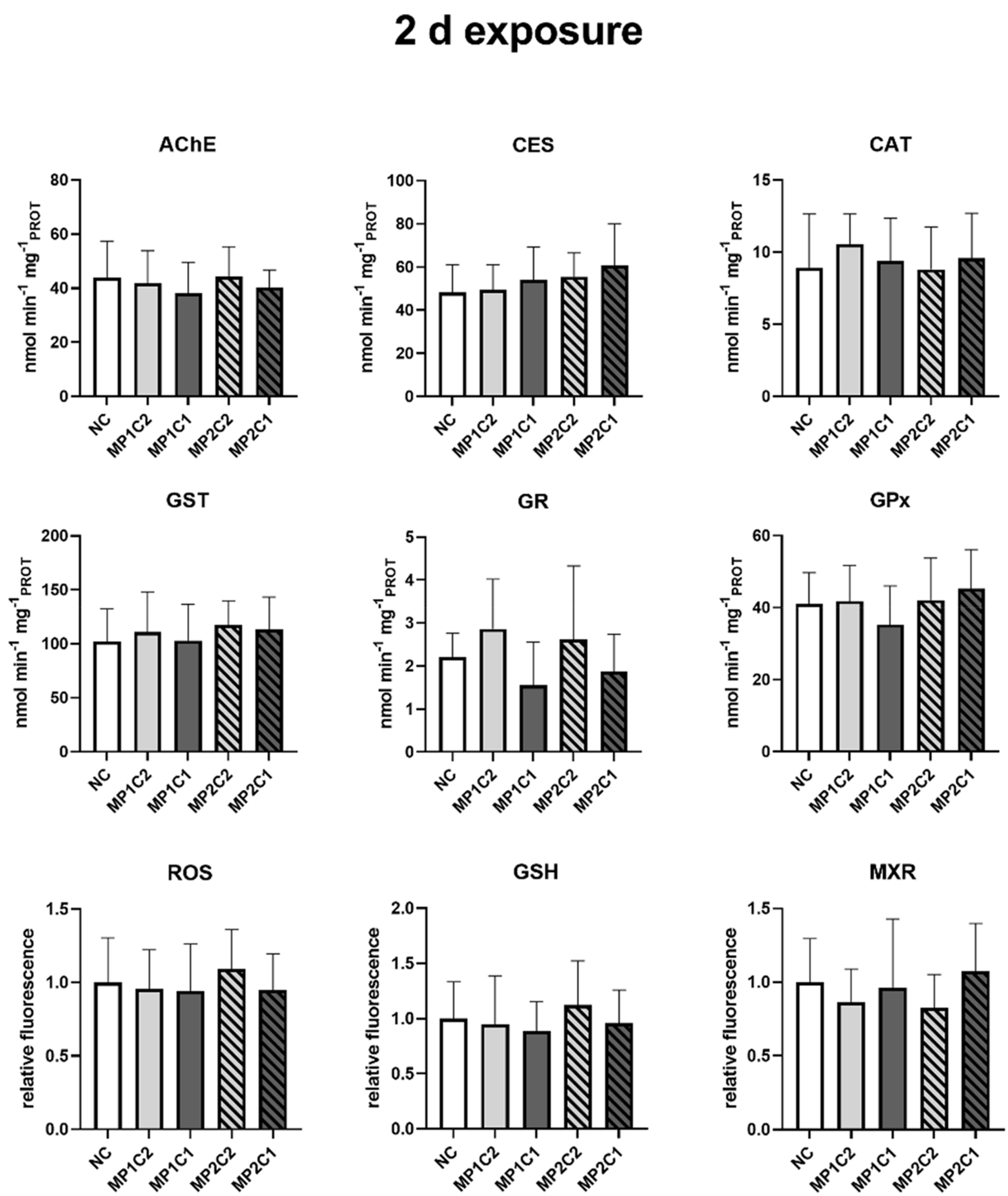

Fig. 1. Subcellular responses after 2 d exposures of earthworm *E. andrei* to two types of microplastics. Specific activities of: acetylcholinesterase (AChE), carboxylesterase (CES), catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx) and relative fluorescence of reactive oxygen species (ROS), reduced glutathione (GSH) and rhodamine B (MXR) after 2 d exposures of car tire abrasions and polystyrence-HBCD to *E. andrei* (mean ± standard deviation; N = 20). Abbreviations: NC: negative control, MP1C1: 1000 mg/kg car tire abrasion; MP1C2: 1 mg/kg car tire abrasion; MP2C1: 100 mg/kg polystyrene-HBCD; MP2C2: 0.1 mg/kg polystyrene-HBCD.

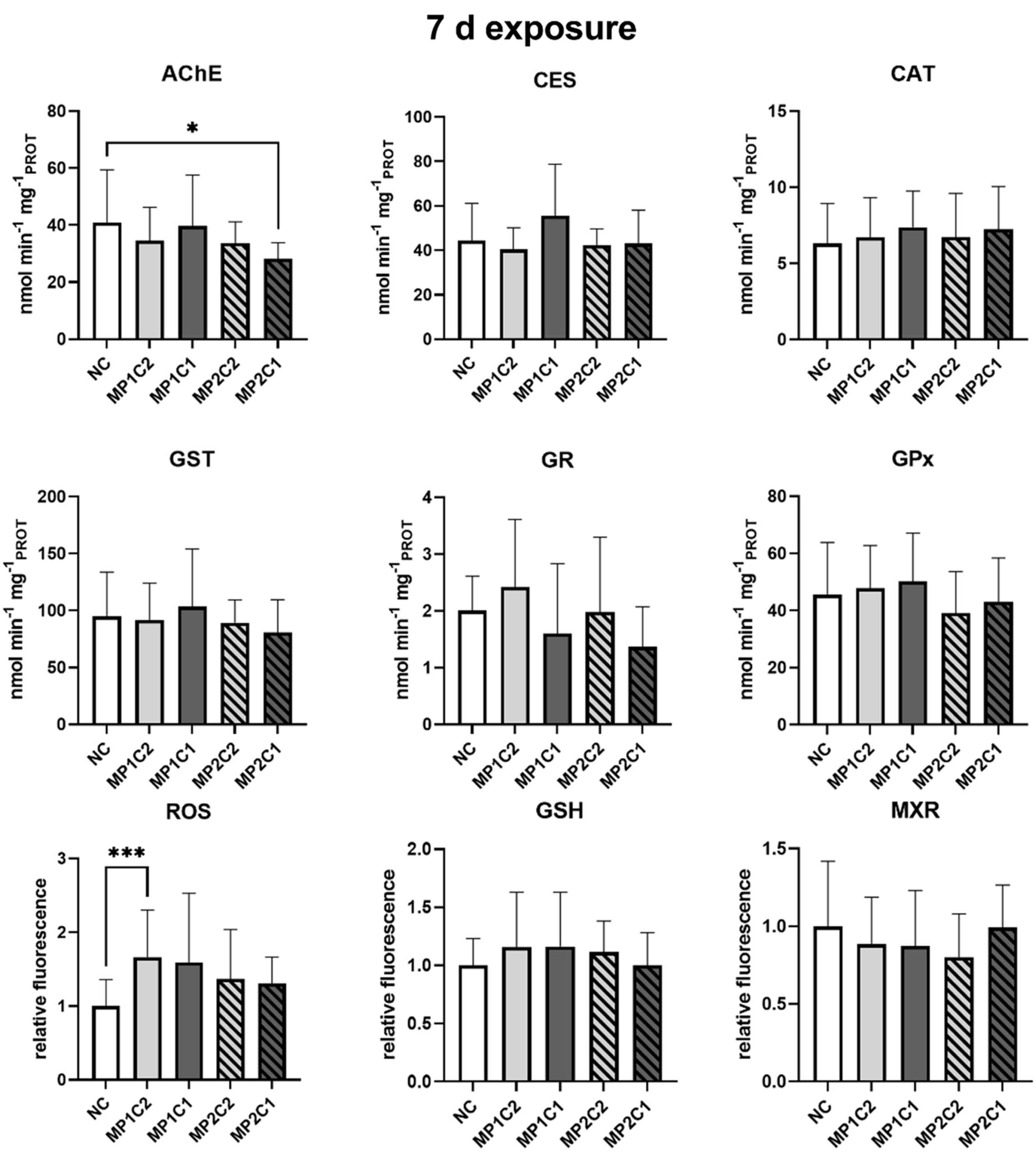

Fig. 2. Subcellular responses after 7 d exposures of earthworm *E. andrei* to two types of microplastics. Specific activities of: acetylcholinesterase (AChE), carboxylesterase (CES), catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx) and relative fluorescence of reactive oxygen species (ROS), reduced glutathione (GSH) and rhodamine B (MXR) after 7 d exposures of car tire abrasions and polystyrence-HBCD to *E. andrei* (mean ± standard deviation; N = 20). Abbreviations: NC: negative control, MP1C1: 1000 mg/kg car tire abrasion; MP1C2: 1 mg/kg car tire abrasion; MP2C1: 100 mg/kg polystyrene-HBCD; MP2C2: 0.1 mg/kg polystyrene-HBCD. Significant differences between control and microplastic treatments (ANOVA followed by Dunnett's multiple comparison test) are labeled with * ($p < 0.05$) and *** ($p < 0.001$).

activity (Fig. 4). The CAT activity was decreased after exposure to both 1 and 1000 mg/kg car tire abrasion, but a significant decrease was only observed for the higher concentration.

3.4. Reproduction

During all exposures for the determination of reproduction success, no mortality was observed after the 28 d of exposure. Results of reproduction success are summarized in Fig. 5. No significant differences occurred in any of the tested concentrations for either cocoons production or juveniles hatched. A decrease in cocoon production could be observed for exposures to 1 mg/kg car tire abrasion, but these changes were not significant compared to the negative control.

4. Discussion

Microplastics have become ubiquitous in soils with terrestrial ecosystems being major sinks for microplastic pollution (Büks & Kaupenjohann, 2020; Hurley & Nizzetto, 2018). Microplastics are highly persistent pollutants, that can interact with the abiotic environment, impact terrestrial organisms directly or indirectly and function as carriers for other contaminants (Baho et al., 2021). Therefore, understanding their toxic mechanisms is important to gain insight into the possible negative effects this global threat poses to the environment. In this study two types of commonly found secondary microplastics were investigated, namely, car tire abrasion and polystyrene-HBCD. As the name suggests, car tire abrasions get emitted in urban areas through

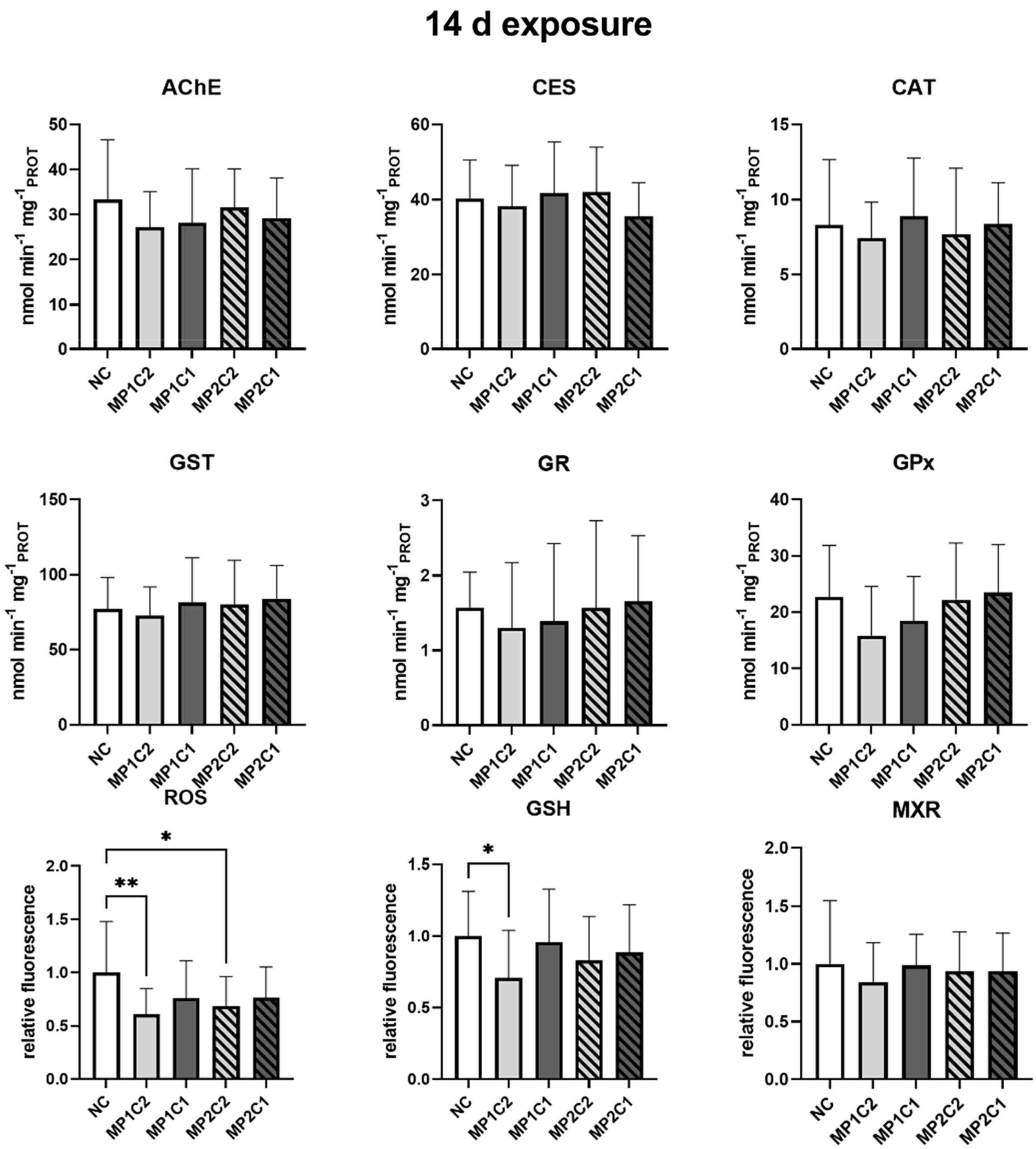

Fig. 3. Subcellular responses after 14 d exposures of earthworm *E. andrei* to two types of microplastics. Specific activities of: acetylcholinesterase (AChE), carboxylesterase (CES), catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx) and relative fluorescence of reactive oxygen species (ROS), reduced glutathione (GSH) and rhodamine B (MXR) after 14 d exposures of car tire abrasions and polystyrence-HBCD to *E. andrei* (mean ± standard deviation; N = 20). Abbreviations: NC: negative control, MP1C1: 1000 mg/kg car tire abrasion; MP1C2: 1 mg/kg car tire abrasion; MP2C1: 100 mg/kg polystyrene-HBCD; MP2C2: 0.1 mg/kg polystyrene-HBCD. Significant differences between control and microplastic treatments (ANOVA followed by Dunnett's multiple comparison test) are labeled with * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

traffic and are mainly a mixture of different polymers, additives, and metals and highly vary in shape, size and chemical composition. In a previous study, it was for example determined that the car tire abrasion, that was also used in the present study, contained around 11.7 g/kg zinc (Müller et al., 2022).

Polystyrene, a main component of insulation boards, is commonly found on construction sites in form of extruded polystyrene foam. While also highly variable in shape and size, it is mostly of a highly pure grade and not mixed with other synthetic polymers. However, it often does contain additives, in the case of the here investigated polystyrene, 1% of the flame retardant HBCD. This 1% HBCD concentration represents a rather high substance concentration. Due to its persistent, bioaccumulative and toxic properties, HBCD is regarded as substance of very high concern and the production, use and marketing of products with contents > 100 mg/kg has been gradually banned in the EU since 2016 (European Commission, 2016). However, materials used before 2016 are still in use and constitute a possible source for microplastics with high HBCD contents. The variable composition and shape of plastics and the possible leaching of chemicals increase the need to thoroughly investigate the potential adverse effects of microplastics, which could be either of physical or chemical nature (Campanale et al., 2020).

4.1. Mortality

None of the investigated microplastic concentrations showed acute or chronic toxicity in earthworms, i.e., no mortality or morphological

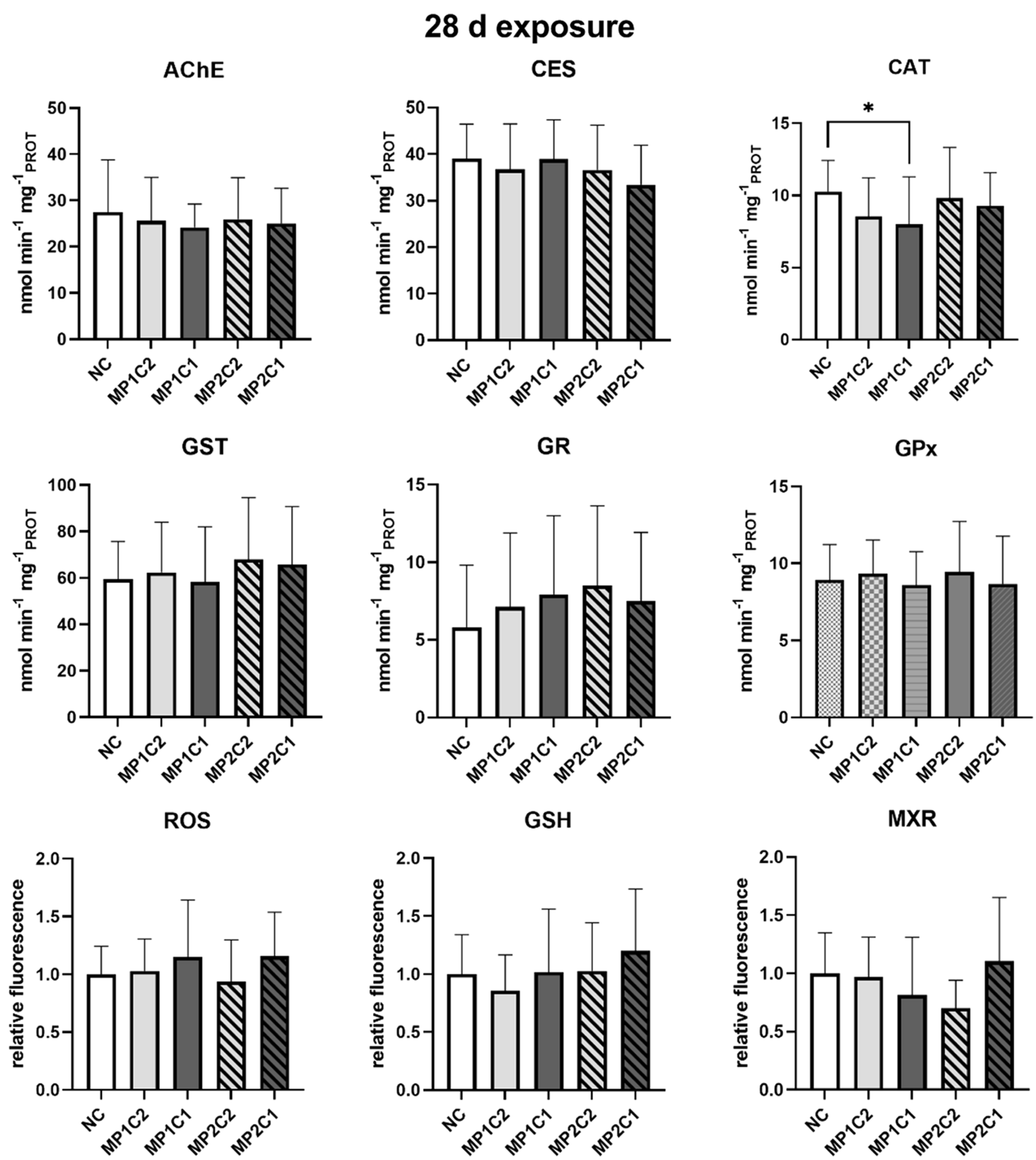

Fig. 4. Subcellular responses after exposures for 28 d of earthworm *E. andrei* to two types of microplastics. Specific activities of: acetylcholinesterase (AChE), carboxylesterase (CES), catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx) and relative fluorescence of reactive oxygen species (ROS), reduced glutathione (GSH) and rhodamine B (MXR) after 28 d exposures of car tire abrasions and polystyrence-HBCD to *E. andrei* (mean ± standard deviation; N = 20). Abbreviations: NC: negative control, MP1C1: 1000 mg/kg car tire abrasion; MP1C2: 1 mg/kg car tire abrasion; MP2C1: 100 mg/kg polystyrene-HBCD; MP2C2: 0.1 mg/kg polystyrene-HBCD. Significant differences between control and microplastic treatments (ANOVA followed by Dunnett's multiple comparison test) are labeled with * ($p < 0.05$).

changes were observed. While research on the ecotoxicological effects of microplastics is limited, a study by (Jiang et al., 2020) has observed mortality of earthworms (*Eisenia fetida*) after exposures to 0.1 mg/kg polystyrene for 14 d. However, this observation was not made after exposures to higher concentrations of 1 mg/kg polystyrene, which fits more with the results of the present study. Other studies confirm this low acute toxicity with no observed mortality after even higher concentrations of PS or exposures to other microplastics such as polyethylene (Rodríguez-Seijo et al., 2018a; 2018b; Wang et al., 2019). A study by (Sheng et al., 2021) investigated effects of car tire abrasions on the earthworm *E. fetida* after exposures of 14 and 28 d in artificial soil and only observed changes on enzymatic biomarkers but no mortality. Overall, this shows that research so far has not observed high mortality rates associated with microplastic exposures, thus suggesting that other endpoints might be more suitable to determine microplastic toxicity.

4.2. Avoidance behavior

Avoidance behavior is considered to be of high ecological relevance and also a highly sensitive endpoint (Hund-Rinke et al., 2003). The range of behavioral responses in earthworms is rather limited but gives important insight into potential effects on the soils habitat function due to earthworms important function as ecosystem engineers (Pelosi et al., 2014). Behavioral changes can be a first response to altered environmental conditions (Wong & Candolin, 2015) and as (Ford et al., 2021) argue should thus be a part of ecotoxicological assessments.

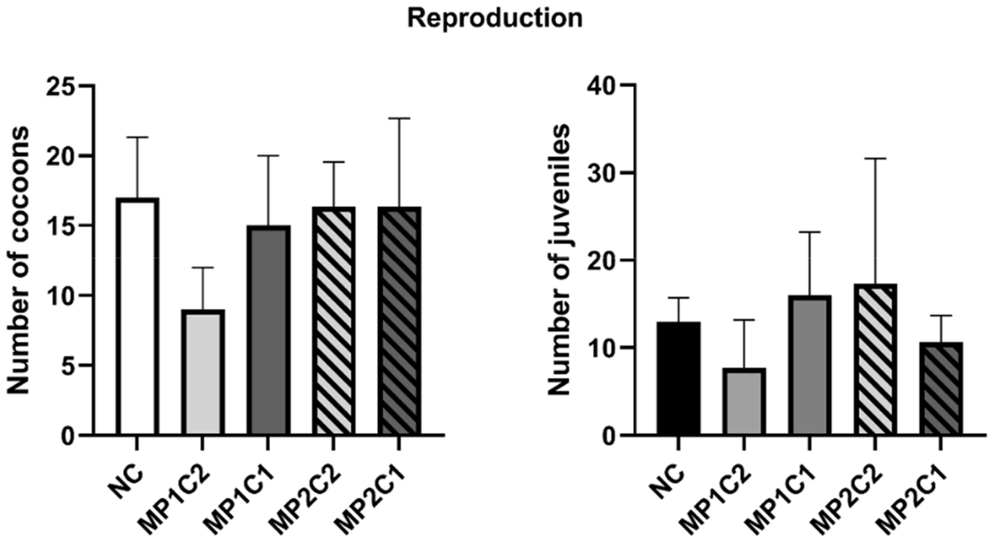

Fig. 5. Results of the earthworm reproduction test after exposure to two types of microplastics. Numbers of cocoons and juveniles after 28 d exposures of earthworm *E. andrei* to car tire abrasion and polystyrene-HBCD in soil. After 28 d, juveniles counted and cocoons removed from soil and hatching observed for another 28 d (mean ± standard deviation; N = 3). Abbreviations: NC: negative control, MP1C1: 1000 mg/kg car tire abrasion; MP1C2: 1 mg/kg car tire abrasion; MP2C1: 100 mg/kg polystyrene-HBCD; MP2C2: 0.1 mg/kg polystyrene-HBCD.

In the present study, no changes of avoidance behavior could be observed for any of the tested microplastics. While studies on avoidance behavior after exposures to more commonly investigated pollutants such as pesticides are rather common (de Sousa & de Andréa, 2011; García-Santos & Keller-Forrer, 2011; Lackmann et al., 2018; Marques et al., 2009; Pereira et al., 2010), there are only a small number of studies on avoidance behavior after microplastic exposures, therefore only allowing limited comparisons to current literature. A study by (Baeza et al., 2020) investigated the effects of microplastic mixtures (2.5%; 5%, and 7% w/w) on the avoidance behavior of *Lumbricus terrestris* and while similarly to the present study the microplastics showed no effects on earthworm behavior, they did cause morphological changes in the earthworm in the form of lesions. This suggests that microplastics might cause mechanical or biological damages when the defense mechanism of avoidance behavior is not triggered in plastic polluted areas. (Judy et al., 2019) investigated the effects of municipal waste organic outputs mixed with microplastic on the avoidance behavior of *E. fetida* and though for some exposure scenarios avoidance behavior was observed, the authors concluded that these behavioral changes are most likely not to be attributed to the addition of the microplastics. Similarly, a study by (Hodson et al., 2017) observed no avoidance behavior after exposures of *L. terrestris* to Zn-adsorbed microplastics.

Avoidance behavior is a crucial defense mechanism in many organisms and the lack of avoidance of microplastics could potentially facilitate the uptake of other pollutants that might otherwise be avoided such as pesticides or leachates of additives. While this shows that there is still an immense knowledge gap regarding the influence on earthworm behavior in plastic-contaminated soils, it is also clear that avoidance behavior can be a valuable endpoint in effect assessments.

4.3. Subcellular markers

In contrary to the lack of effects observed on a whole-organism level, the obtained results show significant changes on multiple subcellular markers during different exposure times. Both enzymatic and non-enzymatic methods were used to investigate sublethal effects and gain insight into potential modes of action of the two investigated types of microplastics. None of the chosen subcellular markers showed any significant changes after the shortest exposure period of 2 d. This might suggest that the microplastics were not ingested in a sufficient amount yet to cause any effects or that the microplastics only affect earthworm after longer exposure periods. Overall, time-dependent changes could be observed with most biomarkers showing a recovery after 28 d.

Acetylcholinesterase, a commonly used enzymatic biomarker for neurotoxicity, was inhibited only after exposure to 100 mg/kg polystyrene-HBCD for 7 d. After an exposure period of 14 and 28 d AChE activity was not affected significantly by any microplastic exposures. In contrary to these results, (Y. Chen et al., 2020) investigated the effects on the AchE activity in earthworm *E. fetida* after exposure to micro-sized low-density polyethylene during similarly chosen exposure periods. They observed increases in AchE activity after exposures to 1 and 1.5 g/kg and only after 21 and 28 d. Studies on aquatic organisms investigating the effects of polystyrene microplastic show an inhibition of AchE activity (e.g. exposures of the Chinese mitten crab *Eriocheir sinensis* to polystyrene microbeads) (Yu et al., 2018) agreeing more with the results in the present study. As the study by (Y. Chen et al., 2020) investigated a different type of microplastic, this might also indicate varying mode of action depending on the plastic type, but also the shape, size and potential influence of additives. The CES activity was investigated as a biotransformation enzyme involved in the xenobiotic metabolism but overall was not significantly affected by the microplastics during investigated exposure periods. Again, only very few studies investigated the effects of microplastics on CES activity. A study on the effects on fish however similarly showed no effect after exposures to polystyrene microplastic (Schmieg et al., 2020). As another enzyme involved in xenobiotic metabolism and part of the glutathione system, GST activity was investigated. However, it also was not significantly affected in the present study suggesting that no mechanisms of the xenobiotic metabolism were induced. A study by (Rodríguez-Seijo, da Costa, et al., 2018) showed an increase in GST activity after exposures of *E. fetida* to low-density polyethylene microplastics, again as already argued for changes in AchE activity, suggesting that the modes of action differ depending on the microplastic type due to their different chemical properties.

As enzymatic biomarkers related to oxidative stress induction, CAT, GR and GPX activity were investigated in the present study. A fairly commonly used oxidative stress biomarker, CAT activity has been investigated after exposures of both aquatic and soil organisms to various environmental pollutants. As the obtained results show, exposures to 1 mg/kg car tire abrasion caused an inhibition of CAT activity, but only after 28 d of exposure. Both increases and decreases of CAT activity have been reported after microplastic exposures (Y. Chen et al., 2020; Rodríguez-Seijo, da Costa, et al., 2018) and show the potential of microplastics to induce oxidative stress. However, both other investigated biomarkers for oxidative stress, GR and GPx, did not show significant changes in activity after any of the exposure periods. In the case of GR activity, this seems to fit with results from (Scopetani et al., 2020) who investigated the effects of polyethylene microplastics on Tubifex

tubifex and observed no significant changes in GR activity. However, using GPx as a biomarker for oxidative stress, (Yu et al., 2018) have indeed observed changes in GPx activity after crabs were exposed to polystyrene microplastic. Results from (Q. Chen et al., 2017) however agree with our findings, as no changes in GPx activity were observed after exposures of *Danio rerio* larvae to micro- and nano-sized polystyrene. As the mentioned studies that investigated changes in GR and GPx activity investigated effects in aquatic organisms, it is not clear whether these different responses could be attributed to different toxicity responses and uptake routes.

As non-enzymatic markers for oxidative stress, a fluorescence-based assay was used to measure ROS and GSH levels that has been shown to be a fairly sensitive method before (Lackmann et al., 2021). The results of the present study showed a decrease in GSH levels after 14 d of exposure to car tire abrasion. However, during all other exposure times GSH levels did not significantly differ from the negative control. We observed no significant effects of polystyrene-HBCD on GSH levels, where in contrary (Jiang et al., 2020) showed increased GSH levels after exposures of lower concentrations of polystyrene to earthworm *E. fetida*. However, due to the complex interaction of antioxidants and oxidants, these differences in observed effects on GSH levels after exposures to the same microplastic are not unlikely.

Observations of ROS levels are important to gain insight into this very sensitive redox balance. In the present study, significant changes in fluorescence and thus ROS levels could be observed for both microplastic types. Namely, after 7 d of exposure ROS levels were increased after the exposure to 1 mg/kg car tire abrasion but afterwards ROS levels stabilized and showed no more significant changes after exposure to car tire abrasion. For exposures to polystyrene-HBCD a decrease was only observed after the 14 d exposure to the concentration of 100 mg/kg. These changes can be explained through upregulations in gene expression or an inhibition of ROS scavengers (antioxidants) and indicate an induction occurrence of oxidative stress. As both ROS and GSH levels were significantly influenced after 14 d exposures to car tire abrasion, there is a clear indication for changes in the normal redox balance of the cells. This conclusion is further supported by the inhibition of catalase activity after 28 d showing the importance of using different methods for the evaluation of molecular effects.

As a first line of defense, MXR activity is an important cellular defense mechanism and can be a valuable endpoint to understand microplastic toxicity. The results of this study show no significant changes in RB concentrations and thus MXR activity. If a decrease in RB content is observed, it indicates an induction of MXR activity, whereas increased RB accumulations indicate an inhibition of MXR activity. To the best of our knowledge, no other study has investigated the effects of microplastics on MXR activity in earthworms or other soil organisms. However, studies on aquatic organisms have shown the inhibition of MXR activity after exposure to micro and nano-sized polystyrene particles of Mediterranean mussels *Mytilus galloprovincialis* and the monogonont rotifer *Brachionus koreanus* (Franzellitti et al., 2019; C. B. Jeong et al., 2018).

4.4. Reproduction

Reproductive success is an ecologically highly relevant and simultaneously sensitive endpoint. Due to the size of microplastic particles and often reported histopathological damages (Jiang et al., 2020; Rodriguez-Seijo et al., 2017), it is likely that microplastics might influence the reproductive output. In this study, however, no significant changes to the reproductive output of *E. andrei* were observed after exposures to the two selected microplastic types. A slight decrease in cocoon number after exposure to car tire abrasion was observed, but it was not statistically significant. As mentioned, due to potential histopathological changes, microplastics have been reported to cause a decrease in reproductive success (Huerta Lwanga et al., 2016; Lahive et al., 2019). (Kwak & An, 2021) observed damaged gonads in *E. fetida* after exposures to polyethylene. As we did not investigate histopathological changes, it is not clear whether the reproductive organs of the earthworms were influenced in any way. However, in agreement with our study, (Rodriguez-Seijo et al., 2017) also did not observe any impact on reproductive success after exposures of *E. andrei* to polyethylene pellets even though histopathological changes were observed. As the exposure period recommended in the OECD guideline can still be considered rather short, it might also be possible that changes in reproductive success will only be observed after longer exposure periods and should thus be reconsidered in future studies.

4.5. Knowledge gaps

Secondary microplastics such as car tire abrasion have been shown to be of highly dynamic particle properties (Klöckner et al., 2020), thus making research on the possible effects even more difficult. However as current literature suggests leachings of the additives or sorbed contaminants to be the major drivers of microplastic toxicity (Capolupo et al., 2020; Zimmermann et al., 2020), it is important to study these complex microplastics compared to pure microplastic particles. Large data sets on soil toxicity are still very scarce, thus various apical and mechanistic endpoints and both short and long-term exposures were chosen in this study. Overall, the results of subcellular markers suggest oxidative stress as a potential mode of toxicity of the investigated microplastics. Also, stronger effects of the car tire abrasion than the polystyrene-HBCD, where a complete recovery of all subcellular markers occurred, were observed after 28 d. The investigated apical endpoints in this study did not show any effects of the microplastics, however the mechanistic endpoints helped to gain insight into effects on a molecular level. The extensive biomarker data over various time points elucidate potential time-dependent toxicity mechanisms and help fill a large data gap concerning terrestrial toxicity. However, so far it can be mostly considered a first step in the right direction. Promising tools such as putative Adverse Outcome Pathways are in desperate need of larger data sets to link toxicity mechanisms and adverse outcomes to understand the potential impact of microplastic pollution on various ecosystems. While first attempts by (J. Jeong & Choi, 2019) suggest ROS formation as the molecular initiating event for observed adverse outcomes (e.g. mortality, decreasing rates of growth, and reproduction failure) which would support the results of our study, our chosen biomarkers were mostly related to oxidative stress, thus future studies should further investigate other potential toxicity mechanisms.

5. Conclusions

In conclusion, this study demonstrated the time-dependent effects of two types of secondary microplastics on the earthworm *E. andrei* on multiple levels of biological organization in a realistic scenario considering previously determined German exposure levels. Our results showed that while there were no significant effects on behavior or reproduction, environmentally relevant concentrations can cause time-dependent changes on a subcellular level which is often viewed as an early-warning signal. Both types of microplastics induced changes on a subcellular level, however, only car tire abrasion caused significant effects after 28 d. The chosen test battery provides a wide range of valuable insight into the effects of microplastics on earthworm and emphasizes the importance of a more integrative ecotoxicological assessment of microplastics, especially considering the immense knowledge gaps of the effect of these emerging contaminants on soil ecosystems. Overall, this study determined only minor, almost negligible, effects of the investigated secondary microplastics during the chosen exposure scenarios. For a further insight into the effects of microplastics on soil ecosystems, future studies should thus include a wider range of microplastic types, composition, shapes and sizes, chose a more diversified biomarker battery and further investigate the main drivers of microplastic toxicity.

Funding

This work was supported by the BAM (Bundesanstalt für Materialforschung und –prüfung) and the Umweltbundesamt (project code number 3717 72 232 0).

7. Availability of data and materials.

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Author contributions

Conceptualization: CL, MV, UB and HH. Investigations: CL, AS, SE, AM. Data analysis: AS and CL. Writing: AS and CL. Review and editing: all. All authors read and approved the final manuscript.

CRediT authorship contribution statement

Carina Lackmann: Conceptualization, Investigation, Data analysis. **Mirna Velki:** Conceptualization, Data analysis. **Antonio Šimić:** Investigation, Data analysis. **Axel Müller:** Investigation. **Ulrike Braun:** Conceptualization. **Sandra Ečimović:** Investigation. **Henner Hollert:** Conceptualization.

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

The authors kindly thank the Deutsche Bundesstiftung Umwelt (DBU, German Federal Environmental Foundation) for a personal scholarship granted to Carina Lackmann.

The Tecan Spark microplate reader was purchased with the Alexander von Humboldt Foundation equipment grant awarded to Dr. Mirna Velki.

References

Baeza, C., Cifuentes, C., González, P., Araneda, A., Barra, R., 2020. Experimental exposure of lumbricus terrestris to microplastics. Water Air Soil Pollut. 231 (6) https://doi.org/10.1007/s11270-020-04673-0.

Baho, D.L., Bundschuh, M., Futter, M.N., 2021. Microplastics in terrestrial ecosystems: moving beyond the state of the art to minimize the risk of ecological surprise. Glob. Change Biol. 27 (17), 3969–3986.

Bläsing, M., Amelung, W., 2018. Plastics in soil: analytical methods and possible sources. Sci. Total Environ. 612, 422–435. https://doi.org/10.1016/j.scitotenv.2017.08.086.

Blouin, M., Hodson, M.E., Delgado, E.A., Baker, G., Brussaard, L., Butt, K.R., Dai, J., Dendooven, L., Peres, G., Tondoh, J.E., Cluzeau, D., Brun, J.-J., 2013. A review of earthworm impact on soil function and ecosystem services. Eur. J. Soil Sci. 64 (2), 161–182. https://doi.org/10.1111/ejss.12025.

Borrelle, S.B., Ringma, J., Law, K.L., Monnahan, C.C., Lebreton, L., McGivern, A., Murphy, E., Jambeck, J., Leonard, G.H., Hilleary, M.A., Eriksen, M., Possingham, H. P., De Frond, H., Gerber, L.R., Polidoro, B., Tahir, A., Bernard, M., Mallos, N., Barnes, M., Rochman, C.M., 2020. Predicted growth in plastic waste exceeds efforts to mitigate plastic pollution. Science 369 (6510), 1515–1518.

Braun, U., Müller, A., Kittner, M., Altmann, K., Meierdierks, J., Grathwohl, P., Lackmann, C., Šimić, A., Weltmeyer, A., Schmitz, M., Tofan, S., Roß-Nickoll, M., Velki, M., & Hollert, H. (2021). *Plastik in Böden – Vorkommen, Quellen, Wirkungen Abschlussbericht*.

Büks, F., Kaupenjohann, M., 2020. Global concentrations of microplastics in soils – a review. Soil 6 (2), 649–662. https://doi.org/10.5194/soil-6-649-2020.

Campanale, C., Massarelli, C., Savino, I., Locaputo, V., Uricchio, V.F., 2020. A detailed review study on potential effects of microplastics and additives of concern on human health. In: International Journal of Environmental Research and Public Health (Vol. 17, Issue 4). MDPI AG. https://doi.org/10.3390/ijerph17041212.

Capolupo, M., Sørensen, L., Jayasena, K.D.R., Booth, A.M., Fabbri, E., 2020. Chemical composition and ecotoxicity of plastic and car tire rubber leachates to aquatic organisms. Water Res. 169, 115270. https://doi.org/10.1016/j.watres.2019.115270.

Chae, Y., An, Y.J., 2018. Current research trends on plastic pollution and ecological impacts on the soil ecosystem: a review. Environ. Pollut. 240, 387–395. https://doi.org/10.1016/j.envpol.2018.05.008.

Chamas, A., Moon, H., Zheng, J., Qiu, Y., Tabassum, T., Jang, J.H., Abu-Omar, M., Scott, S.L., Suh, S., 2020. Degradation rates of plastics in the environment. ACS Sustain. Chem. Eng. 8 (9), 3494–3511. https://doi.org/10.1021/acssuschemeng.9b06635.

Chen, Q., Gundlach, M., Yang, S., Jiang, J., Velki, M., Yin, D., Hollert, H., 2017. Quantitative investigation of the mechanisms of microplastics and nanoplastics toward zebrafish larvae locomotor activity. Sci. Total Environ. 584–585, 1022–1031. https://doi.org/10.1016/j.scitotenv.2017.01.156.

Chen, Y., Liu, X., Leng, Y., Wang, J., 2020. Defense responses in earthworms (Eisenia fetida) exposed to low-density polyethylene microplastics in soils. Ecotoxicol. Environ. Saf. 187, 109788. https://doi.org/10.1016/j.ecoenv.2019.109788.

Claiborne, A.I., 1985. Catalase activity, In: Handbook of methods in oxygen radical research, pp. 283–284.

de Sousa, A.P.A., de Andréa, M.M., 2011. Earthworm (Eisenia andrei) avoidance of soils treated with cypermethrin. Sensors 11 (12), 11056–11063. https://doi.org/10.3390/s111211056.

Souza Machado, A.A., Kloas, W., Zarfl, C., Hempel, S., Rillig, M.C., 2018. Microplastics as an emerging threat to terrestrial ecosystems. Glob. Change Biol. 24 (4), 1405–1416. https://doi.org/10.1111/gcb.14020.

Duan, J., Bolan, N., Li, Y., Ding, S., Atugoda, T., Vithanage, M., Sarkar, B., Tsang, D.C.W., Kirkham, M.B., 2021. Weathering of microplastics and interaction with other coexisting constituents in terrestrial and aquatic environments. Water Res. 196, 117011. https://doi.org/10.1016/j.watres.2021.117011.

Edwards, C.A., Bohlen, P.j., 1996. Biology and Ecology of Earthworms. Chapman and Hall.

Eisenhauer, N., 2010. The action of an animal ecosystem engineer: Identification of the main mechanisms of earthworm impacts on soil microarthropods. Pedobiologia 53 (6), 343–352. https://doi.org/10.1016/j.pedobi.2010.04.003.

Ellman, G.L., Courtney, K.D., Andres Jr., V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95. https://doi.org/10.1016/0006-2952(61)90145-9.

Commission, E., 2016. COMMISSION REGULATION (EU) 2016/293. Official Journal of the European Union 68, 48–119.

Ford, A.T., Ågerstrand, M., Brooks, B.W., Allen, J., Bertram, M.G., Brodin, T., Dang, Z., Duquesne, S., Sahm, R., Hoffmann, F., Hollert, H., Jacob, S., Klüver, N., Lazorchak, J. M., Ledesma, M., Melvin, S.D., Mohr, S., Padilla, S., Pyle, G.G., Maack, G., 2021. The role of behavioral ecotoxicology in environmental protection. Environ. Sci. Technol. 55 (9), 5620–5628. https://doi.org/10.1021/acs.est.0c06493.

Franzellitti, S., Capolupo, M., Wathsala, R.H.G.R., Valbonesi, P., Fabbri, E., 2019. The Multixenobiotic resistance system as a possible protective response triggered by microplastic ingestion in Mediterranean mussels (Mytilus galloprovincialis): Larvae and adult stages. Comparative Biochem. Physiol. Part - C: Toxicol. Pharmacol. 219 (January), 50–58. https://doi.org/10.1016/j.cbpc.2019.02.005.

Fründ, H.C., Graefe, U., Tischer, S., 2010. Earthworms as Bioindicators of Soil Quality. In Biology of Earthworms, Springer, Berlin Heidelberg https://doi.org/https://doi.org/10.1007/978-3-642-14636-7_16.

Ganesh Kumar, A., Anjana, K., Hinduja, K., Sujitha, K., Dharani, G., 2020. Review on plastic wastes in marine environment – Biodegradation and biotechnological solutions. Mar. Pollut. Bull. 150(May 2019), 110733. https://doi.org/10.1016/j.marpolbul.2019.110733.

García-Santos, G., Keller-Forrer, K., 2011. Avoidance behaviour of Eisenia fetida to carbofuran, chlorpyrifos, mancozeb and metamidophos in natural soils from the highlands of Colombia. Chemosphere 84 (5), 651–656. https://doi.org/10.1016/j.chemosphere.2011.03.036.

Geyer, R., Jambeck, J.R., Law, K.L., 2017. Production, use, and fate of all plastics ever made. Sci. Adv. 3 (7), 3–8. https://doi.org/10.1126/sciadv.1700782.

Gigault, J., ter Halle, A., Baudrimont, M., Pascal, P.Y., Gauffre, F., Phi, T.L., El Hadri, H., Grassl, B., Reynaud, S., 2018. Current opinion: what is a nanoplastic? Environ. Pollut. 235, 1030–1034. https://doi.org/10.1016/j.envpol.2018.01.024.

Habig, W.H., Jakoby, W.B., 1981. Assays for differentiation of glutathione S-transferases. Methods Enzymol. 77, 398–405. https://doi.org/10.1016/S0076-6879(81)77053-8.

Hackenberger, B.K., Velki, M., Stepic, S., Hackenberger, D.K., 2012. First evidence for the presence of efflux pump in the earthworm Eisenia andrei. Ecotoxicol. Environ. Saf. 75, 40–45. https://doi.org/10.1016/j.ecoenv.2011.06.024.

He, D., Luo, Y., Lu, S., Liu, M., Song, Y., Lei, L., 2018. Microplastics in soils: Analytical methods, pollution characteristics and ecological risks. TrAC - Trends Anal. Chem. 109, 163–172. https://doi.org/10.1016/j.trac.2018.10.006.

He, D., Bristow, K., Filipović, V., Lv, J., He, H., 2020. Microplastics in terrestrial ecosystems: a scientometric analysis. Sustain. (MDPI) 12 (20), 1–15. https://doi.org/10.3390/su12208739.

Helmberger, M.S., Tiemann, L.K., Grieshop, M.J., 2020. Towards an ecology of soil microplastics. Funct. Ecol. 34 (3), 550–560. https://doi.org/10.1111/1365-2435.13495.

Hidalgo-Ruz, V., Gutow, L., Thompson, R.C., Thiel, M., 2012. Microplastics in the marine environment: a review of the methods used for identification and quantification. Environ. Sci. Technol. 46 (6), 3060–3075. https://doi.org/10.1021/es2031505.

Hodson, M.E., Duffus-Hodson, C.A., Clark, A., Prendergast-Miller, M.T., Thorpe, K.L., 2017. Plastic bag derived-microplastics as a vector for metal exposure in terrestrial invertebrates. Environ. Sci. Technol. 51 (8), 4714–4721. https://doi.org/10.1021/acs.est.7b00635.

Hosokawa, M., Satoh, T., 2001. Measurement of Carboxylesterase (CES). Current Protocols in Toxicology, 10, 4.7.1-4.7.14. https://doi.org/https://doi.org/10.1002/0471140856.tx0407s10.

Huerta Lwanga, E., Gertsen, H., Gooren, H., Peters, P., Salánki, T., Van Der Ploeg, M., Besseling, E., Koelmans, A.A., Geissen, V., 2016. Microplastics in the Terrestrial Ecosystem: Implications for Lumbricus terrestris (Oligochaeta, Lumbricidae). Environ. Sci. Technol. 50 (5), 2685–2691. https://doi.org/10.1021/acs.est.5b05478.

Hund-Rinke, K., Achazi, R., Römbke, J., Warnecke, D., 2003. Avoidance test with Eisenia fetida as indicator for the habitat function of soils: Results of a laboratory comparison test. J. Soils Sediments 3 (1), 7–12. https://doi.org/10.1007/BF02989462.

Hurley, R.R., Nizzetto, L., 2018. Fate and occurrence of micro(nano)plastics in soils: knowledge gaps and possible risks. Curr. Opin. Environ. Sci. Health 1, 6–11. https://doi.org/10.1016/j.coesh.2017.10.006.

Jeong, C.B., Kang, H.M., Lee, Y.H., Kim, M.S., Lee, J.S., Seo, J.S., Wang, M., Lee, J.S., 2018. Nanoplastic ingestion enhances toxicity of persistent organic pollutants (POPs) in the monogonont rotifer brachionus koreanus via multixenobiotic resistance (MXR) disruption. Environ. Sci. Technol. 52 (19), 11411–11418. https://doi.org/10.1021/acs.est.8b03211.

Jeong, J., Choi, J., 2019. Adverse outcome pathways potentially related to hazard identification of microplastics based on toxicity mechanisms. In *Chemosphere* (Vol. 231, pp. 249–255). Elsevier Ltd. https://doi.org/10.1016/j.chemosphere.2019.05.003.

Jiang, X., Chang, Y., Zhang, T., Qiao, Y., Klobučar, G., Li, M., 2020. Toxicological effects of polystyrene microplastics on earthworm (Eisenia fetida). Environ. Pollut. 259 https://doi.org/10.1016/j.envpol.2019.113896.

Jones, C.G., Lawton, J.H., Shachak, M., 1994. Organisms as ecosystem engineers. Oikos 69 (3), 373–386. https://doi.org/10.2307/3545850.

Judy, J.D., Williams, M., Gregg, A., Oliver, D., Kumar, A., Kookana, R., Kirby, J.K., 2019. Microplastics in municipal mixed-waste organic outputs induce minimal short to long-term toxicity in key terrestrial biota. Environ. Pollut. 252, 522–531. https://doi.org/10.1016/j.envpol.2019.05.027.

Klöckner, P., Seiwert, B., Eisentraut, P., Braun, U., Reemtsma, T., Wagner, S., 2020. Characterization of tire and road wear particles from road runoff indicates highly dynamic particle properties. Water Res. 185 https://doi.org/10.1016/j.watres.2020.116262.

Kwak, J. Il, An, Y.J., 2021. Microplastic digestion generates fragmented nanoplastics in soils and damages earthworm spermatogenesis and coelomocyte viability. J. Hazardous Mater., 402(September 2020), 124034. https://doi.org/10.1016/j.jhazmat.2020.124034.

Lackmann, C., Velki, M., Bjedov, D., Ečimović, S., Seiler, T.-B., Hollert, H., 2021. Commercial preparations of pesticides exert higher toxicity and cause changes at subcellular level in earthworm Eisenia andrei. Environ. Sci. Europe 33 (1). https://doi.org/10.1186/s12302-021-00455-5.

Lackmann, C., Velki, M., Seiler, T.-B., Hollert, H., 2018. Herbicides diuron and fluazifop-p-butyl affect avoidance response and multixenobiotic resistance activity in earthworm Eisenia andrei. Chemosphere 210, 110–119. https://doi.org/10.1016/j.chemosphere.2018.07.008.

Lahive, E., Walton, A., Horton, A.A., Spurgeon, D.J., Svendsen, C., 2019. Microplastic particles reduce reproduction in the terrestrial worm Enchytraeus crypticus in a soil exposure. Environ. Pollut. 255, 113174 https://doi.org/10.1016/j.envpol.2019.113174.

Lavelle, P., Pashanasi, B., Charpentier, F., Gilot, C., Rossi, J.-P., Derouard, L., Andre, J., Ponge, J.-F., & Bernier, F. (1998). Large-scale effects of earthworm on soil organic matter and nutrient dynamics. In C. A. Edwards (Ed.), *arthworm Ecology* (pp. 103–122.). CRC Press.

MacLeod, M., Arp, H.P.H., Tekman, M.B., Jahnke, A., 2021. The global threat from plastic pollution. Science 373 (6550), 61–65. https://doi.org/10.1126/science.abg5433.

Marques, C., Pereira, R., Gonçalves, F., 2009. Using earthworm avoidance behaviour to assess the toxicity of formulated herbicides and their active ingredients on natural soils. J. Soils Sediments 9 (2), 137–147. https://doi.org/10.1007/s11368-009-0058-0.

Müller, A., Kocher, B., Altmann, K., Braun, U., 2022. Determination of tire wear markers in soil samples and their distribution in a roadside soil. Chemosphere 294, 133653. https://doi.org/10.1016/j.chemosphere.2022.133653.

Ng, E.L., Huerta Lwanga, E., Eldridge, S.M., Johnston, P., Hu, H.W., Geissen, V., Chen, D., 2018. An overview of microplastic and nanoplastic pollution in agroecosystems. Sci. Total Environ. 627, 1377–1388. https://doi.org/10.1016/j.scitotenv.2018.01.341.

OECD, 1984. OECD Guideline 207: Earthworm, Acute Toxicity Tests. OECD Publishing, Paris. https://doi.org/10.1787/9789264070042-en.

OECD, 2016. OECD guideline 222: Earthworm reproduction test (Eisenia fetida/ Eisenia andrei) (Issue July). OECD Publishing. https://doi.org/10.1787/9789264264496-en.

Pelosi, C., Barot, S., Capowiez, Y., Hedde, M., Vandenbulcke, F., 2014. Pesticides and earthworms. A review. Agron. Sustain. Dev. 34 (1), 199–228. https://doi.org/10.1007/s13593-013-0151-z.

Pereira, J.L., Antunes, S.C., Ferreira, A.C., Gonçalves, F., Pereira, R., 2010. Avoidance behavior of earthworms under exposure to pesticides: is it always chemosensorial? J. Environ. Sci. Health - Part B Pesticides Food Contaminants Agric. Wastes 45 (3), 229–232. https://doi.org/10.1080/03601231003613625.

Rochman, C.M., Hoellein, T., 2020. The global odyssey of plastic pollution. Science 368 (6496), 1184–1185. https://doi.org/10.1126/science.abc4428.

Rodríguez-Seijo, A., da Costa, J.P., Rocha-Santos, T., Duarte, A.C., Pereira, R., 2018a. Oxidative stress, energy metabolism and molecular responses of earthworms (Eisenia fetida) exposed to low-density polyethylene microplastics. Environ. Sci. Pollut. Res. 25 (33), 33599–33610. https://doi.org/10.1007/s11356-018-3317-z.

Rodriguez-Seijo, A., Lourenço, J., Rocha-Santos, T.A.P., da Costa, J., Duarte, A.C., Vala, H., Pereira, R., 2017. Histopathological and molecular effects of microplastics in Eisenia andrei Bouché. Environ. Pollut. 220, 495–503. https://doi.org/10.1016/j.envpol.2016.09.092.

Rodríguez-Seijo, A., Santos, B., Ferreira Da Silva, E., Cachada, A., Pereira, R., 2018b. Low-density polyethylene microplastics as a source and carriers of agrochemicals to soil and earthworms. Environ. Chem. 16 (1), 8–17. https://doi.org/10.1071/EN18162.

Schmieg, H., Burmester, J.K.Y., Krais, S., Ruhl, A.S., Tisler, S., Zwiener, C., Köhler, H.R., Triebskorn, R., 2020. Interacting effects of polystyrene microplastics and the antidepressant amitriptyline on early life stages of brown trout (Salmo trutta f. fario). Water (Switzerland) 12 (9). https://doi.org/10.3390/W12092361.

Sheng, Y., Liu, Y., Wang, K., Cizdziel, J.V., Wu, Y., Zhou, Y., 2021. Ecotoxicological effects of micronized car tire wear particles and their heavy metals on the earthworm (Eisenia fetida) in soil. Sci. Total Environ. 793, 148613 https://doi.org/10.1016/j.scitotenv.2021.148613.

Stubbins, A., Law, K.L., Muñoz, S.E., Bianchi, T.S., Zhu, L., 2021. Plastics in the Earth system. Science 373 (6550), 51–55. https://doi.org/10.1126/science.abb0354.

Wang, J., Coffin, S., Sun, C., Schlenk, D., Gan, J., 2019. Negligible effects of microplastics on animal fitness and HOC bioaccumulation in earthworm Eisenia fetida in soil. Environ. Pollut. 249, 776–784. https://doi.org/10.1016/j.envpol.2019.03.102.

Wendel, A., 1980. Glutathione Peroxidase. In: Jakoby, W.B. (Ed.), Enzymatic Basis of Detoxication. Academic Press.

Wong, B.B.M., Candolin, U., 2015. Behavioral responses to changing environments. Behav. Ecol. 26 (3), 665–673. https://doi.org/10.1093/beheco/aru183.

Yu, P., Liu, Z., Wu, D., Chen, M., Lv, W., Zhao, Y., 2018. Accumulation of polystyrene microplastics in juvenile Eriocheir sinensis and oxidative stress effects in the liver. Aquat. Toxicol. 200 (April), 28–36. https://doi.org/10.1016/j.aquatox.2018.04.015.

Zimmermann, L., Göttlich, S., Oehlmann, J., Wagner, M., Völker, C., 2020. What are the drivers of microplastic toxicity? Comparing the toxicity of plastic chemicals and particles to Daphnia magna. Environ. Pollut. 267 https://doi.org/10.1016/j.envpol.2020.115392.