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Microplastics from cigarette filters: Comparative effects on selected terrestrial and aquatic invertebrates☆

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ABSTRACT

Cigarette filters comprise plasticised cellulose acetate, a synthetic polymer categorized as bioplastic. They represent a significant source of microplastics (MPs), in particular microfibers, and associated chemicals, yet their impact on organisms, especially soil invertebrates, is not well-studied. This research examines the effects of MPs milled from smoked (SCF-MPs) and unsmoked cigarette filters (UCF-MPs) on terrestrial invertebrates (*Porcellio scaber, Tenebrio molitor*) and aquatic invertebrates (*Daphnia magna, Brachionus calyciflorus*). SCF-MPs and UCF-MPs were found to be 9.06 ± 4.1 μm and 12.71 ± 6.82 μm in size, respectively. Both samples contained triacetin and potentially toxic metals, while SCF-MPs also contained nicotine and a larger number of trace organic compounds. While exposure to SCF-MPs or UCF-MPs (up to 1.5 % MPs, w/w in soil) did not affect the survival of either terrestrial invertebrate, several physiological responses were observed. These included changes in immune parameters, energy-related biomarker levels, and altered glutathione S-transferase and acetylcholinesterase activities. Both types of MPs were acutely toxic to aquatic invertebrates, reducing the survival rates of *B. calyciflorus* (10 mg L^{-1} of either particle after 48h exposure) and *D. magna* (100 mg L^{-1} of SCF-MPs after 48h exposure). SCF-MPs generally caused more pronounced effects than UCF-MPs. This study highlights the need for effective environmental management to address both smoked and unsmoked cigarette filters.

1. Introduction

Cigarette butts represent one of the most significant sources of microplastics (MPs) due to their global occurrence and the substantial quantities of them are found in the environment across the globe (Green et al., 2022; Lian et al., 2024). According to estimates, more than 6 trillion cigarettes are consumed worldwide annually, of which 4.5 trillion end up as cigarette butt litter in the environment (World Health Organisation, 2024; Vanapalli et al., 2023), accounting for up to 33–91.5 % of total global litter (Cigarette butt pollution project, 2013; GBD 2019 Tobacco Collaborators, 2021). Studies have reported different densities of cigarette butt litter pollution: 0.03–38.1 butts/m^2 in urban areas (Dobaradaran et al., 2018; Nasab et al., 2022; Ribeiro et al., 2022), 1–9 butts/m^2 on two beaches in Brazil (da Silva et al., 2023) and up to 0.3 butts/m^2 in China (Lian et al., 2024). Awareness of cigarette butt pollution is increasing, with many local communities undertaking various initiatives aimed at the general public to reduce this problem (Fig. S1). Furthermore, cigarette butts are listed as one of the plastic litter items of concern in the documents of the ongoing Intergovernmental Negotiating Committee on plastic pollution (UNEP, 2024), and World Health Organisation is taking an active role in alarming about plastic pollution linked to cigarette butt disposal (World Health Organisation, 2024).

Cigarette butts are composed of a cigarette filter and the remaining tobacco. The major component is plasticised cellulose acetate, a synthetic polymer that is categorized as a bioplastic, because it is based on plant-derived cellulose that has been treated with acetic acid (Torkashvand et al., 2020). Cellulose acetate fibres are treated with

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titanium dioxide and packed firmly together with triacetin (glycerol triacetate) as a binding agent to produce a single filter (Slaughter et al., 2011). Furthermore, plasticizers such as polyethylene glycol are added. In the environment, cigarette butts are exposed to biota and various abiotic conditions, such as rain, abrasion by solid particles, salt, temperature fluctuations, and especially UV radiation, which significantly accelerate the degradation of the polymer into smaller particles, contributing to the problem of MP pollution (Bonanomi et al., 2020; Erdal and Hakkarainen, 2022). Microfibers are a predominant form of MP released from cigarette filters (Belzagui et al., 2021). It was found that smoked fibers detach approximately 100 small microfibers (<0.2 mm) per day, leading to about 0.3 million tons of potential microfibers annually reaching aquatic environments from this source (Belzagui et al., 2021). What makes MPs from cigarette butts a unique environmental challenge is the fact that they contain various chemicals (e.g., nicotine, tobacco-specific nitrosamines, tar, polycyclic aromatic hydrocarbons, metals, and metalloids (Beutel et al., 2021; Moerman and Potts, 2011; Torkashvand et al., 2020) that accumulate in the butts during smoking and can later leach into the environment, leading to serious negative effects on organisms (Lucia et al., 2023; Xi et al., 2023).

Initially, the studies concerning the environmental hazard of cigarette butts focused on the effects of the whole cigarette butts (not microplastics) or their leachates (Gill et al., 2018; Xi et al., 2023; Koroleva et al., 2021; Green et al., 2019, 2020; 2021; Oliva et al., 2021; Mohamed, 2023; Lian et al., 2024). However, data for MP derived from cigarette filters are limited. One study has reported the effects of MP from cigarette butts on marine crustacean (*Penaeus vannamei*) (Kim et al., 2023). Furthermore, to the best of our knowledge, there is currently no study on the effects of MPs from cigarette butts on terrestrial organisms. However, several studies have shown the toxic potential and ingestion of microplastics, including microfibers, from other plastic materials (Jemec et al., 2016; Jemec Kokalj et al., 2018; Jemec Kokalj et al., 2022a). Hence, the ecotoxicological investigation of cigarette microplastics is of interest.

This study aimed to investigate the environmental hazard of MPs milled from unsmoked and smoked cigarette filters. We focussed primarily on the response of two soil invertebrates (the crustacean *Porcellio scaber* and the insect *Tenebrio molitor*). Biochemical stress response including enzyme activities of acetylcholinesterase (AChE), detoxification enzyme glutathione S-transferase (GST) and electron transfer system (ETS) as a measure of metabolic activity was investigated in both terrestrial invertebrates. The immune response of *P. scaber* was analyzed by examining the total number of circulating haemocytes, their viability, and the proportion of different haemocyte types. Haemocytes play a crucial role in the crustacean innate immune system, performing various functions in response to immune challenges (Söderhäll, 2016). In *P. scaber*, three distinct haemocyte types have been identified based on their morphology and function: semi-granular cells (also referred to as semigranulocytes), granular cells (e.g. granulocytes), and hyaline cells (e.g. hyalinocytes) (Dolar et al., 2020). The selected soil invertebrates play an important role in the decomposition of organic matter in the ecosystem. In addition, previous studies with MPs and other pollutants have confirmed that these two soil species are suitable model organisms for ecotoxicological experiments (Dolar et al., 2021, 2022a,b; Jemec Kokalj et al., 2024). As a comparison, we also evaluated the acute toxicity for two standard aquatic (freshwater) invertebrates commonly used in ecotoxicity testing (the rotifer *Brachionus calyciflorus* and the crustacean *Daphnia magna*) (ASTM E-1440-91, 2012; EN ISO 6341, 2012; OECD TG211, 2012).

2. Materials and methods

2.1. Microplastic production and characterisation

Microplastics from unsmoked cigarette filters (UCF-MPs) and smoked cigarette butts (hereafter referred to as smoked cigarette filters, SCF-MPs) were produced by cryo-milling, as described previously (Dolar et al., 2021). Conventional cigarettes from a commonly sold brand were purchased in a shop. Microplastics from unsmoked cigarette filters were produced from the filters of the same unsmoked cigarettes, while SCF-MPs were produced from the filters of smoked cigarettes provided by a regular smoker (Fig. 1). In brief, the outer paper wrapping (tipping paper) of each cigarette filter was removed, after which the filters were cut in half and placed in a milling ball (diameter 25 mm), which was placed in liquid nitrogen and frozen for 4 min. The samples were then milled with a horizontal homogenizer (Milli Mix 20; Domel, Slovenia) at a horizontal frequency of 28 Hz for 2.5 min.

The shapes of the obtained particles (UCF-MPs and SCF-MPs) were characterized using a field emission scanning electron microscope (FE-SEM, ThermoScientific Apreo 2, Thermo Fisher Scientific, USA). The samples were fixed on an aluminium holder and sputtered with a 10 nm thick layer of Au/Pd before imaging. The particle size distributions were measured using a Microtrac S3500 Bluewave laser diffraction analyzer in wet mode (Microtrac, Germany). Wet measurements were performed in water at room temperature. For every sample, a final particle size distribution and statistical parameters (mean value, SD) were calculated as average values of three repeatedly performed analyses. The main material of the produced MPs was determined using attenuated total reflection Fourier transform infrared spectroscopy (diamond crystal, FTIR Spectrum Two spectrometer; PerkinElmer). The spectra were recorded from 400 cm^{-1}–4000 cm^{-1} with an average of four scans at a resolution of 4 cm^{-1}.

Elemental analysis of the UCF-MP and SCF-MP samples (4 replicates each) was performed after acidic mineralization in a microwave system (Ethos Up, Milestone, Italy). Approximately 120 mg of sample was weighed directly into a Teflon vessel and 5.0 mL of 67 % (w/w) HNO_3 (Suprapur, Carlo-Erba) was added before heating the samples to 220 °C over a 40 min period and then holding at this temperature for 20 min. After cooling to <60 °C (approx. 40 min), the samples were quantitatively transferred into 20 mL flasks and diluted with ultrapure water (Milli-Q, resistivity of >18.2 MΩ cm). Analysis of the prepared samples was performed using a quadrupole inductively-coupled plasma mass spectrometer (ICP-MS Agilent 7900ce, Agilent Technologies, Palo Alto-CA, USA). A forward RF power of 1.5 kW was used with Ar gas flows: carrier 0.85 L/min, makeup 0.28 L/min, plasma 1.0 L/min, cooling 15 L/min, and sample flow rate 0.2 mL/min, measuring one point per mass and acquiring the following isotopes: ^{7}Li, ^{9}Be, ^{11}B, ^{23}Na, ^{24}Mg, ^{27}Al, ^{31}P, ^{39}K, ^{44}Ca, ^{47}Ti, ^{51}V, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{69}Ga, ^{75}As, ^{78}Se, ^{85}Rb, ^{88}Sr, ^{95}Mo, ^{107}Ag, ^{111}Cd, ^{115}In, ^{118}Sn, ^{121}Sb, ^{125}Te, ^{133}Cs, ^{137}Ba, ^{205}Tl, ^{208}Pb, and ^{209}Bi. The calibration curves were based on nine calibration multi-standards in the concentration range 0.1–1000 µg/L ($R^2 > 0.999$), prepared by the dilution of a certified reference material multi-standard solution (Periodic table mix 1 for ICP, TraceCERT $^{(R)}$, Sigma-Aldrich, Germany).

Gas chromatography with mass spectrometry (GC/MS) (Model 7890B, Agilent Technologies, USA) under Argon (≥99.99 %) was used for the identification of organic compounds in the samples. The samples were prepared as specified in ISO 11890-2, 2020. Approximately 1 g of sample was weighed into a vial and diluted with a suitable volume of the extraction solvent, methanol.

2.2. Test organisms

Woodlice *Porcellio scaber* (Crustacea: Isopoda) were obtained from an uncontaminated compost heap near Kamnik, Slovenia (46° 11′ 35.878″ N; 14° 35′ 38.342″ E) and cultured for at least 2 weeks in glass containers filled with loamy sand and sterilized dry leaves of common hazel (*Corylus avellana*) under controlled conditions (20 ± 2 °C, light: dark = 16/8) at the University of Ljubljana. Animals of both sexes (>25 mg) were used, while moulting individuals, females with marsupia, and infected individuals were excluded from the experiments.

Larvae of the mealworm *Tenebrio molitor* (Insecta: Coleoptera) were

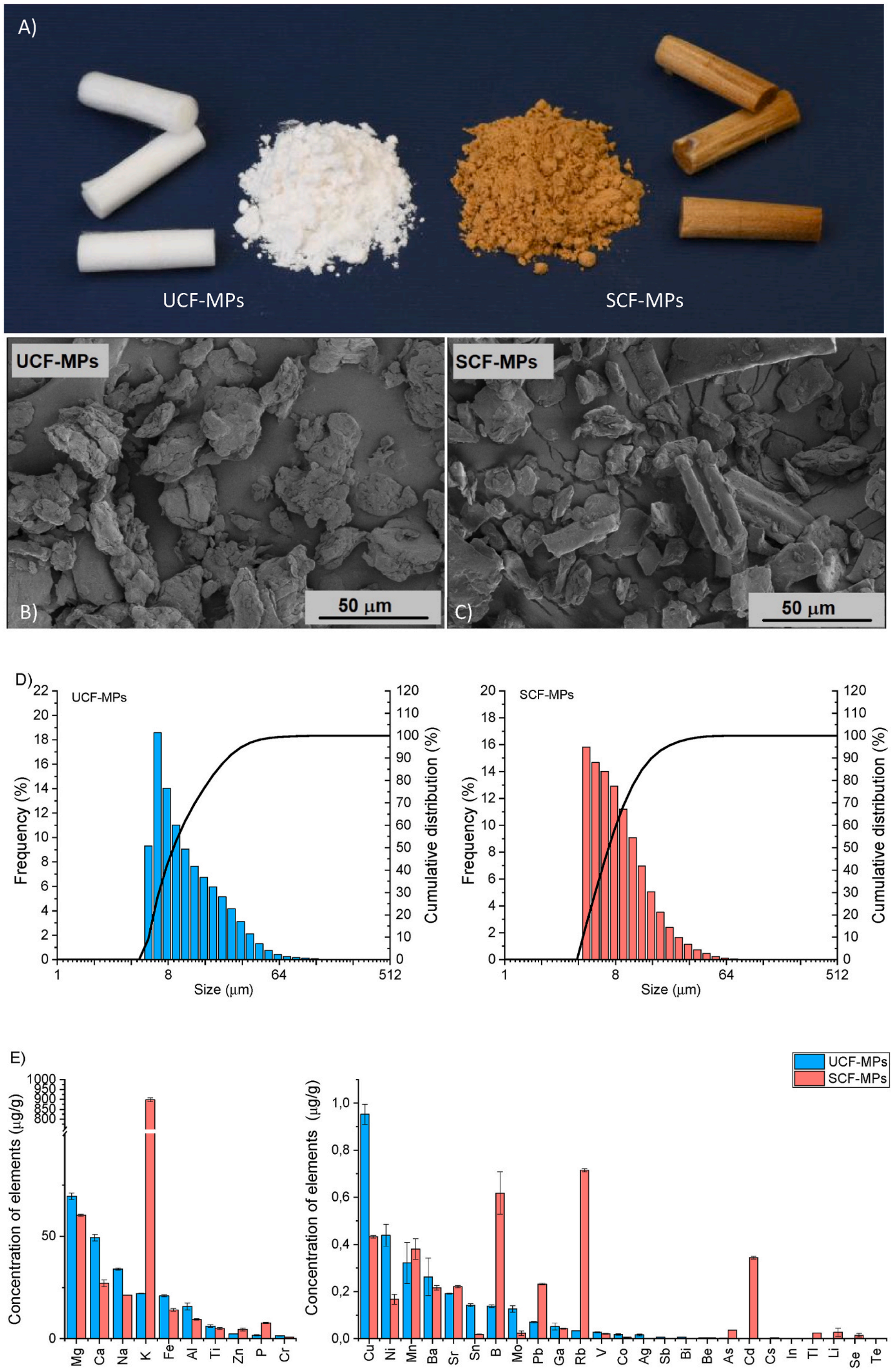

Fig. 1. Characteristics of microplastics milled from unsmoked (UCF-MPs) and smoked cigarette filters (SCF-MPs). A) photo of cigarette filters and microplastics; B, C) shape of particles analyzed by scanning electron microscope; D) particle size distribution, expressed as number distribution, according to laser diffraction particle size analysis; E) metals and other selected elements detected in concentration ranges >1.5 μg/g (left), and <1.5 μg/g (right). Elements are shown in descending order of SCF-MP values. Mean values and standard errors are shown.

purchased at a pet shop (Ljubljana, Slovenia) and raised in plastic containers on a diet of wheat bran and flour at 20 ± 2 °C in the dark for at least one generation (minimum 12 weeks). For the experiment, we used larvae of the new generation with a mass between 60 and 90 mg, which were bred from the parent generation (purchased organisms).

The aquatic organisms: the rotifer *Brachionus calyciflorus* (Rotifera: Monogononta) and the water flea *Daphnia magna* (Crustacea: Branchiopoda), were both obtained from commercially available kits (Rotoxkit F and Daphtoxkit F™ magna; Microbiotests, Belgium) and hatched according to the provided instructions.

2.3. Experiments with terrestrial invertebrates

Woodlice and mealworms were exposed to standard agricultural soil (Lufa 2.2, Speyer, Germany), which was contaminated with either UCF-MPs or SCF-MPs at final concentrations of 0.06 %, 0.5 %, and 1.5 %, w w^{-1} dry soil. These concentrations are commonly tested in soil MP research as they represent environmentally relevant concentrations (Jemec Kokalj et al., 2024; Kumar and Sheela, 2020; Selonen et al., 2020). Prior to exposure, MPs were mixed into the dry soil using a metal spoon, and the moisture content was adjusted to 40 % of the water holding capacity by adding deionised water, as described by Dolar et al. (2021) and van Loon et al. (2025).

The experiments with woodlice were carried out for 14 days. Animal survival, immune system response, and enzyme activities of AChE and GST in the whole animal were evaluated after a 7-day and 14-day exposure to either UCF-MPs or SCF-MPs in soil. Twenty grams of the moist test soil was placed into a 100-mL glass jar, and five woodlice were introduced per jar. Five jars were prepared for each treatment and control group. Altogether, 25 woodlice per treatment and control were exposed. Dry, sterilized leaves of common hazel (*Corylus avellana*) were added to each container as food. The moisture content of the soil was checked every 2–3 days, and the common hazel leaves were replaced every week. The exposures were performed at 20 ± 2 °C in the dark. After 7 and 14 days of exposure, haemolymph was collected from individual woodlice using a sterile syringe needle and a glass capillary micropipette (Brand). The haemolymph was immediately diluted at a ratio of 1:5 with Dulbecco's phosphate-buffered saline (pH 7.1–7.5; Sigma) and 0.4 % trypan blue (Sigma), which stains dead haemocytes while leaving viable haemocytes unstained. Ten μL of the haemocyte suspension was pipetted into a haemocytometer (Neubauer) to determine THC, the proportion of the three main haemocyte types (SGC: semi-granular cells, GC: granular cells, and HC: hyaline cells) and viability of the haemocytes under a light microscope (Axio Imager Z1, Zeiss; Dolar et al., 2020). At least 10 animals (i.e., 10 samples) were analyzed for each treatment and control and each time point (7 and 14 days). AChE and GST activities were measured according to Madžarić et al. (2018) (as described in **Supplementary information**).

The experiments with mealworm larvae were carried out for 28 days. The survival and mass of the animals were assessed after each week of exposure, while the whole animal activities of AChE, GST, and the energy-related biomarker, ETS, were assessed after 14 and 28 days of exposure to either UCF-MPs or SCF-MPs in soil. Twenty grams of the moist test soil were placed into 100 ml jars and five mealworms were placed in each jar. Six jars were prepared for each microplastic treatment and the control group, resulting in 30 mealworms per exposure group. Each week, four flakes of oat bran (~20 mg each) were added to each jar as food and the moisture content of the soil was checked. Exposure was carried out at 20 ± 2 °C in the dark. On the 14th and 28th day of exposure, the whole animal was homogenized. The obtained supernatant was used to measure the activities of AChE and GST (according to Madžarić et al., 2018), and the energy-related biomarker, ETS (according to Selonen et al., 2021) (**Supplementary information**). Ten samples were analyzed for each biomarker and exposure group per time point.

2.4. Experiments with aquatic invertebrates

Both *D. magna* and *B. calyciflorus* were exposed to UCF-MPs and SCF-MPs in standard freshwater media at final concentrations of 1 mg L^{-1}, 10 mg L^{-1}, and 100 mg L^{-1}. The selected concentrations of MPs have previously been used in testing with *D. magna* (Jemec Kokalj et al., 2018; Zimmermann et al., 2020).

The acute toxicity test with rotifer *B. calyciflorus* was performed according to slightly modified standard guidelines (ASTM E-1440-91, 2012). Rotifer larvae were hatched from dormant eggs (cysts) in a standard freshwater medium (96 mg $NaHCO_3$, 120 mg $CaSO_4 2H_2O$, 123 mg $MgSO_4 7H_2O$, and 4 mg KCl per 1 L dH_2O) after 18h incubation at 25 °C under continuous illumination. The UCF-MP and SCF-MP suspensions were prepared in a standard freshwater medium and vortex-stirred prior to dilution and pipetting. The experiment was performed using a multi-well test plate. For each test concentration, six test wells were prepared with 0.3 mL of test medium and five rotifer larvae were added to each test well. A standard freshwater medium was used for the control group. The rotifer larvae were incubated for 24h at 25 ± 1 °C in the dark. After 24h, mortality was determined by the absence of observed movement within 5 s of gently shaking the liquid.

Daphnids were hatched from dormant eggs (cysts) in M4 medium (OECD TG211, 2012) after 48h incubation at 20 °C under a 16:8 light/dark regime. Hatched daphnids less than 24 h old were fed with algae (*Desmodesmus subspicatus*; density 5×10^4 cells/ml) for 1.5 h prior to microplastic exposure. UCF-MPs and SCF-MPs were added to the M4 medium (OECD TG211, 2012) containing 0.0024 % (v/v) Tween 40, and the suspensions were vortex stirred before dilution and pipetting according to Zidar et al. (2024). For each test concentration, four Petri dishes containing 10 mL of the test medium were prepared and five neonates were added to each Petri dish. Controls containing only the test medium with 0.0024 % (v/v) Tween 40 were used in all experiments. The daphnids were exposed for 96h at 20 ± 2 °C under a 16:8 light/dark regime. After 48h of exposure, the daphnids were fed with algae. Exposure during the toxicity tests was static, without mixing the test suspensions. After 24h, 48h, 72h, and 96h of exposure, the daphnids were analyzed for mobility (according to ISO 6341:2012), and their mortality was determined by the absence of a heartbeat. During the exposure (after 48h) daphnids were fed by adding 50 μL of spirulina green algae (11 mg/mL) to the test medium.

2.5. Data analysis

The data were analyzed and visualized using OriginPro v2023 software (OriginLab Corporation, Northampton, MA, USA). All data were first tested using the Kolmogorov-Smirnov test and Levene's test, to determine whether they met the assumptions for normality and homoscedasticity, respectively. In the case of a normal distribution and homoscedasticity, a Two-way ANOVA with interaction was performed (fixed factors: test material and test concentrations), followed by Tukey tests; otherwise, a non-parametric analysis of variance of Aligned Rang Transform (ART) ANOVA was used. Differences between the control group and the treatment group were considered statistically significant if $p < 0.05$ (*). The common baseline viability of haemocytes in the control population of woodlice estimated from our previous studies is 85 % (n = 371; i.e., 95 % of woodlice with a viability greater than 85 %; Dolar et al., 2024). Therefore, we considered haemocyte viability to be significantly affected if it was less than 85 %.

3. Results

3.1. Microplastic characteristics

Both microplastics (UCF-MPs and SCF-MPs) consisted of mostly irregularly shaped fragments (Fig. 1B and C). However, in both samples, we also detected fibrous structures (Fig. S2), the proportion of which cannot be determined precisely using the techniques mentioned. The SEM micrograph (Fig. 1 C) clearly shows that the SCF-MPs sample contains a higher proportion of fibrous structures, which are characterised by a diameter corresponding to that observed in the finer sample fractions. The results obtained with the laser particle size diffraction instrument (Fig. 1D) show that the SCF-MPs sample has a narrower number size distribution with an upper limit of 64 mm. While the UCF-MPs sample contains a fine fraction where the larger particles reach up to 100 μm, which corresponds to an agglomerate size composed of fine MPs. The particle size distribution (mean ± SD; numerical distribution) of the UCF-MPs was 12.7 ± 6.8 μm, while that of the SCF-MPs was 9.1 ± 4.1 μm (Fig. 1D). The chemical composition of both MPs was confirmed to be cellulose acetate (**Supplementary information,** Fig. S3). The spectra of UFC-MPs and SCF-MPs are identical, indicating that the sample was not chemically altered by smoking.

The elemental profile of UCF-MPs and SCF-MPs is shown in Fig. 1E and in the **Supplementary information,** Table S1. Some elements were present in very high concentrations, although most of them were in the range of <1.5 μg/g (Fig. 1E). According to the classification proposed by Pourret and Hursthouse (2019), the following potentially toxic elements were identified in both MPs: Zn, Cr, Cu, Mn, Cd, Pb, Ni, As, Co, and Ag.

The highest concentrations were measured for Zn (2.31 ± 0.05 and 4.51 ± 0.68 μg/g for UCF-MPs and SCF-MPs, respectively), Cr (1.43 ± 0.075 and 0.69 ± 0.052 μg/g for UCF-MPs and SCF-MPs, respectively), and Cu (0.952 ± 0.043 and 0.43 ± 0.0057 μg/g, for UCF-MPs and SCF-MPs, respectively). We further found that the concentrations of some of these potentially toxic metals were higher in SCF-MPs compared with UCF-MPs, including Zn (2-times), Cd (~190-times), Pb (~3-times) and As (~15 times). However, the rest of the metals showed the opposite trend. In particular, Cr and Cu were found to be two times lower in SCF-MPs.

The identification of organic compounds in both samples was performed using GC/MS analysis. The list of organic compounds identified in UCF-MPs and SCF-MPs and the peak area are shown in the **Supplementary information,** Table S2. The results indicated that both samples contained triacetin, while SCF-MPs also contained nicotine and several trace compounds, such asphenol, butyl citrate, n-hexadecanoic acid, and 1,2-propanediol.

3.2. *Response of woodlice Porcellio scaber*

Microplastics from unsmoked and smoked cigarette filters did not affect the survival of *P. scaber* after 14 days of exposure in soil. However, both test materials significantly altered the total number of haemocytes in the haemolymph (referred to as THC) (Fig. 2). Compared to the control, MPs from UCFs induced a statistically significant reduction of THC at 0.06 % ($p = 0.010$) and 0.5 %, w w^{-1} ($p = 0.001$) after a 7-day exposure in soil, while no change was observed after 14 days of exposure to UCF-MPs in soil. On the other hand, MPs from SCF caused a statistically significant increase in THC already 7 days after exposure to 1.5 % SCF-MPs ($p < 0.001$), as well as after 14 days to 0.5 % ($p = 0.018$) and 1.5 % SCF-MPs ($p = 0.005$) (Fig. 2). The interaction term for test material and concentration was statistically significant ($F_{3,\ 140} = 9.02$; $p < 0.001$).

Compared to the control, the proportion of the three main haemocyte types and the viability of haemocytes in the haemolymph of *P. scaber* did not change at any exposure time (7 and 14 days), irrespective of the test material (UCF-MPs and SCF-MPs; Fig. S4).

The whole-body enzyme activities of acetylcholinesterase (AChE)

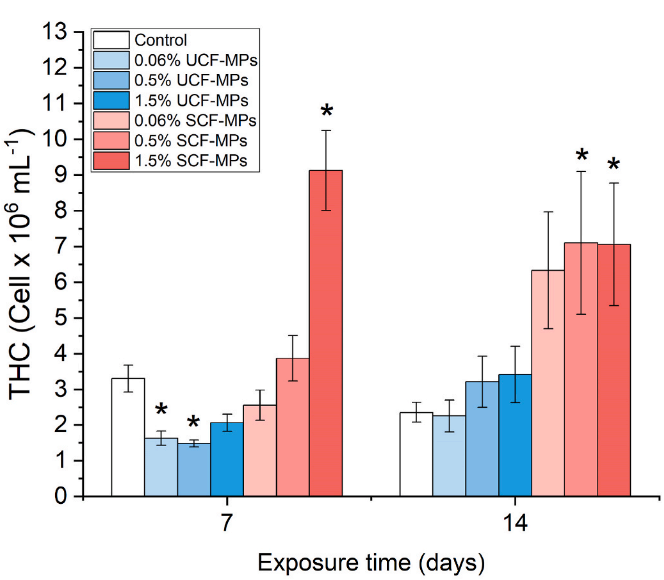

Fig. 2. Total haemocyte count (THC) in the haemolymph of the isopod *Porcellio scaber* exposed to microplastics from unsmoked (UCF-MPs) and smoked cigarette filters (SCF-MPs) in Lufa 2.2 soil (0, 0.06, 0.5, 1.5 % w w^{-1} dry weight) for **A)** 7 days and **B)** 14 days. Each column represents the mean ± standard error. An asterisk indicates a statistically significant difference compared to the respective control (*, $p < 0.05$).

and glutathione S-transferase (GST), which were measured in *P. scaber* after 14 days of exposure to UCF-MPs and SCF-MPs, were significantly different compared to the control (Fig. 3). Exposure to UCF-MPs in soil resulted in a significant increase in AChE activity at 1.5 % ($p < 0.001$) (Fig. 3A) and a significant decrease in GST activity at 0.5 % ($p < 0.001$) and 1.5 % ($p = 0.008$) (Fig. 3B). In contrast to UCF-MP exposure, exposure to SCF-MPs led to a significant increase in AChE activity at 0.06 % ($p < 0.001$), 0.5 % ($p < 0.001$), and 1.5 % ($p < 0.001$) (Fig. 3A), while GST activity was increased at 0.06 % ($p = 0.003$) and 1.5 % ($p = 0.022$) (Fig. 3B). The interaction between test material and concentration was statistically significant for AChE ($F_{3,\ 85} = 10.43$, $p < 0.001$) and GST activity ($F_{3,\ 85} = 7.14$, $p < 0.001$).

3.3. *Response of mealworm Tenebrio molitor*

Microplastics from UCFs and SCFs did not affect the survival of *T. molitor* after 28 days of exposure in soil, but larval growth was significantly reduced (Fig. 4A). Exposure to SCF-MPs reduced growth already after 7 days at 0.5 % ($p = 0.016$) and 1.5 % ($p = 0.026$); after 14 days at 0.06 % ($p = 0.039$), 0.5 % ($p = 0.027$) and 1.5 % ($p = 0.038$); after 21 days at 1.5 % ($p = 0.049$); and after 28 days at 0.5 % ($p = 0.007$) and 1.5 % ($p = 0.027$). For UCF-MP exposure, the reduction in growth was first observed after 21 days at 0.5 % ($p = 0.048$), while the most pronounced was after 28 days at all three exposure concentrations, i.e. 0.06 % ($p = 0.047$), 0.5 % ($p = 0.013$), and 1.5 % ($p = 0.035$). The interaction between test material and concentration proved to be statistically insignificant ($F_{3,\ 244} = 1.25$, $p = 0.292$).

In general, the activity of the electron transfer system (ETS) was reduced for both materials and time points (14 and 28 days; Fig. 4B). ETS activity was significantly reduced after 14 days of exposure to 0.06 % ($p < 0.001$), 0.5 % ($p = 0.048$) and 1.5 % UCF-MPs ($p = 0.012$), and for the same material also after 28 days of exposure at all three test concentrations, i.e. 0.06 % ($p = 0.038$), 0.5 % ($p = 0.009$) and 1.5 % ($p = 0.009$). On contrary, the effects on ETS activity after 14-day exposure to SCF-MPs were significant at 0.06 % ($p = 0.032$) and 0.5 % ($p = 0.046$), while after 28-day exposure they were significant at 0.06 % ($p = 0.034$) and 1.5 % ($p = 0.049$). The interaction between the type of MPs and the test concentration was not significant ($F_{3,\ 92} = 1.03$, $p = 0.382$). The activity of GST was significantly reduced after 14-day exposure to UCF-MPs at 0.06 % ($p < 0.001$), 0.5 % ($p = 0.046$), 1.5 % ($p < 0.001$) and SCF-MPs at ($p = 0.032$), 0.5 % ($p = 0.011$), 1.5 % ($p < 0.001$) after 14 days, but not after 28 days (except for decreased GST at 0.06 % SCF-MPs; $p = 0.026$; Fig. 4C). AChE activity was not altered in mealworms exposed to UCF-MPs and SCF-MPs (Fig. 4D). No significant interaction between MPs type and test concentration was found for GST ($F_{3,\ 152} = 0.92$, $p = 0.429$) and AChE activity ($F_{3,\ 150} = 0.63$, $p = 0.59$).

3.4. *Effects on water flea Daphnia magna and rotifer Brachionus calyciflorus*

The survival of *D. magna* was significantly reduced after 48h (by 20 %) ($p < 0.001$) and 96h (by 90 %) ($p = 0.006$) exposures to 100 mg L^{-1} SCF-MPs. In addition, a non-significant trend towards decreased survival over time was also observed at all exposure concentrations of SCF-MPs (1, 10, and 100 mg L^{-1}). UCF-MPs induced no statistically significant effect on the survival of *D. magna*; however, a non-significant trend towards decreased survival over time was also observed at all exposure concentrations (Fig. 5A). The interaction between MP type and test concentration was found to be statistically significant ($F_{3,\ 200} = 5.50$, $p = 0.001$). On the other hand, both microplastic types (UCF-MPs and SCF-MPs) significantly reduced the survival of *B. calyciflorus* already at 10 mg L^{-1} (UCF-MPs: $p = 0.001$; SCF-MPs: $p = 0.047$), while survival at the highest concentration (100 mg L^{-1}) was significantly lower only in the case of SCF-MPs ($p = 0.046$) (Fig. 5B). The interaction term for MP type and concentration was not significant ($F_{3,\ 18} = 1.35$, $p = 0.290$). Overall, the effects on both organisms were more significant when

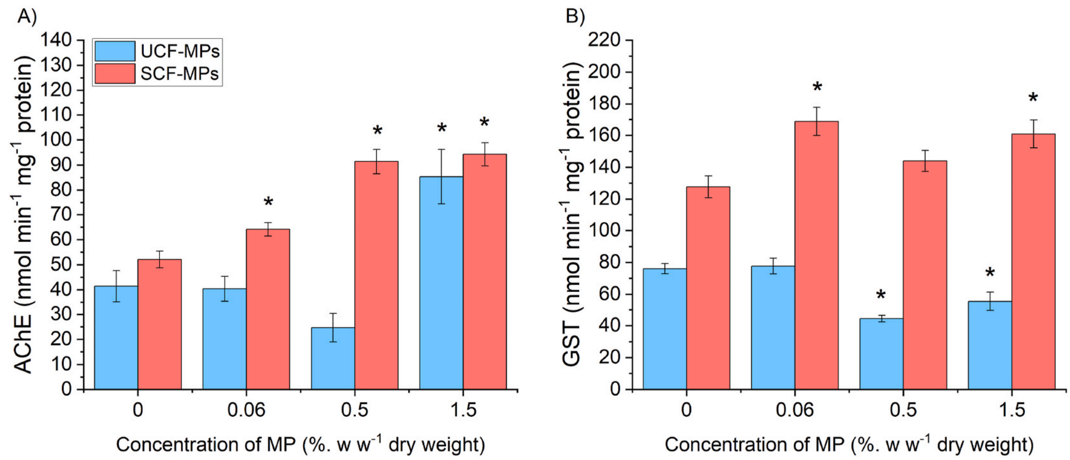

Fig. 3. Activities of **A)** acetylcholinesterase (AChE) and **B)** glutathione S-transferase (GST) in the whole body of *Porcellio scaber* exposed to microplastics from unsmoked (UCF-MPs) and smoked cigarette filters (SCF-MPs) in Lufa 2.2 soil (0, 0.06, 0.5, 1.5 % w w^{-1} dry weight) for 14 days. Each column represents the mean ± standard error. An asterisk indicates a statistically significant difference compared to the respective control (*, p < 0.05).

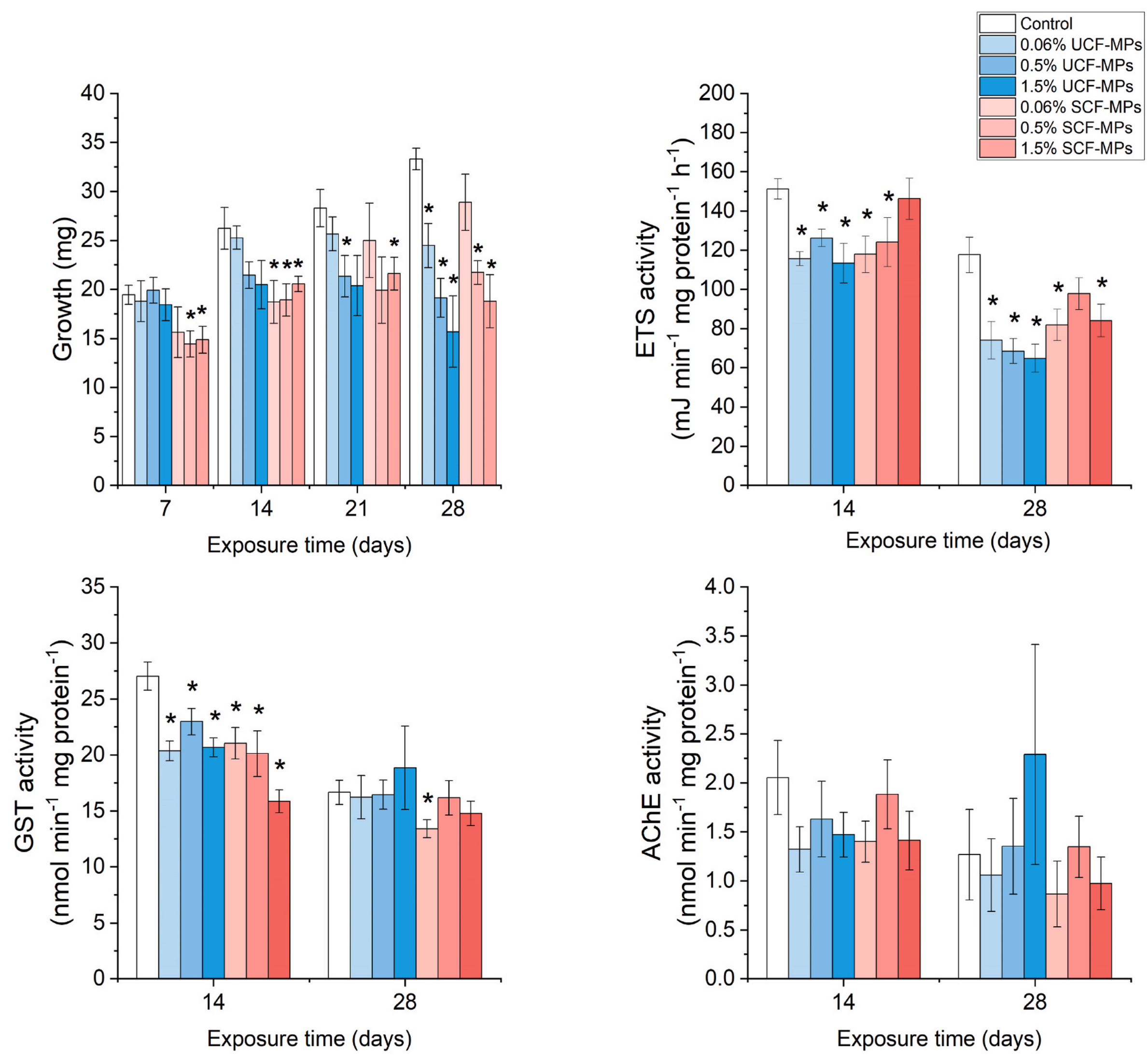

Fig. 4. Mealworm *Tenebrio molitor* larval **A**) growth and activities of **B)** the Electron transfer system (ETS)**, C)** glutathione S-transferase (GST), and **D)** acetylcholinesterase (AChE) following exposure to microplastics from unsmoked (UCF-MPs) and smoked cigarette filters (SCF-MPs) in Lufa 2.2 soil (0, 0.06, 0.5, 1.5 % w w^{-1} dry weight) for 28 days. Each column represents the mean ± standard error. An asterisk indicates a statistically significant difference compared to the respective control (*, p < 0.05).

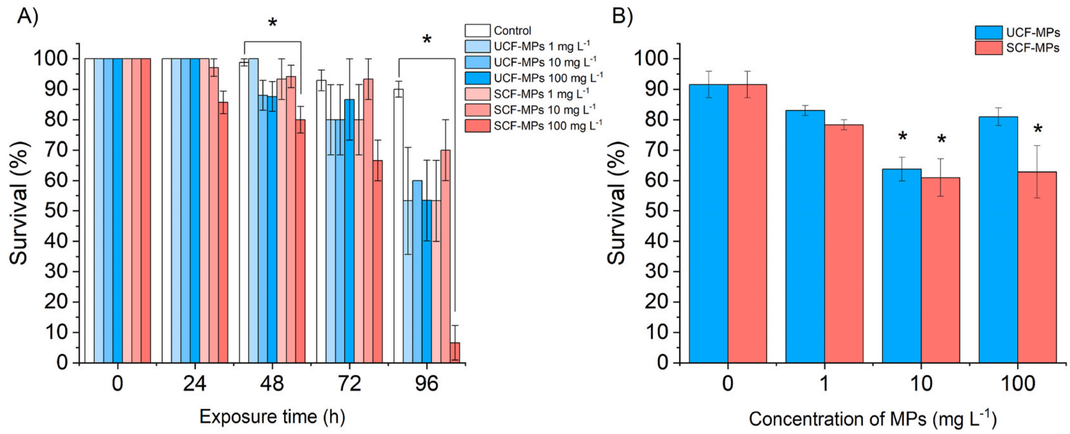

Fig. 5. Survival of A) water flea *Daphnia magna* and B) rotifer *Brachionus calyciflorus* exposed to microplastics (MPs) from unsmoked (UCF-MPs) and smoked cigarette filters (SCF-MPs) in water media (0, 1, 10, 100 mg L^{-1}). The survival of daphnids was estimated after 24, 48, 72, and 96 h, and that of rotifers after 24 h. The columns represent the mean ± standard error (n = 7–10). An asterisk indicates a statistically significant difference compared to the respective control (*, $p < 0.05$; **, $p < 0.01$).

exposed to SCF-MPs than to UCF-MPs.

4. Discussion

This study provides comprehensive evidence that MPs milled from smoked and unsmoked cigarette filters have various effects on the tested terrestrial invertebrates, *P. scaber* and *T. molitor* and aquatic invertebrates, *D. magna* and *B. calyciflorus*. Before discussing the effects of SCF-MPs and UCF-MPs on the organisms, it is relevant to describe the complexity of these types of MPs. First, the cigarette filters were easy to grind, resulting in a relatively small particle size (9.1 ± 4.1 μm and 12.7 ± 6.8 μm for SCF-MPs and UCF-MPs, respectively) compared to other types of MPs that we have previously obtained using the same milling process (polypropylene, ~70–90 μm; polyethylene, ~200 μm; tyre particles, ~60 μm) (Zidar et al., 2024). This implies that mechanical degradation of cigarette filters in the environment may result in quicker fragmentation to small-sized MPs compared to other polymers. The potentially toxic metals (as classified by Pourret and Hursthouse, 2019) detected in SCF-MPs and UCF-MPs were similar to those previously detected in whole cigarette filters (Koutela et al., 2020; Torkashvand et al., 2020) and/or their leachates (Moerman and Potts, 2011; Lian et al., 2024). When comparing between the SCF-MPs and UCF-MPs used in this study, some metal concentrations (Zn, Cd, Pb and As) were higher in SCF-MPs than in UCF-MPs, while Cr, Al, and Cu were higher in UCF-MPs. Various types and concentrations of metals in cigarette filters have been reported so far (Koutela et al., 2020; Moerman and Potts, 2011; Lian et al., 2024). As the metal concentrations depend on the relative contamination of the agricultural soils used for tobacco cultivation, as well as on the packaging and storage processes used after harvesting the tobacco plants (Koutela et al., 2020; Moerman and Potts, 2011; Pinto et al., 2017) it is difficult to generalize the metal types and concentrations for MPs obtained from different cigarette brands.

The analysis of organic compounds identified triacetin in both UCF-MPs and SCF-MPs, while MPs from the smoked cigarette filters also contained nicotine, cotinine (a metabolite of nicotine) and a greater variety of trace organic compounds (Table S2). Triacetin is used as a binding agent to produce cigarette filters (Slaughter et al., 2011) and is hence present in both UCF-MPs and SCF-MPs. As expected, we found a high peak of nicotine and cotinine only in SCF-MPs. In addition, several phenolic compounds were identified in SCF-MPs, which is in line with Si et al. (2022), who reported several of these substances in cigarette smoke and tobacco smokers' saliva. Several other toxic organic chemicals have been previously reported in cigarette filters, such as polycyclic aromatic hydrocarbons (PAHs; Dobaradaran et al., 2019; Micevska et al., 2006; Talhout et al., 2011; Torkashvand et al., 2020) and various pesticides (López Dávila et al., 2020). Altogether this indicates that SCF-MPs and UCF-MPs are very complex, containing a mixture of potentially toxic metals and organic chemicals.

The survival of the terrestrial organisms, woodlice (*P. scaber*) and mealworms (*T. molitor*) was not affected by any of the MPs tested. This is in line with our previous studies, where we found that MPs did not affect the survival of terrestrial invertebrates (Dolar et al., 2021, 2022a,b; Jemec Kokalj et al., 2024). A similar finding was reported in studies investigating the effects of leachate from cigarette butts (4 butts/L) on the land snail *Anguispira alternata* after 32 days (Gill et al., 2018) and whole cigarette butts on the earthworm *Eisenia fetida* after 70 days (3 butts in the soil; Korobushkin et al., 2020). In contrast, Xi et al. (2023) found increased mortality of the earthworm *E. fetida* exposed to leachate from smoked cigarette butts for 7 and 14 days, but at very high concentrations (100 butts/L).

Although no mortality of *P. scaber* was observed, we noted a marked immune response to exposure to SCF-MPs and UCF-MPs, as evidenced by changes in total haemocyte counts. The nature of the THC change was different between the two types of MPs (also confirmed by statistically significant interaction between the MP concentration and type). In the case of SCF-MPs, a significant increase in THC levels was observed after 7 days at the highest concentration (1.5 %) and after 14 days at 0.5 % and 1.5 %. When exposed to UCF-MPs, a transient but statistically significant reduction in the THC levels of *P. scaber* haemolymph was only observed after 7 days of exposure to 0.5 % UCF-MPs in soil, while no change was observed 14 days after exposure compared to the control. Similar types of changes in immune parameters (increased or decreased THC levels) were previously detected in *P. scaber* when exposed to MPs (Dolar et al., 2022a; Jemec Kokalj et al., 2022a). An altered haemocyte count is often associated with stress (Coates and Söderhäll, 2021; Hirt and Body-Malapel, 2020). As innate immunity is a crucial system of homeostasis, changes in THC could be an early response to soil changes caused by MPs to restore homeostasis in *P. scaber*. Previous studies on other invertebrates, such as mussels, have also reported immune responses to cigarette butt exposure (Lucia et al., 2023). In addition, a study on vertebrate model organisms treated with an aqueous extract of tobacco leaves has shown that nicotine exposure induces an immune response (Andong et al., 2021).

We also analyzed two biochemical biomarkers: glutathione S-transferase (GST) and acetylcholinesterase (AChE). Glutathione S-transferase is a stress-induced detoxification enzyme that plays a role in the cellular detoxification of xenobiotic substances and endogenous products generated during lipid peroxidation, maintaining redox homeostasis, and in many signalling pathways that govern cell proliferation and apoptosis (Mazari et al., 2023). The most well-known role of AChE is in cholinergic synapses where it terminates synaptic signal transmission via rapid hydrolysis of the neurotransmitter acetylcholine. AChE is thus commonly regarded as a biomarker for neurotoxicity (Abouda et al., 2022; Bouraoui et al., 2009; Milivojević et al., 2015), but several non-neuronal roles of AChE in cell proliferation, differentiation, and locomotion, immune function, wound healing, and metabolic regulation have been reported as well (reviewed by Glavan et al., 2018, Karczmar et al., 2010). In woodlice, GST activities were increased in the case of SCF-MPs but decreased in the case of UCF-MPs. In mealworms, GST was significantly decreased from exposure to both types of MPs (UCF-MPs and SCF-MPs) after 14 days, but not after 28 days. Both increases and decreases of GST have been previously reported in the literature (Abouda et al., 2022; Bouraoui et al., 2009). A very extensive review of invertebrate responses to MPs showed that responses of antioxidant biomarkers, such as antioxidant and detoxification enzymes, are very complex showing both increase and decrease of enzyme activities (Trestrail et al., 2020). An increase in GST activity is usually associated with the activation of detoxification processes and indicates the occurrence of oxidative stress (Zhang et al., 2021). However, suppression of the detoxification system due to the production of ROS could cause oxidative damage to proteins and lead to inactivation of the GST enzyme (Avio et al., 2015; Ribeiro et al., 2017; Tang et al., 2018). A 14-day exposure to both types of MPs (UCF-MPs and SCF-MPs) led to significantly increased activity of AChE in the body of *P. scaber*. As AChE is a part of the cholinergic system, its activity also depends on the concentrations of the neurotransmitter acetylcholine and nicotinic acetylcholine receptors (nAChRs). As the name suggests, nicotine can bind to nAChRs as an antagonist to acetylcholine (Rufener et al., 2020). The effect was more significant with SCF-MPs (already at 0.06 %), which contain nicotine. The observed increase in AChE is consistent with similar observations in studies with MPs and other invertebrates (Choi et al., 2022; Malafaia et al., 2020; Silva et al., 2021), where this type of change is usually explained by an increased release of acetylcholine, leading to regulatory overcompensation by an increase in AChE (Choi et al., 2022; Gambardella et al., 2017; Milivojević et al., 2015; Silva et al., 2021). However, as AChE is involved in many different physiological roles, it is difficult to elucidate the mechanisms behind the observed change in activity.

The mealworms were further analyzed for energy-related endpoints, including electron transfer system (ETS) activity and larval growth. Both parameters were reduced in the case of SCF-MPs and UCF-MPs, particularly after 28 days of exposure. After 14 days, the effect was more pronounced with SCF-MP exposure. Electron transfer system (ETS) activity is a measure of oxygen consumption and cellular respiration rate and is therefore often used to assess metabolic activity and energy expenditure during exposure to pollutants (Ferreira et al., 2015; Silva et al., 2021). Reduced ETS activity is consistent with decreased mealworm growth. A similar decrease in ETS activity after exposure to MPs was also observed in larvae of the dipteran *Chironomus riparius* (Silva et al., 2021).

In contrast to terrestrial invertebrates, acute tests with the freshwater organism's rotifers *B. calyciflorus* and daphnids *D. magna* showed that both SCF-MPs and UCF-MPs reduced their survival, but that the effect of SCF-MPs was greater (confirmed by statistically significant interaction between the MP concentration and type). SCF-MPs caused mortality of these organisms after only 48 h, with a LOEC of 10 mg L^{-1} and 100 mg L^{-1} for rotifers and daphnids, respectively. With a prolonged exposure of up to 96 h to SCF-MPs, mortality of the daphnids at 100 mg L^{-1} was exceptionally high (90 %), and almost 50 % of daphnids died at 1 mg L^{-1}. On the other hand, UCF-MPs did not affect the daphnids after 48 h, but after 96 h, ~50 % of the daphnids died at all exposure concentrations (1 mg L^{-1} - 100 mg L^{-1}). To date, only Kim et al. (2023) reported the effects of MPs from cigarette butts (mean size ± SE: 185.47 ± 125.04 μm; test concentration: 1.86 mg L^{-1}) on gill tissue and moulting cycle of marine crustacean (*Penaeus vannamei*) (Kim et al., 2023. Oliva et al. (2021) tested leachate from whole cigarette butts (20 butts/L, 24 h leaching time in the dark) on *D. magna*. They found no effects of leachate from unsmoked cigarette filters on the survival of *D. magna*, but smoked cigarette filters led to daphnid mortality already at 10 per cent by volume of the leachate. Furthermore, daphnids experienced an increased heart rate from exposure to both smoked and unsmoked cigarette filters. High mortality rates of various aquatic invertebrates (mussel *Dreissena polymorpha*, flatworm *Polycelis nigra*, snail *Planorbis planorbis*, snail *Bithynia tentaculata*) were measured in a mesocosm study following 24h exposure to the leachates of smoked cigarette filters (5 butts/L, 18 h leaching under room conditions; Green et al., 2020). The SCF-MPs and UCF-MPs tested in our study are more toxic to *D. magna* than any other type of MP we have previously tested; formerly, daphnid mortality was never observed, even at MP concentrations of up to 100 mg MP/L (Jemec Kokalj et al., 2018, 2022b). Considering the metal profile and organic chemicals of SCF-MPs and UCF-MPs, we hypothesize that the leaching of substances from the MPs could be the main reason for the observed effects on *B. calyciflorus* and *D. magna*. This has already been suggested by Micevska et al. (2006), who found a clear link between the nicotine content in cigarettes and their toxic effects on the daphnid *Ceriodaphnia dubia*. This would also explain the greater effect of SCF-MPs on *B. calyciflorus* and *D. magna* compared to UCF-MPs.

We observed a stronger response induced by SCF-MPs than UCF-MPs. These two types of MPs differ slightly in size (SCF-MPs are smaller) but have different elemental and organic compositions. The metal composition cannot be the only reason for the observed differences in toxicity between SCF-MPs and UCF-MPs, because both MPs contain potentially toxic metals. However, SCF-MPs contain a high proportion of nicotine and various organic compounds produced during tobacco combustion. Considering the complexity of the analyzed MPs, the observed changes are probably due to a combined effect of the microplastic particles and, in particular, the possible leaching of chemicals into the water and soil or in the organism's gut microenvironment. As the leaching of chemicals depends on the conditions of the test medium (such as pH, ionic strength, presence of complexing ligands, etc.) (Koutela et al., 2020), further studies should be performed to elucidate how the leaching of MPs milled from cigarette filters in different test media (e.g., freshwater, soil pore water) affects the tested organisms.

Cigarette filter-derived MPs tested in this study were mostly in the form of fragments obtained during cryo-milling, however a small share of fibers was also observed in particular for SCF-MPs. In the environment, however, microfibers are the predominant form of microplastics released from cigarette filters (Belzagui et al., 2021). These are represented in a whole range of sizes including longer fibers exceeding the size of MPs in this work. These might induce a different effect on organisms as some previous studies have shown a microfiber size-dependent ecotoxicity (Kwak and An, 2022). Furthermore, it would be interesting to test MPs derived from cellulose acetate (CA) which is widely used to produce cigarette filter tow but does not yet contain the additives added during cigarette filter production. It was previously suggested that different processing of CA affects its biodegradability and finally environmental hazard (Yadav and Hakkarainen, 2021).

This study provides clear evidence that both SCF-MPs and UCF-MPs can impact the environment. Therefore, it is crucial to minimize environmental pollution throughout the entire life cycle of cigarette filters. Various solutions have been proposed, including recycling used cigarette butts (see Yadav and Hakkarainen, 2021; Marinello et al., 2020). However, several questions remain unanswered, particularly regarding the economic viability and ethical challenges of recycling processes and the potential toxicity of the resulting products (Marinello et al., 2020). Above all, greater efforts should be directed toward preventing cigarette smoking and preventing the production of cigarette butt waste through product changes and other regulatory interventions (Marinello et al., 2020; Novotny and Hamzai, 2024).

CRediT authorship contribution statement

Andraž Dolar: Writing – review & editing, Writing – original draft, Visualization, Methodology, Conceptualization. **Branka Mušič:** Writing – review & editing, Methodology. **Tina Skalar:** Writing – review & editing, Methodology, Funding acquisition. **Gregor Marolt:** Writing – review & editing, Methodology, Funding acquisition. **Damjana Drobne:** Writing – review & editing. **Andrijana Sever Škapin:** Writing – review & editing, Funding acquisition. **Anita Jemec Kokalj:** Writing – review & editing, Writing – original draft, Visualization, Funding acquisition, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2025.126199.

Data availability

Data will be made available on request.

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