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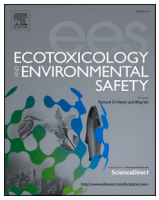

Toxicological effects of microplastic fibers from different disposable face masks on *Caenorhabditis elegans*

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ABSTRACT

During the COVID-19 pandemic, the widespread use of disposable face masks generated substantial waste, much of which ends up in terrestrial environments. However, the impact of this discarded material on soil ecosystems remains poorly understood. This study investigated the toxicological effects of microplastics derived from three types of disposable face masks (KF94, medical, and particulate respirator) and a polypropylene reference material on the soil nematode *Caenorhabditis elegans* (*C. elegans*). Exposure to 0.3 % (w/w, microplastic/soil) microplastics from KF94 masks and particulate respirators significantly reduced offspring numbers. Mass spectrometry (MS)-based metabolomic analysis revealed distinct metabolic alterations in *C. elegans* exposed to microplastics. Both KF94 masks and particulate respirators commonly disrupted the polyamine biosynthesis pathway but exhibited differing impacts on associated metabolites. High-resolution MS analysis of plastic additives extracted from the masks suggested that these differential metabolic changes could be attributed to various additives, including phthalates known to exhibit reproductive toxicity in *C. elegans*. These findings highlight the potential risk of plastic additives from disposed face masks disrupting soil ecosystems, raising concerns about their long-term environmental impact on soil health and biodiversity.

1. Introduction

The COVID-19 pandemic has led to widespread adoption of single-use personal protective equipment as a crucial preventive measure (Wu et al., 2020). Disposable face masks, in particular, became mandatory in public areas to prevent virus transmission. Since the pandemic's onset, the annual global usage of disposable face masks has been estimated in the hundreds of billions (Li et al., 2022; Prata et al., 2020). This unprecedented consumption has created significant waste management challenges (Adyel, 2020; Li et al., 2022), with discarded masks causing physical obstruction (Selvaranjan et al., 2021) and generating organic pollutants (Fernandez-Arribas et al., 2021; Knicker and Velasco-Molina, 2022). Additionally, growing evidence suggests that mask waste represents a significant source of microplastic pollution

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(Aragaw, 2020; Chen et al., 2021; Fadare and Okoffo, 2020).

Disposable face masks, constructed from polymer fibers, readily deteriorate in environmental conditions. Research has shown that various mask types can release 25–172 fiber-like microplastic particles during just two hours of simulated breathing (Li et al., 2021). This particle release increases when masks undergo treatments like washing or sunlight disinfection. With discarded masks increasingly visible in urban waterways and streets (Ardusso et al., 2021; Chowdhury et al., 2021), they represent an emerging source of microplastic fiber pollution. The environmental impact of these mask-microplastic fibers (MMF) remains poorly understood. Like typical microplastics, MMF can potentially transport pollutants (Anastopoulos and Pashalidis, 2021). Face masks incorporate various chemical additives and antimicrobial polymers (Zuniga and Cortes, 2020), with composition varying by intended use. These chemicals can leach into the environment during degradation (Rillig et al., 2021). Since extractable additives significantly contribute to microplastic toxicity (Kim et al., 2020b; Zimmermann et al., 2020), the chemical toxicity of MMF is anticipated to be a significant environmental concern.

While face masks generate substantial plastic waste in soil environments, few studies have examined how microplastics from polypropylene (PP) surgical masks affect terrestrial organisms. Meszaros *et al.* demonstrated that mask-derived microplastics inhibit lateral root formation in *Brassica napus*, with effects varying by fragment size and concentration (Meszaros et al., 2022). Kwak and An revealed that inner melt-blown face mask filters adversely affect soil organisms, particularly springtails and earthworms (Kwak and An, 2021). Similarly, Kokalj *et al.* found that different mask layers exhibit varying toxicity levels toward soil organisms (Jemec Kokalj et al., 2022). Collectively, these studies highlight how the physical properties of microplastics influence their toxicity on soil plants and animals. However, the metabolic changes induced in soil organisms by toxic chemicals leaching from mask waste remain unexplored, despite evidence that disposable face masks can release chemical pollutants (Sullivan et al., 2021).

This study investigates the soil toxicity and metabolic alterations induced by MMFs in the model organism *Caenorhabditis elegans* (*C. elegans*). We evaluated the soil toxicity of four microplastic fiber samples derived from three different commercial disposable face masks and a reference PP raw material. The impact of these microplastic fibers on *C. elegans* was assessed by measuring offspring production during a short-term exposure period. To gain deeper insights into the biological metabolic disruptions caused by microplastic exposure, liquid chromatography-mass spectrometry (LC-MS)-based metabolomic analysis was conducted. Furthermore, chemical analysis of MMF extracts using LC-MS was performed to identify toxic compounds potentially contributing to the observed soil toxicity in nematodes.

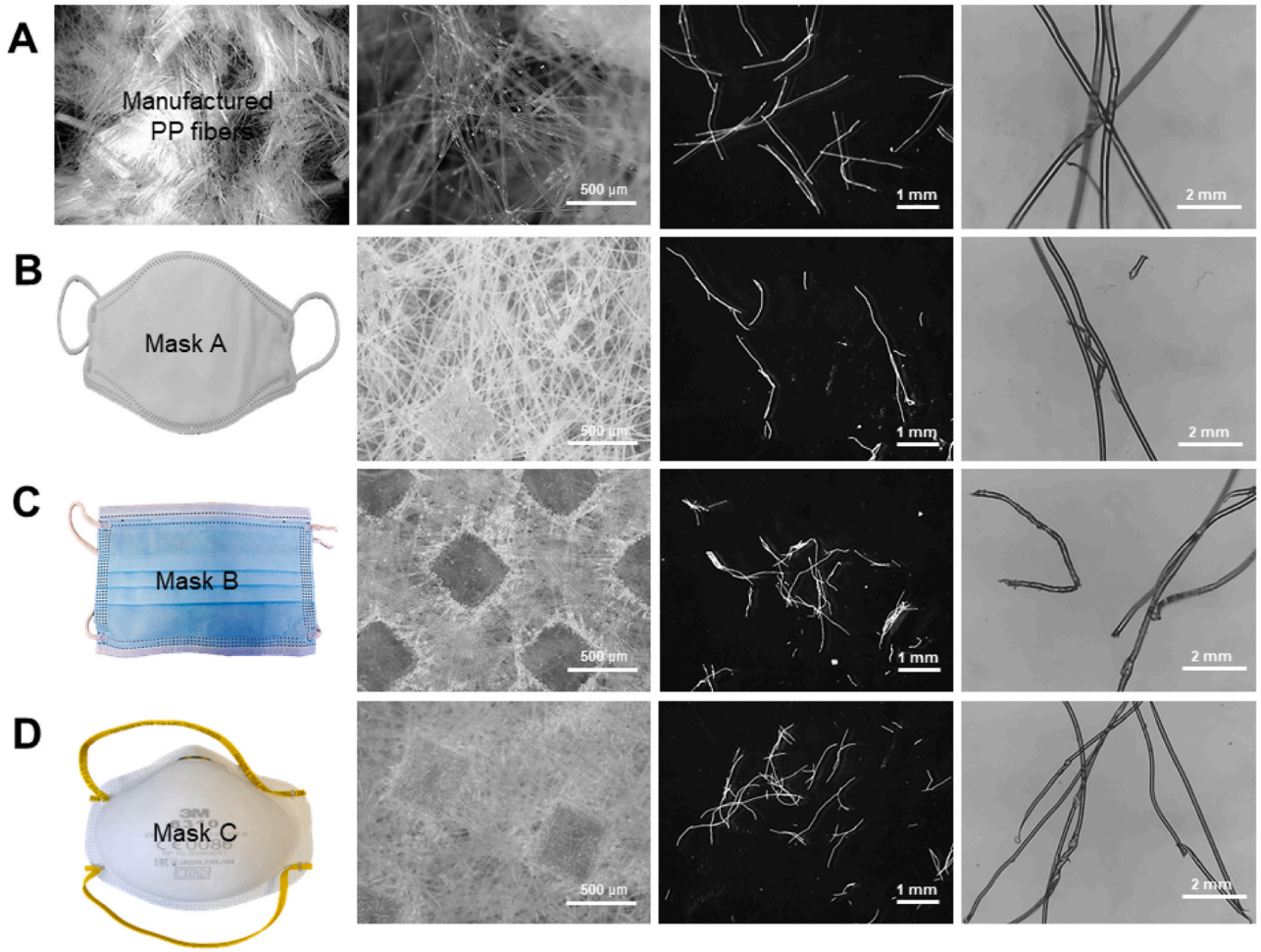

Fig. 1. Morphological characteristics of microplastic fibers derived from different disposable face masks. (A) Manufactured PP fibers and three types of masks; (B) mask A (a public-health care mask), (C) mask B (a medical face mask), and (D) mask C (a particulate respirator). The first column shows the macroscopic appearance of each microplastic fibers. The second column presents microscope images of the (A) manufactured PP and (B-D) fiber networks on mask surfaces (scale bar = 500 μm). The third and fourth columns display microscope images of extracted microplastic fibers at different magnifications (scale bars = 1 mm and 2 mm, respectively).

2. Materials and methods

2.1. Target materials and organism

Manufactured PP fiber (MPPF) was obtained from Türkiye (100 % virgin PP, Polipropilen Elyaf San., Istanbul, Türkiye). Three different disposable face masks (referred to as masks A-C) were acquired from public markets in Germany and South Korea. Detailed information of each disposable face mask can be found in Table S1. Although there were some structural variations in different mask brands, they all appeared to be manufactured using polymeric-fiber fabrication (Fig. 1). To create mask microplastic fibers (MMFs), we shaved the filter layer of each mask using a razor blade (Fig. S1). The obtained fibers were passed through a 1 mm-sieve. We observed each fiber sample under a microscope and captured close-up photographs for measuring the average sizes using image analysis software (ImageJ, 1.52a, National Institutes of Health, USA). The average widths of MPPF, MMF-A, MMF-B, and MMF-C were 49.3 ± 5.0, 38.4 ± 6.7, 35.0 ± 3.2, and 27.0 ± 2.6 μm, respectively. The average lengths were 2.24 ± 0.36, 1.47 ± 0.68, 1.45 ± 0.62, and 1.51 ± 0.58 mm, respectively ($n = 200$). For spectroscopic characterization, a spectrophotometer (Jasco, model FT/IR-4100, ATR mode) was used. Each sample was scanned 32 times from 4000 to 600 cm^{-1} with a resolution of 4 cm^{-1}. The spectra of MPPF and the three MMFs exhibited characteristic peaks of PP.

C. elegans (wild type, Bristol strain N2) used in this study was obtained from the Berlin Institute for Medical Systems Biology at the Max Delbrück Center for Molecular Medicine (Berlin, Germany). The nematodes were maintained on nematode growth medium (NGM; NaCl 3 g/L, peptone 2.5 g/L, agar 17 g/L, 1 M potassium phosphate 25 mL/L, 1 M $CaCl_2$•2 H_2O 1 mL/L, 1 M $MgSO_4$•7 H_2O 1 mL/L, cholesterol 1 mL/L) at 20 ± 2°C in the dark. *Escherichia coli* (*E. Coli*, strain OP50) was grown in Luria-Bertani (LB) medium (LB broth 2.5 g/L) overnight at 36 ± 2°C and provided as a food source for the nematodes. To synchronize the developmental stages of the worms before conducting the soil toxicity test, we treated at least 3 d-cultured adult worms with a Clorox solution (1 N NaOH:5 % NaOCl, 1:1) for 20 min. The resulting suspension was centrifuged at 5000 rpm for 2 min. The embryo pellets were washed with K-medium (0.032 M KCl, 0.051 M NaCl) three times. The embryos were then placed on a new NGM plate with a food source and incubated for 60–65 h to reach young adult stage for the soil toxicity tests.

2.2. Soil toxicity tests

Untreated field soil was collected from a site of Freie Universität Berlin, Berlin, Germany (52.45676 N, 13.30240E) on January 20, 2020. The test soil was prepared by sieving through a 2 mm-mesh and subsequently drying at 60°C for 24 h. The test soil exhibited a sandy texture (sand 93.3 %, silt 5.0 %, and clay 1.7 %), with a pH of 6.7 ± 0.2 and water holding capacity (WHC) of 0.34 ± 0.10 mL/g ($n = 3$). To prepare MPPF and MMF-amended soils, 30 mg of each fiber sample was thoroughly mixed with 9.97 g of test soil to create stock mixtures, which were serially diluted with additional test soil to achieve final concentrations of 0.1 % and 0.3 % (w/w, dry weight basis). Control treatments containing only test soil (no fibers) were included for comparison. These concentrations were selected based on environmentally relevant levels. Similar to other microplastics, MMFs can enter industrial and agricultural lands through contaminated water and sewage sludge application (Edo et al., 2020). Previous studies have reported microplastic concentrations ranging from 8 to 67,500 mg kg^{-1} in such environments (Fuller and Gautam, 2016; Liu et al., 2018; Scheurer and Bigalke, 2018). We also considered the upper threshold (0.1–0.4 %) at which microplastic fibers have been shown to exert minimal effects on soil properties (Machado et al., 2019; Rillig et al., 2019).

Standard soil toxicity tests often involve extracting *C. elegans* from the soil medium using methods like colloidal silica flotation with LUDOX solution (ASTM, 2002; ISO, 2010), sometimes followed by staining (e.g., with Rose Bengal in the ISO method) to aid visualization. However, given the presence of microplastic fibers in our test soils, we were concerned that these fibers could interfere with the efficiency of flotation-based nematode collection and potentially lead to misinterpretation during staining procedures. Our experiment therefore employed an alternative validated method (Kim et al., 2018) in which nematode offspring in test soil were attracted by a microbial food source under no-food conditions in the test soil itself. For the toxicity test, 0.3 g of each prepared soil (control, MPPF, or MMF-contaminated) was added to individual wells of a 24-well plate, along with 82 μL of K-medium (80 % of WHC). Ten age-synchronized worms were added to each well and incubated at 20 ± 2°C in the dark (control, $n = 24$; each mask treatment $n = 12$). After 24 h, the soil containing nematodes was transferred onto soil-agar isolation plates (Kim et al., 2020a). These plates were prepared by culturing E. coli strain OP50 in LB medium at 37°C overnight, with 75 μL of cell suspension spread on each side of a NGM agar plate. Each test soil was arranged linearly in the central area of the soil-agar isolation plate, allowing the offspring to move from the test soil toward the bacterial food source on either side. The number of offspring migrating to each side was then quantified, and the data were expressed as a percentage (%) of the average value of the control group.

2.3. Metabolite extraction

For the metabolomics analysis, 60 nematodes were prepared per sample with three replicates. The harvested worms were washed with M9 buffer three times and then immediately placed in 500 μL of cold methanol at 0°C in a glass tube to quench metabolism. All the samples were subsequently stored at −80°C until the extraction process. For extraction, we added 1.7 mL of methyl *t*-butyl ether (MTBE) to the frozen sample glass tube placed on ice, followed by vigorous vertexing. The nematodes were sonicated in an ice-cold bath for 30 min. After adding 420 μL of water (H_2O), the mixture was further sonicated for 15 min. The resulting sample was then centrifuged at 4°C with 4600 rpm for 15 min. The upper organic phase was transferred to a new glass vial, while the remaining lower phase underwent an additional extraction step with 650 μL of MTBE, followed by 15 min of sonication. After centrifugation, the organic layers were combined, and the solvent was evaporated using nitrogen gas in a fume hood.

2.4. LC-MS/MS for untargeted metabolomics

The dried nematode extract samples were reconstituted in a mixture of acetonitrile (ACN)/isopropanol/H_2O (65/30/5, v/v/v) with a volume of 50 μL. Then 15 μL of each sample was injected into an Agilent 1290 Infinity II ultra-high performance liquid chromatography (UHPLC) coupled to an Agilent 6550 iFunnel quadrupole time-of-flight (Q-TOF) instrument, which was equipped with an Agilent Jet Stream electrospray ionization (ESI) source. Metabolites were separated using a reversed-phase Agilent ZORBAX Eclipse Plus C18 RRHD column (2.1 mm × 50 mm, 1.8 μm). The mobile phases for the chromatographic separation were composed of H_2O (mobile phase A) and a mixture of ACN and H_2O (95:5, v/v) both containing 10 mM ammonium formate and 0.125 % formic acid (mobile phase B). The binary gradient elution employed the following changes in mobile phase B: 1 % at 0 −1 min, 1 −20 % at 1 −2.5 min, 20 −30 % at 2.5 −4 min, 30–50 % at 4 −5 min, 50 −76 % at 5 −6 min, 76–99 % at 6 −8 min, 99 % at 8 −10.25 min, 99–1 % at 10.25 −12.5 min, and maintained at 1 % for 5 min after the run. The flow rate of the LC system was set to 0.400 mL/min. The capillary voltage for ESI was adjusted to 3.5 kV. For untargeted LC-MS/MS in both positive and negative ion modes, the top five abundant ions in the MS scan were automatically selected for collision-induced dissociation in with an active exclusion time of 0.15 min. The precursor isolation width was 4 Da, and the collision energy applied was 20.0 V. The mass ranges for the MS and MS/MS scans were 200–1000 and 50–1000 m/z, respectively.

The raw MS data was processed in three steps: feature detection, identification, and statistical analysis. The raw MS data files were converted to mzXML files and then processed using XCMS Online. This involved steps such as feature detection, peak detection, peak alignment, and peak grouping, with the parameters of XCMS Online set to the UHPLC/Q-TOF preset. The detected features were initially matched to the KEGG pathway database of *C. elegans* using mummichog program in MetaboAnalyst 5.0 (Lu et al., 2023; Xia et al., 2009)), with an *m/z* threshold of 30 ppm. Significantly changed features were selected through statistical analysis methods such as analysis of variance (ANOVA) and hierarchical clustering analysis (HCA). To address multiple testing issues, false discovery rate (FDR) adjusted p-values were computed using the Benjamini-Hochberg procedure, and post-hoc p-values were determined using Tukey's honestly significant difference test. For confident identification, MS/MS spectra of significantly changed features were matched to METLIN databases. When the KEGG database proved insufficient, additional identification was performed using NIST 20. From the NIST 20 spectral library results, identifications with reverse-dot product scores below 800 were excluded, and among duplicate identifications, those with higher scores and abundances were selected.

2.5. Chemical analysis using ultra-high resolution MS

MPPF and MMF samples were cut into small pieces using sterilized scissors. A volume of 2 mL of extraction solvent (either H_2O or ACN) was added to 0.1 g of each mask sample, respectively. This dual-solvent approach was implemented to account for the diverse soil chemistry and multiple potential leaching pathways that exist in natural environments (Ramanayaka et al., 2023). H_2O was selected to simulate polar compound extraction in aqueous soil solutions, while ACN was employed to capture less polar additives that might interact with soil organic matter components. The samples were sonicated for 1 h and subsequently centrifuged at 10,000 × g for 10 min. The resulting supernatants were transferred to new glass tubes and concentrated to dryness in a SpeedVac overnight. The dried extracts were then resuspended in 100 μL of their respective original extraction solvents (H_2O or ACN) for subsequent chemical analysis.

Microfiber extracts were analyzed with an UHPLC coupled with Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer (SolariX 2XR, Bruker Daltonics, Germany) equipped with an ESI source. Elemental compositions were calculated using DataAnalysis (version 4.4, Bruker Daltonics, Germany) and Composer64 (version 1.5.6, Sierra analytics Inc., USA) with ± 1 ppm *m/z* tolerance. The obtained elemental compositions were compared to the plastic_additive databases in CompTox using a batch search feature (https://comptox.epa.gov).

3. Results and discussion

3.1. Effects of MMFs on nematodes in soil

The mean value of offspring number in control treatment was measured as 94 ± 14 (mean ± standard deviation), while those observed in MMF treatments varied considerably depending on mask type, ranging from 49 ± 10–97 ± 14. Exposure of *C. elegans* to MMFs at the low concentration (0.1 %) exhibited no significant effects on the number of offspring (Fig. 2). The normalized offspring counts relative to the control were 93 ± 8 %, 97 ± 9 %, 99 ± 9 %, and 92 ± 7 % for MPPF, MMF-A, MMF-B, and MMF-C treatments, respectively. However, at a higher concentration (0.3 %), significant reductions in offspring were observed for MMF-A (67 ± 10 %) and MMF-C (54 ± 10 %) compared to the control. In contrast, MPPF (94 ± 8 %) and MMF-B (85 ± 15 %) were relatively unaffected. Despite all tested materials being classified as PP fibers, only MMF-A and MMF-C caused a notable reduction in offspring at the higher concentration.

The number of offspring in the control treatment reached 9.4 ± 1.4 per worm. Considering that *C. elegans* can produce approximately 17.8 embryos hatching within 7.3 h (Muschiol et al., 2009), and that the hatching rate of about 79.6 % at 20°C (Skantar et al., 2005), our test conditions appear to support normal reproduction. Moreover, because nematodes were not provided with food during exposure, potential confounding effects such as food dilution—typically caused by particles too large for ingestion—can be excluded in this study. However, the absence of food may influence responses specifically in the MMF

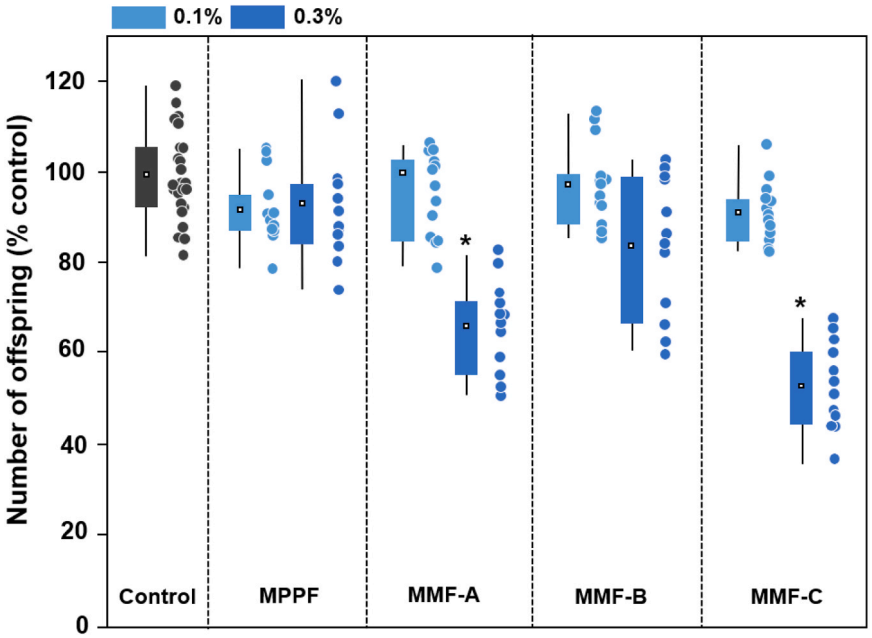

Fig. 2. Effects of microplastic exposure on *C. elegans* reproduction. Number of offspring of *C. elegans* exposed to different types of microplastics: manufactured polypropylene fibers (MPPF) and three types of mask-microplastic fibers (MMF-A, MMF-B, and MMF-C). Nematodes were exposed to two concentrations: 0.1 % and 0.3 % (w/w). Replicates of control ($n = 24$) and each MMF treatment ($n = 12$) are represented by dots. Data are presented as mean ± standard error of the mean and normalized to the control group. Asterisks (*) indicate statistically significant differences compared to the control treatment ($p < 0.05$).

treatments, and further studies could help clarify how dietary conditions interact with toxic effects. Given that the sizes of all PP fibers used (width, 27.0–49.3 μm; length 1.45–2.24 mm) exceeded the edible size for *C. elegans* (≤ 3.4 μm) (Mueller et al., 2020), the observed differences in toxicity are likely attributed to variations in the chemical composition of additives within the microplastics, rather than their physical properties. This interpretation aligns with previous studies highlighting a link between the chemical composition of microplastic extracts and their potential to harm organisms (Kim et al., 2020b; Zimmermann et al., 2019, 2020).

Face mask filtration typically relies on either electrospun nanofiber or melt-blown microfiber mats, which capture particulate matter through physical sieving or electrostatic adsorption (Choi et al., 2021). Electrospinning utilizes polymer solutions, while melt-blown processes involve melting and extruding polymers. Reducing the viscosity of these materials is crucial for obtaining smaller diameter fibers, which enhance filtration performance (Zhiyuan et al., 2014). PP, being non-polar, presents manufacturing challenges due to its poor organic solvent solubility, necessitating melt processing (Haigh et al., 2017). Manufacturers commonly incorporate additives to enhance melt plasticity or polarizability. Previous research has demonstrated that additives such as irgatec CR 76 can reduce PP fiber diameter from 35 μm to 0.8 μm, while stearic acid or sodium stearate achieve reductions from 5.4 μm to 1.6–4.9 μm (Dalton et al., 2007; Zhiyuan et al., 2014). This relationship between smaller fiber diameter and increased additive content may explain heightened toxicity in finer fibers. In our study, MPPF showed a larger diameter (49.3 μm) compared to other MMFs (27.0–38.4 μm), supporting this connection between fiber diameter, additive content, and toxicity.

Additionally, face mask filters often incorporate performance-enhancing materials such as carbon compounds, metal-organic frameworks, and antibacterial nanoparticles (*e.g.*, silver), though their toxicological impacts remain poorly understood (Choi et al., 2021; Kharaghani et al., 2018; Xiong et al., 2017). During our nematode recovery procedure, we observed inhibited bacterial colony formation in soil-agar isolation plates (Fig. S2), which provides indirect evidence for the presence of biologically active compounds leaching from the mask fibers. These bacterial colonies, present on all test soil plates, showed consistent growth inhibition across all replicates (n = 12). While specific antibacterial compounds were not chemically identified, this observation suggests the release of substances with potential antibacterial properties or other bioactive components from the MMFs. This phenomenon supports our broader finding that mask-derived materials contain additives capable of exerting biological effects, which may contribute to the observed toxicity in nematodes through various mechanisms. Further chemical characterization would be necessary to identify the specific compounds responsible for these effects.

3.2. Untargeted metabolomic analysis

Untargeted metabolomic analysis revealed molecular-level insights into how microplastics affect *C. elegans* metabolism. Initial feature screening using mummichog identified 173 LC-MS features from the KEGG *C. elegans* metabolic pathway database. Among these, 61 features showed significant changes across the different microplastic treatments based on univariate unpaired ANOVA (FDR < 0.05). HCA revealed distinct patterns of metabolite changes across the different microplastic exposures. The metabolite features segregated into four distinct clusters based on their quantitative responses to microplastic exposure (Fig. 3).

Cluster 1 features showed high signals in controls but decreased with

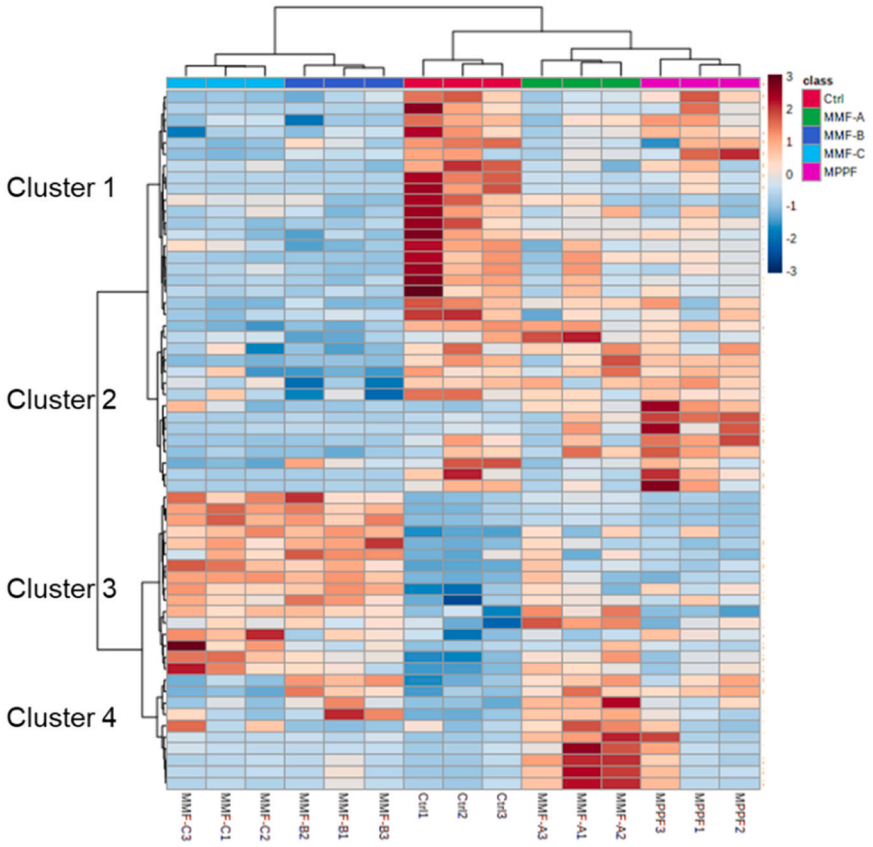

Fig. 3. Hierarchical clustering analysis of differentially abundant metabolites in *C. elegans* exposed to Microplastics. Metabolites exhibiting significant changes (FDR < 0.05) following exposure to MMFs and MPPF were clustered and visualized as a heatmap. Metabolites with similar patterns of change across experimental groups were grouped into four distinct clusters.

both MPPF and MMF exposure. Cluster 2 was characterized by uniquely elevated signal intensities in the MPPF exposure group, likely reflecting the influence of manufacturer-added substances in the PP fiber. Cluster 3 comprised features specifically altered by MMF-B and MMF-C exposures, with MMF-C inducing stronger metabolomic changes despite similar response patterns. Cluster 4 contained features uniquely modified by MMF-A exposure, displaying patterns distinct from all other experimental groups. These metabolomic findings aligned with offspring count data, revealing non-identical response patterns among MMF exposure groups. Notably, while MMF-A and MMF-C both reduced offspring numbers, they induced different metabolomic changes. MMF-B, despite showing no significant effect on offspring numbers, demonstrated metabolomic changes similar to MMF-C. All MMF-exposed groups exhibited metabolite profiles distinctly different from MPPF, supporting the hypothesis that PP fiber toxicity stems from chemical rather than physical effects. This metabolomic approach provided insights beyond traditional phenotypic endpoints, suggesting that various mask manufacturing additives can induce distinct metabolomic signatures.

Further analysis using commercial METLIN MS/MS spectral libraries identified spermidine with high confidence (METLIN score 0.969) among the significantly altered features. Expanded analysis using the NIST 20 MS/MS library revealed 358 additional features in positive ion mode and 161 in negative ion mode, with 39 features showing significant changes (p-value < 0.05). Two metabolites in the polyamine biosynthetic pathway were identified with high confidence: agmatine and spermidine. MMF-A exposure significantly affected agmatine levels (Fig. 4A), while MMF-B and MMF-C exposure altered spermidine levels (Fig. 4B). The MS/MS spectra of both agmatine and spermidine were validated using their respective authentic standards (Fig. S3), confirming their identity. The full list of all detected and significantly altered features is available in Table S2.

Both metabolites are involved in the polyamine biosynthetic pathway, which plays essential roles in cellular processes such as protein and nucleic acid synthesis (Tabor and Tabor, 1976), cell proliferation, and reproduction (Lefevre et al., 2011). Polyamines, which are fully protonated at physiological pH, interact strongly with anionic macromolecules like nucleic acids, proteins, and phospholipids, and are particularly critical for gametogenesis and reproduction. Changes in polyamine levels under stress conditions, such as oxidative stress induced by triclosan exposure in *C. elegans*, have been previously documented (Kim et al., 2019a,b). Similarly, exposure of lettuce to Cu $(OH)_2$ nanopesticides led to upregulation of spermidine and putrescine, which mitigated oxidative stress by scavenging free radicals and enhancing plant tolerance (Zhao et al., 2016).

Alterations in the polyamine biosynthetic pathway likely contribute directly to the observed reduction in *C. elegans* offspring, affecting reproduction and development. Interestingly, while both MMF-A and MMF-C exposures impacted this pathway, the metabolic responses differed: MMF-A exposure elevated agmatine, whereas MMF-B and MMF-C exposures reduced spermidine levels. These findings suggest that while microplastics may share common effects on polyamine biosynthesis, their specific toxic mechanisms differ depending on the additives used during manufacturing.

3.3. Chemical analysis of MMF extracts

Chemical features suspected to be plastic additives were analyzed using high-resolution MS. Features from microplastic extracts were detected, and their elemental compositions were identified within a 1 ppm m/z tolerance. This high-accuracy analysis enabled selective assignment of elemental compositions, which were matched to the CompTox database. Features of the H_2O and ACN extracts of microplastics are presented in Fig. 5. More than half of the chemical features in MMF and MPPF extracts were unique to each microplastic, highlighting the distinct chemical properties of the materials. Among the groups, the H_2O extract of MMF-C displayed the highest number of features, whereas its ACN extract exhibited the fewest. These results suggest that the observed differences in toxicity in MMF-A and MMF-C from the metabolomic analysis may stem from their distinct chemical properties.

Previous research has demonstrated that leached chemicals from plastic products can induce cytotoxicity (Zimmermann et al., 2021), with extracted additives showing toxicity comparable to raw microplastics in nematodes (Kim et al., 2020b). To identify potential compounds responsible for reduced *C. elegans* offspring numbers, unique features in MMF-A and MMF-C extracts were matched against CompTox plastic additive databases (Table S3). This analysis identified 15 chemicals in MMF-A and 17 in MMF-C. In the MMF-A extract, trihexyl acetylcitrate was identified. While this compound is considered a safer

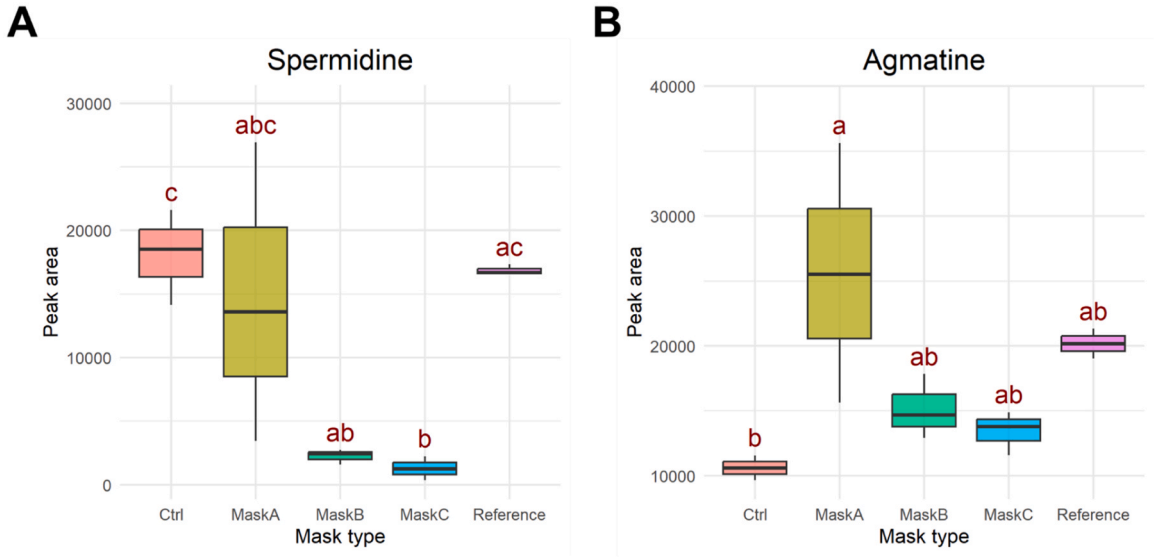

Fig. 4. Boxplots showing the peak areas of significantly altered metabolites in *C. elegans* exposed to different microplastic treatments. (A) Spermidine and (B) agmatine. The different letters above the whiskers (*e.g.*, a, ab, and abc) indicate statistical significance based on Tukey's post-hoc multiple comparison tests following ANOVA. Data points sharing the same letter are not significantly different from each other, whereas data points with different letters indicate statistically significant differences.

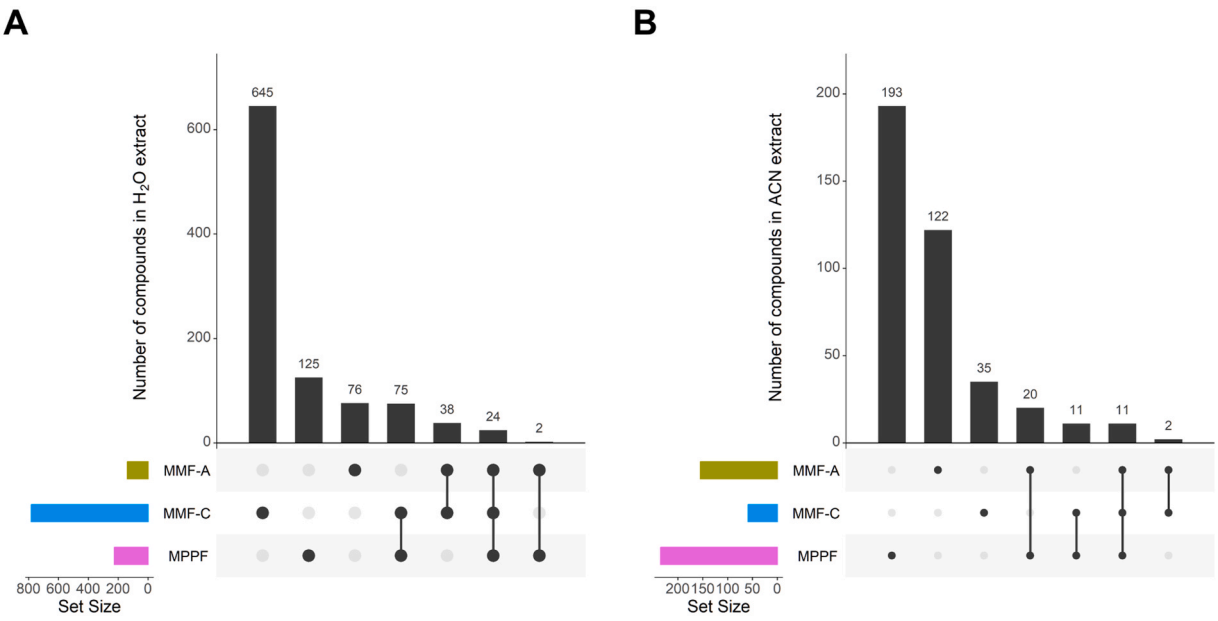

Fig. 5. Chemical feature distribution in microplastic extracts analyzed by CompTox database matching. UpSet plots showing overlapping and unique chemical features detected in MMFs and MPPF extracts using (A) H_2O and (B) ACN as solvents. Each vertical bar represents the number of compounds found at the intersection of the sets indicated below it, while each horizontal bar indicates the total number of compounds detected in each individual microplastic sample.

alternative to phthalates (Johnson, Jr, 2002) due to its lower toxicity in skin and chromosome damage tests (Kim et al., 2019a,b), recent studies indicate potential hazards. For example, trihexyl acetylcitrate has been shown to induce apoptosis and necrosis in Xenopus embryos under oxidative stress (Xu and Gye, 2018). These findings suggest organism-specific toxicity with heightened effects on developing embryos.

MMF-C extracts contained multiple phthalates and acrylates, consistent with previous reports of phthalate presence in disposable face masks (Xie et al., 2022). Phthalates, widely used as plasticizers to enhance polymer flexibility, are known endocrine disruptors with reproductive and developmental toxicity (Hlisnikova et al., 2020). For example, di(2-ethylhexyl) phthalate has been shown to reduce oocyte numbers and damage DNA in *C. elegans* (Yin et al., 2018) and cause multigenerational reproductive toxicity in this model (Li et al., 2018). Similarly, butyl benzyl phthalate decreased reproduction timing and numbers in *Daphnia magna* (Li et al., 2021). These findings support the connection between observed offspring reduction and phthalate exposure. The high proportion of polar chemicals in the H_2O extract of MMF-C likely relates to surface acrylates, though direct evidence linking acrylates to reproductive effects remains limited (Greim et al., 1995). In summary, distinct chemical additives were identified for each mask type, with some potentially affecting reproductive functions.

4. Conclusion

This study demonstrates the potential toxicity of microplastic fibers derived from three different types of face masks to the soil nematode *C. elegans*. We observed significant reductions in offspring production in nematodes exposed to two of the three mask types. Notably, no toxicity was observed with the control PP fibers, suggesting that additives introduced during mask manufacturing are likely responsible for the observed effects. To investigate the underlying mechanisms, we conducted an untargeted metabolomic analysis, revealing distinct metabolic perturbations in nematodes exposed to different mask types. These findings, coupled with the chemical analysis of mask extracts, which identified potential toxicants such as phthalates and trihexyl acetylcitrate, further support the hypothesis that additives play a crucial role in the toxicity of face mask-derived microplastics.

In this study, manufactured PP fibers were used as control materials to distinguish additive-related toxicity from potential physical effects of fibrous microplastics. However, we acknowledge that even synthetic control fibers may retain residual chemicals from production, potentially influencing biological responses. As an alternative, natural material-based fibers (e.g., cellulose or cotton) could be explored in future ecotoxicological studies to better isolate and evaluate the physical impacts of microplastic-like particles. The development and standardization of such inert reference materials would contribute to improving the interpretability and reproducibility of microplastic toxicity assessments.

This study highlights the potential ecological risks associated with the increasing accumulation of face mask waste. However, several limitations should be considered. The use of soil media, while ecologically relevant, can present analytical challenges due to the complex soil matrix. Additionally, while we employed a comprehensive approach to metabolite identification, further validation with authentic standards is crucial to enhance the confidence of metabolite assignments. Furthermore, this study focused on short-term exposure, and long-term studies are necessary to fully assess the chronic effects of face mask-derived microplastics on soil ecosystems. Importantly, while we demonstrated potential toxicity linked to chemical additives, the actual concentrations of leached compounds in soil and their bioavailability to soil organisms were not quantified. Given that leaching behavior is highly dependent on various environmental factors—including soil composition, moisture, and microbial activity—future studies should aim to characterize these dynamics more precisely. Despite these limitations, this study provides valuable insights into the potential ecological risks associated with the increasing use and disposal of face masks. Further research is warranted to fully understand the long-term environmental impacts of these ubiquitous microplastics and to develop strategies for their sustainable management.

CRediT authorship contribution statement

Jonghyun Kim: Writing – original draft, Investigation. **Shin Woong Kim:** Writing – original draft, Investigation, Conceptualization. **Maria K. Parr:** Resources. **Matthias C. Rillig:** Supervision. **Tae-Young Kim:** Writing – review & editing, Supervision, Conceptualization. **Waldman**

Walter W.: Conceptualization. **Felix Bredendiek:** Investigation. **Seungwoo Son:** Investigation. **Sunghwan Kim:** Resources.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT and Claude in order to improve readability and language of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.118451.

Data availability

Data will be made available on request.

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