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Article

Effects of Different Microplastics on Nematodes in the Soil Environment: Tracking the Extractable Additives Using an Ecotoxicological Approach

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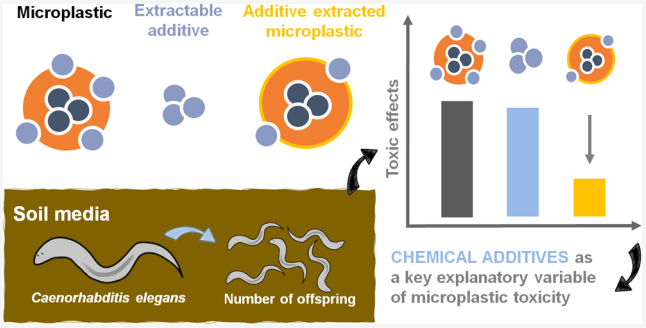
ABSTRACT: With increasing interest in the effects of microplastics on the soil environment, there is a need to thoroughly evaluate the potential adverse effects of these particles as a function of their characteristics (size, shape, and composition). In addition, extractable chemical additives from microplastics have been identified as an important toxicity pathway in the aquatic environment. However, currently, little is known about the effects of such additives on the soil environment. In this study on nematodes (*Caenorhabditis elegans*), we adopted an ecotoxicological approach to assess the potential effects of 13 different microplastics (0.001−1% of soil dry weight) with different characteristics and extractable additives. We found that poly(ethylene terephthalate) (PET) fragments and polyacrylicnitrile (PAN) fibers show the highest toxicity, while high-density polyethylene (HDPE), polypropylene (PP), and polystyrene (PS) fragments induced relatively less adverse effects on nematodes. In addition, low-density polyethylene (LDPE) induced no toxicity within our test concentration range for the acute period. Acute toxicity was mainly attributed to the extractable additives: when the additives were extracted, the toxic effects of each microplastic disappeared in the acute soil toxicity test. The harmful effects of the LDPE films and PAN fibers increased when the microplastics were maintained in the soil for a long-term period with frequent wet−dry cycles. We here provide clear evidence that microplastic toxicity in the soil is highly related to extractable additives. Our results suggest that future experiments consider extractable additives as key explanatory variables.



INTRODUCTION

Plastic polymers have been widely used for the past 70 years, and an enormous amount of plastic litter has been spread in the environment.[1,2] Primary plastics have been fragmented into a smaller size (<5 mm, microplastics), and these tiny particles are ubiquitously detected in a broad range of environmental compartments including oceans,[1] freshwater bodies,[3] soils,[4] atmospheres,[5] and even drinking water sources.[6] Although microplastic pollution in the soil environment has not received significant media and research attention, microplastic abundance is estimated to be up to 23 times larger than that in the ocean.[7] Soils have various input sources including amendments with compost or sludge containing microplastics, application of agricultural films, surface runoff, and landfill leachates.[8−11] Previous studies have reported that 8−67 500 mg/kg microplastics can be observed in industrial,[12] pristine floodplain,[13] and agricultural lands.[14]

A key concern of microplastic pollution is whether it poses a risk to ecosystems. Although a lack of available data and methodological issues still hinder the progress,[15] several previous studies have provided laboratory-scale evidence of harmful effects on living organisms.[4,16] In the soil environment, invertebrates and agricultural plants can experience adverse effects, such as mortality increase and growth decrease,[17−20] and negative effects on microbial and enzymatic activities have also been reported.[21,22] With an increasing number of these studies, it is becoming necessary to systematically test for parameters that can control these effects,[4] and microplastic characteristics (size, shape, and composition) have been highlighted as an important factor to consider.[23,24] While the database on microplastic effects is much rich for aquatic environments,[25−28] fewer such studies have been reported for soils. Several studies have reported size- or composition-dependent effects on plants, nematodes, and soil properties,[17,29−34] but part of these studies were performed in nonsoil media or using spherical beads.[29,30,32,33]

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Chemical effects may serve as an important mediator of microplastic toxicity. A central hypothesis is that microplastics can carry harmful hydrophobic organic pollutants with strong sorption capacity[35] and that leaching of chemical additives from microplastics can be expected.[36] These additives are intentionally added to plastic products to improve their functionality (*e.g.*, functional additives, colorants, fillers, and reinforcements) and are optimized for the first use phase, not for recycling.[37,38] These incorporated chemicals can be continually released into the environment during the decomposition or fragmentation process and may be partially responsible for any microplastic toxicity.[37,39] For example, bisphenol A, which is authorized under Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as a stabilizer, is regarded as an estrogen agonist.[38] Phthalates are another common organic species in plastic manufacturing[40] and are considered endocrine disruptors at very low environmental levels (in the range of ng/L).[41] Several experiments have found that leaching solutions from microplastics can induce severe damage to the aquatic organisms including water fleas,[42,43] microalgae,[44,45] copepods,[46] and brown mussels,[47] but the evidence is sparse for the soil environment.

Since additive leaching from plastics is highly related to both chemical equilibria and diffusion kinetics, a partition constant (K_D) between the plastic and surrounding media can be the most important factor to understand the leaching mechanism.[35,36] Nevertheless, K_D is mostly calculated with pure solvents or food simulants and nondegraded polymers, having limited information about K_D for microplastic research, considering secondary microplastics or environmental conditions.[35,48,49] Furthermore, the immediate surrounding of microplastics in aquatic environments is dynamic, constantly changing due to physicochemical and biological parameters.[50,51] Several pieces of evidence have also suggested that microplastics might be transported from the surface to the soil system through cracking or movement of living organisms,[52,53] and the physicochemical properties of surrounding media are varied and complicated similar to aquatic environments.[54−57] Since we have no knowledge about predicting the effects of chemical additives in such soil media from first principles, data from experimental studies are needed.

Here, we conducted soil toxicity tests using the nematode *Caenorhabditis elegans* as a model organism, and 13 microplastics were chosen as target materials; six different compositions (high-density polyethylene, HDPE; poly(ethylene terephthalate), PET; polypropylene, PP; polystyrene, PS; low-density polyethylene, LDPE; polyacrylonitrile, PAN) and three different shapes (fragments, film, fibers) with one to three different size ranges (Table S1). To evaluate the potential effects of extractable additives from each microplastic, we adopted an ecotoxicological approach instead of prediction by chemical analysis. The additives were extracted with water as the solvent using two different methods. The most efficient method was used to follow the influence of microplastics size and concentration on the ecotoxicological assessment. Finally, microplastic ecotoxicity was tested in two situations: after removing additives from particle surfaces, to correlate toxicity and the presence of the additives, and an ecotoxicological assessment as a function of time with soil experiencing wet−dry cycles. We explain the rationale for this experiment in greater detail in the Materials and Methods section. In addition, untargeted liquid chromatography−mass spectrometry (LC−MS) was performed in an attempt to screen the extractable additives of microplastics.

MATERIALS AND METHODS

Target Microplastics and Organisms. Target microplastic fragments were prepared by cryomilling as reported in our previous study.[17] The polymers, including HDPE, PET, PP, and PS were obtained from Bundesanstalt für Materialforschung und -prüfung (Berlin, Germany), and they were ground in an ultracentrifugal mill (using a 2 mm ring sieve) after embrittlement with liquid nitrogen. After drying, the fragments were passed through a 1000 μm sieve and stored at room temperature. To obtain different-sized fragments, sieving (630 and 250 μm) was additionally performed in the present study. HDPE, PP, and PS were prepared in three different size ranges (<250, 250−630, and 630−1000 μm), and PET was separated into two size ranges (<250 and 250−630 μm) due to smaller size distribution than others.[17] In a previous paper,[58] we have monitored microplastic fragments using scanning electron microscopy to determine whether they contained any nanosized particles and found that nanosized particles were present on the fragment surface. Since preparing nanoplastic-free samples is nearly impossible, we can expect that there is an unknown quantity of nanosized plastics in all samples. The LDPE films and PAN fibers were prepared using commercial mulching films (LDPE; thickness, 13.66 ± 2.32 μm, Ihlshin Chemical Co., Ltd., Ansan, South Korea) and knitting wool (100% PAN, DIKTAS Sewing & Knitting Yarns Co., Turkey), respectively.[59] Each material was cut using sterilized scissors, then passed through a 630 μm sieve (<630 μm), and stored at room temperature. For the spectroscopic characterization, we used a spectrophotometer (Jasco, model FT/IR-4100, attenuated total reflection (ATR) mode). Each sample was scanned 32 times, from 4000 to 600 cm^{-1}, with a resolution of 4 cm^{-1} (Figure S1).

C. elegans (wild type, Bristol strain N2) was obtained from Berlin Institute for Medical Systems Biology at the Max Delbrück Center for Molecular Medicine (Berlin, Germany). They 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 $CaCl_2 \cdot 2H_2O$ 1 mL/L, 1 M $MgSO_4 \cdot 7H_2O$ 1 mL/L, cholesterol 1 mL/L) at 20 ± 2 °C in the dark, and *Escherichia coli* (strain OP50) was supplied as a food source.[60] To synchronize the developmental stage before the experiment, the culture plates that were maintained for at least 3 days were treated with a Clorox solution (1 N NaOH/5% NaOCl, 1:1) for 20 min, and then, the suspension containing embryos was centrifuged at 4500 rpm for 2 min. Subsequently, the embryo pellets were washed thrice with K-medium (0.032 M KCl, 0.051 M NaCl)[61] and placed onto a new NGM plate with *E. coli* strain OP50. The culture plates were incubated for 60−65 h (young adult) for soil toxicity tests.

Soil Toxicity Tests. Test soil samples were collected from Linde, Märkisch Luch, Germany (52.545529N, 12.661135E) on April 18, 2018. The soil was passed through a 2 mm sieve and then dried at 60°C for 24 h. The texture of our test soil was sandy (sand 89.3%, silt 8.3%, and clay 2.4%), and the pH and water-holding capacity (WHC) were measured as 5.7 ± 0.2 and 0.32 ± 0.10 mL/g, respectively (n = 3). To prepare test soils for microplastic fragments (HDPE, PET, PP, and PS) and films (LDPE), 100 mg of each microplastic was first mixed with 9.9 g of dry soil (1%) and then these initial mixtures were diluted using the same soil 10 and 100 times. The final test

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concentrations were determined as 0.01 (n = 4, fragments; n = 10, film), 0.1 (n = 4, fragments; n = 10, film), and 1 (n = 8, fragments; n = 10, film) % (based on the dry weight in soil), and control sets (no microplastic added) were prepared with a matching equal number of replicates for every microplastic treatment set. For PAN fibers, 10 mg was mixed with 9.99 g of dry soil, and the final test concentrations were 0.001 (n = 10), 0.01 (n = 10), and 0.1 (n = 10)%. Soil toxicity tests were performed as reported in previous studies.[31,62,63] We added 0.3 g of microplastic-laced soil to each well of a 24-well plate, together with 76 μL of K-medium (80% of WHC). Ten age-synchronized worms were added to each well and maintained at 20 ± 2 °C in the dark. After 24 h, soil containing nematodes was placed onto soil−agar isolation plates.[31,62,63] To prepare these plates, *E. coli* strain OP50 was cultured in Luria−Bertani medium (25 g/L) at 37 °C for overnight, and 75 μL of cell suspension was spread on each side of an NGM agar plate. Each test soil was arranged linearly in the central area of the soil−agar isolation plate, and the number of offspring moving from the test soil to each side was counted. We expected that toxicity would be captured by fewer nematodes moving out from the test soil to the fresh food resource. The data were expressed as a percentage (%) of the average value of the control group.

Preparation of Extractable Additive Solutions. Eactractable additive solutions were prepared from 13 different microplastics (Table S1), and two methods were investigated using only liquid (method 1) and glass beads (method 2) (Figure S2). To obtain the extractable additive solution using method 1, 118.4 mg of each microplastic was placed into 10 mL glass vials containing 3 mL of K-medium. Although microplastics either floated or sank depending on their different densities, hydrophobicity, or interaction with surface tension of microplastic, we did not attempt to immerse the particles in the solution. The vials were maintained at 20 ± 2 °C in the dark for 24 h, conditions similar to the soil toxicity test, and then, the solutions were passed through a syringe filter (pore size 0.45 μm; D-76185, ROTILABO, Carl Roth GmbH & Co., Karlsruhe, Germany). For method 2, glass beads (1−2 mm) were washed ten times with deionized water, autoclaved at 121 °C for 15 min, and then dried at 60 °C for 24 h. Each microplastic (118.4 mg) was added to a 10 mL glass vial containing 5 g of glass beads, and they were gently mixed using a spatula. Then, 5 g of additional glass beads were placed on top of this bead−microplastic mixture and afterward 3 mL of K-medium was added. The microplastics were immersed in the solution, similar to what the situation in soil water films inside of pores would be. The vials were maintained for 24 h at 20 ± 2 °C in the dark, and the mixtures were moved to a 50 mL syringe. The syringes were carefully pumped to obtain the extractable solution, and the obtained solution was passed again through the glass bead−microplastic system in the syringe two times and then filtered using a syringe filter. As a result, we obtained the 24 h extractable additive solution from the 0.04 microplastic mg/μL K-medium. We added 76 μL of this solution to each well of the 24-well plate containing 0.3 g of soil, and the final concentration of our 24 h extractable additive solution in soil (3.0 mg/0.3 g) corresponds to approximately 1% of microplastic in soil. The numbers of replicates were 4 (for method 1), 8 (HDPE, PET, PP, and PS fragments for method 2), and 4 (LDPE films and PAN fibers for method 2), and control sets were prepared with a matching equal number of replicates for every microplastic treatment set. Soil toxicity tests were performed, and negative control sets (without microplastics) were implemented for each method. The data were expressed as a percentage (%) of the average value of each control group.

Preparation of Microplastics with Easily Extractable Materials Removed. The additive-extracted microplastics were prepared using 13 different microplastics (Table S1). We expected that microplastics can lose their harmful effects when the extractable additives are removed, and the soil toxicity test was conducted using these extracted microplastics to test our hypothesis. We added 100 (for fragments and film) or 10 (for fibers) mg of each microplastic into 25 mL glass vials containing 5 mL of ethanol (96%), and these were maintained at 20 ± 2 °C in the dark. We chose ethanol to remove extractable additives. Since the additives used in plastic products are mostly apolar, ethanol, which is slightly more apolar than water, could be better to extract from microplastics.[64] We omitted stirring or shaking to avoid changing of size distributions of the microplastics. After 24 h, 4 mL of supernatant was removed, and 20 mL of deionized water was added to wash the microplastics. The suspensions were stabilized for 1 h, and 20 mL of supernatant (upper layer) or subnatant (middle layer) was removed again by careful pipetting. This washing process was repeated three times, and then, the vials containing microplastics were dried at 65 °C for 24 h. To ensure that every available extractable additive is partitioned into the ethanol solution from the microplastic surface, these extraction procedures including ethanol-extraction and water-washing were repeated twice. These extracted microplastics with one and two extractions were mixed with soil, and the final concentration was determined as 1% (for fragments and film) or 0.1% (for fibers). We then added 0.3 g of each microplastic-laced soil to each well of a 24-well plate, and 76 μL of K-medium was poured into each well. The numbers of replicates were 8 (HDPE, PET, PP, and PS fragments for one time-extraction), 4 (LDPE films and PAN fibers for one time-extraction), and 4 (HDPE, PET, PP, and PS fragments for two times extraction), and control sets were prepared with a matching equal number of replicates for every microplastic treatment set. Soil toxicity tests were performed, and negative controls (no microplastic added) were also implemented for the whole process. The data were expressed as a percentage (%) of the average value of the control group. We also performed untargeted LC−MS of the ethanol extractable addition solution. The experimental procedures and the data processing for the untargeted LC−MS are described in the Supporting Information.

Simulation of Wet−Dry Cycles in the Soil Environment. We selected LDPE films and PAN fibers as target materials for our extended experiment. LDPE is a common material used for the plastic mulching film, which is a main source of microplastics in farmlands,[65] and the fibers are among the most commonly observed shapes in soils.[66,67] Wet−dry cycles are important scenarios in agricultural and climate research fields, and these have often been used to assess the influence of variables on the chemical and physical properties of soil systems.[68] We used wet−dry cycle treatment to maintain target microplastics in environmentally relevant test condition for a longer time. To simulate wet−dry cycles in soil, 24-well plates containing each microplastic-laced soil were prepared using the same procedures as used for the soil toxicity test (0, 0.01, 0.1, and 1% for LDPE films; 0, 0.001, 0.01, and 0.1% for PAN fibers) and 76 μL of deionized water was added

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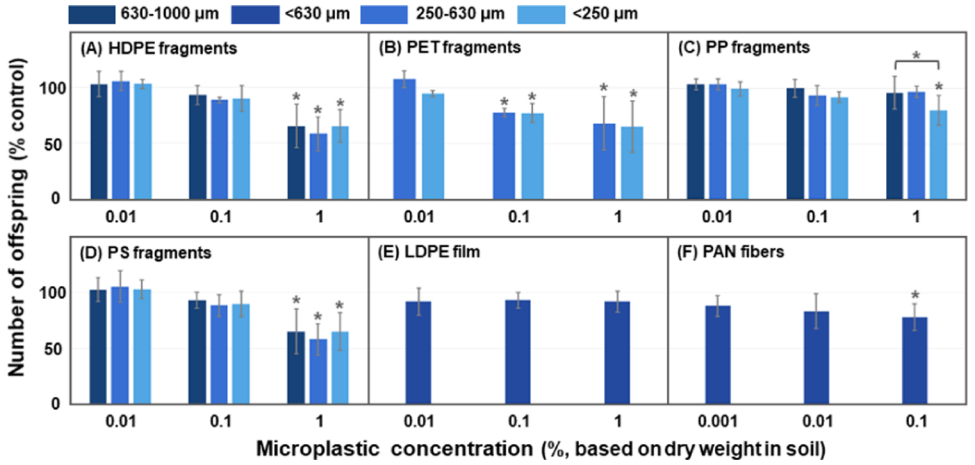

Figure 1. Offspring number of *C. elegans* exposed to (A) high-density polyethylene (HDPE) fragments, (B) poly(ethylene terephthalate) (PET) fragments, (C) polypropylene (PP) fragments, (D) polystyrene (PS) fragments, (E) low-density polyethylene (LDPE) film, and (F) polyacrylicnitrile (PAN) fibers in soil. Each microplastic contains one to three different size ranges (<250, 250−630, <630, and 630−1000 μm), and test concentrations are expressed as the percentage (%) based on the dry weight in soil. All data are normalized to each control group, and error bars indicate standard deviations. The asterisks (*) indicate significant ($p < 0.05$) differences compared to the control or the other different sizes.

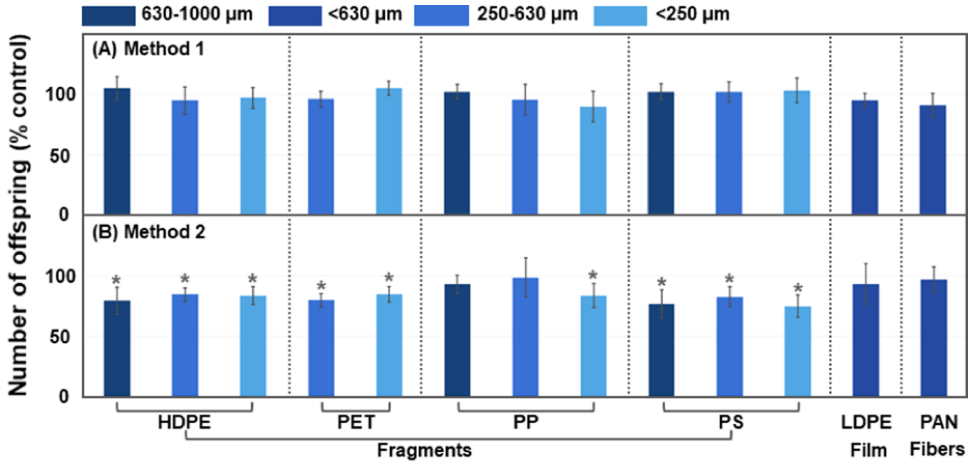

Figure 2. Offspring number of *C. elegans* exposed to 24 h extractable additive solutions that are obtained by (A) method 1 (only liquid) and (B) method 2 (glass beads). Each 24 h extractable additive solution was prepared using high-density polyethylene (HDPE) fragments, poly(ethylene terephthalate) (PET) fragments, polypropylene (PP) fragments, polystyrene (PS) fragments, low-density polyethylene (LDPE) film, and polyacrylicnitrile (PAN) fibers, and final concentrations were determined with an approximate level of the additive concentration from 1 or 0.1% (PAN fibers) based on the dry weight in soil (see the Materials and Methods section). Each microplastic contains one to three different size ranges (<250, 250−630, <630, and 630−1000 μm). All data are normalized to each control group, and error bars indicate standard deviations. The asterisks (*) indicate significant ($p < 0.05$) differences compared with the control.

to each well ($n = 4$). We prepared three plates (first, second, and third) for both microplastics, and each plate was covered and maintained at 20 ± 2 °C in the dark. After 6 days, all soil samples dried because water had evaporated. Seventy-six microliters of K-medium was added into each well of the first plate, and the same amount of deionized water was added to the second and third plates. The first plate was used for soil toxicity tests (6 days, first wet−dry cycle), and the others were maintained at 20 ± 2 °C in the dark for an additional 6 days. Subsequently, the second plate was used for soil toxicity tests (12 days, second wet−dry cycle), and the third plate was used after an additional 6 days (18 days, third wet−dry cycle). Negative controls (no microplastic added) were also prepared and handled the same way.

Statistical Analyses. Data were analyzed using SPSS statistical software (version 24.0, SPSS Inc., Chicago, IL). One-way analysis for variance (ANOVA) and Turkey's tests were conducted to determine the significance ($p < 0.05$) of multiple comparisons.

RESULTS

Effects of Microplastics on Nematodes in Soil. *C. elegans* showed vigorous reproductive activity in our soils, and an average value of offspring number per replicate was calculated as 171 ± 50 worms ($n = 26$) in control soil, which is comparable to the international standards.[69] Microplastic exposure showed that HDPE and PS fragments induced a significant effect on nematodes at a higher concentration, 1% (Figure 1A,D). By comparison, PET fragments started to be significantly harmful at 0.1% (Figure 1B), and PP influenced nematode offspring only for microplastics smaller than 250 μm, at a higher concentration of 1% (Figure 1C). There was no

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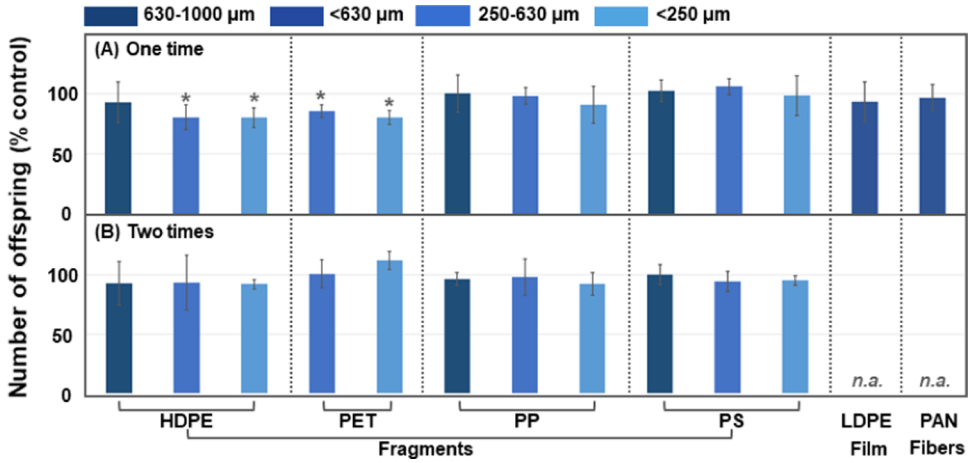

Figure 3. Offspring number of *C. elegans* exposed to extracted microplastics for (A) one extraction round and (B) two rounds of extraction. Each extracted microplastic was prepared using high-density polyethylene (HDPE) fragments, poly(ethylene terephthalate) (PET) fragments, polypropylene (PP) fragments, polystyrene (PS) fragments, low-density polyethylene (LDPE) films, and polyacrylicnitrile (PAN) fibers, and final concentrations were determined as 1 or 0.1% (polyacrylate (PA) fibers) based on the dry weight in soil. Each microplastic contains one to three different size ranges (<250, 250−630, <630, and 630−1000 μm). All data are normalized to each control group, and error bars indicate standard deviations. The asterisks (*) indicate significant ($p < 0.05$) differences compared with the control.

effect of the LDPE film (Figure 1E), and PAN fibers induced significant reproduction decrease at 0.1% (Figure 1F). In summary, microplastics mostly influenced nematodes at 1% concentration, and the number of offspring decreased to 78−80% (for PP and PAN) and 56−68% (HDPE, PET, and PS) compared with that for the control group. The harmful effects of PET fragments and PAN fibers seem to appear at lower concentrations compared to others, while HDPE, PP, and PS fragments induced relatively lower toxicity. In addition, low-density polyethylene (LDPE) induced no toxicity within our test concentration range (0.01−1%) for the acute period (24 h). PP fragments were the only plastic inducing a size-dependent effect.

Effects of Extractable Additive Solutions. The 24 h extractable additive solution was acquired using two methods (method 1 with liquid and 2 with glass beads). Average values of offspring were 174 ± 24 (n = 8) and 161 ± 12 (n = 12) worms in each negative control soil (no microplastic added) for methods 1 and 2, respectively. As shown in Figure 2A, additives extracted using method 1 had no effects, while method 2 led to a significant percentage decline of the number of offspring to 79 ± 11 (HDPE fragments, 630−1000 μm), 84 ± 5 (HDPE fragments, 250−630 μm), 84 ± 7 (HDPE fragments, <250 μm), 80 ± 5 (PET fragments, 250−630 μm), 84 ± 6 (PET fragments, <250 μm), 84 ± 10 (PP fragments, <250 μm), 77 ± 12 (PS fragments, 630−100 μm), 83 ± 8 (PS fragments, 250−630 μm), and 75 ± 9 (PS fragments, <250 μm), compared with the control group (Figure 2B). There were no significant effects of larger PP fragments (630−1000 and 250−630 μm), LDPE films, and PAN fibers. These toxicity trends were similar to the results of each microplastic at 0.1 (PAN fibers) or 1% (fragments and film) concentration, as presented in Figure 1.

Effects of the Additive-Extracted Microplastics. The average values of offspring were 166 ± 35 (n = 12) and 165 ± 22 (n = 4) in each negative control experiencing extraction procedures (without microplastics) for one and two times, respectively. Extracting the microplastics once (Figure 3A) led to the disappearance of the toxic effects of PP and PS fragments. Still, HDPE fragments (250−630 and <250 μm) significantly reduced the offspring number to 80 ± 10 and 80 ± 8% compared to that of the control, respectively. PET fragments (250−630 and <250 μm) also still showed a toxic effect to 86 ± 5 and 80 ± 6% of the control, respectively. When the extraction procedures were repeated twice, there were no more toxic effects for any the microplastics (Figure 3B).

Untargeted Chemical Screening of the Extractable Additive Solution. Ethanol-extractable additives from 13 microplastics and the solvent blank (a total of 14 samples) were analyzed in both positive and negative ion modes of LC−MS. Across the 14 samples, the number of molecular features ranged from 12 to 76 for each LC−MS spectrum. Ethanol-extractable additives from PAN fibers showed 38 and 15 features in positive and negative ion modes, respectively. After applying the filtering criteria described in the Supporting Information, PAN fibers revealed 13 significantly higher abundant molecular features (6 and 7 for positive and negative modes, respectively) compared with those from the other microplastics (Table S2). The 13 features include ethanone, 1,2-diphenyl-2-[(tetrahydro-2*H*-pyran-2-yl)oxy]-, β-estradiol 17-propionate, mirfentanil, 2,2′-(1,4-butanediylbis(oxy-4,1-phenylene))bis(4,5-dihydro-1*H*-imidazole), 2-[2-(4-methoxybutylamino)ethoxy]ethylcarbamic acid, 2-[3-(trifluoromethyl)piperidin-1-yl]cyclohexan-1-ol, bis(5-fluoropentyl)borinic acid, 4-[[[1-[(2-methylpropan-2-yl)-oxycarbonyl]pyrrolidine-2-carbonyl]amino]methyl]-cyclohexane-1-carboxylic acid, 4-[[[3-methyl-2-[(2-methylpropan-2-yl)oxycarbonylamino]pentanoyl]amino]methyl]-cyclohexane-1-carboxylic acid, 2-(benzenesulfonamido)-9-(5,6,7,8-tetrahydro-1,8-naphthyridin-2-yl)nonanoic acid, and 5-[(1-octanoylpyrrolidine-2-carbonyl)amino]pentanoic acid. When the features of 250 μm PP were compared with those of larger PP, two features showed significant differences (*N*-(2-ethylphenyl)acridin-9-amine and (3*R*,5*R*)-7-[(1*S*,2*R*,5*R*,6*S*,8*S*,8Ar)-8-(2,2-dimethylbutanoyloxy)-3,5,6-trihydroxy-2,6-dimethyl-2,3,5,7,8,8a-hexahydro-1*H*-naphthalen-1-yl]-3,5-dihydroxyheptanoic acid for positive and negative ion modes, respectively). Information on the use or function of

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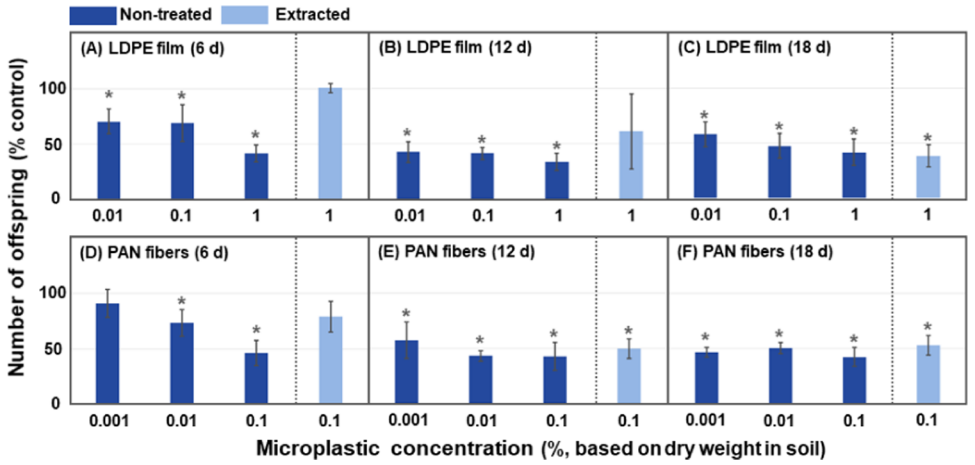

Figure 4. Offspring number of *C. elegans* exposed to (A) LDPE films and (B) PAN fibers in soil. Each soil was maintained for (A, B) 6 days, (B, E) 12 days, and (C, F) 18 days, and experienced one wet−dry cycle every 6 days. Test concentrations are expressed as the percentage (%) based on the dry weight in soil. All data are normalized to each control group, and error bars indicate standard deviations. The asterisks (*) indicate significant ($p < 0.05$) differences compared with the control.

these tentatively annotated compounds was not available in PubChem.

Simulation of Wet−Dry Cycle in the Soil Environment. After one wet−dry cycle (6 days), the number of offspring significantly decreased for the LDPE film at all concentrations (0.01−1%), and the average values were 70 ± 11, 69 ± 17, and 41 ± 8% compared to the control, respectively (Figure 4A). Toxic effects were intensified to 43 ± 9, 41 ± 5, 34 ± 8% at each concentration after two wet−dry cycles (12 days) (Figure 4B), and these effects were maintained at 42−58% after three wet−dry cycles (18 days) (Figure 4C). When LDPE films were extracted before the experiment, significant effects did not appear until two wet−dry cycles (Figure 4A,B), and 39% of the reproduction level was observed after three wet−dry cycles (Figure 4C). In the case of PAN fibers, the number of offspring significantly decreased at 0.01 and 0.1% after one wet−dry cycle (6 days) with average values of 74 ± 12 and 46 ± 11 compared to the control, respectively (Figure 4D). These effects were intensified at all concentrations (0.001−0.1%) after two and three wet−dry cycles, and 42−57% of the reproduction level was found (Figure 4E,F). When the PAN fibers were extracted, significant effects started to appear after two wet−dry cycles (Figure 4E), and 49−53% of the reproduction level was maintained until three wet−dry cycles (Figure 4F). Figure S3 shows that the toxic effects of LDPE films (1%) and PAN fibers (0.1%) increased as a function of the repetition of the wet−dry cycle toward a plateau of around 34−56%. Extraction procedures slowed down the appearance of toxic effects, but both treated and nontreated microplastics showed a trend to the same plateau after three wet−dry cycles, at 18 days.

DISCUSSION

Effects of Microplastics on Nematodes in Soil. *C. elegans* is one of the most extensively studied species for microplastic toxicity research, and 26 scientific papers have been published until March 31, 2020 (Table S3). These studies provide reliable, initial information aiding our understanding of microplastic toxicity on nematodes, but they also left open many important points. Notably, most of these studies, except only four papers,[32,70−72] have adopted spherical PS particles as the target material, and only two papers are utilizing field-collected or secondary-treated particles instead of purchased beads or pellets.[70,72] Although *C. elegans* has been suggested as a standard soil test species,[69,73] there is only one study conducting tests in soil media,[31] and 25 studies were performed using liquid media such as K-medium and M9 buffer solution (Table S3). On the other hand, six papers report size-dependent inhibitory effects of microplastic on *C. elegans*,[30−33,74,75] showing a tendency toward toxic effects that can be increased by smaller sizes in ranges of 0.05−0.2,[30] 0.1−6.0,[75] and 0.1−5.0 μm.[32] Lei et al.[33] reported that the effects of microplastics in this smaller size range might not be linear since the intermediate-sized group (1.0 μm) had the lowest survival rate, compared to smaller and larger sizes (0.1−0.5 and 2.0−5.0 μm), and Mueller et al.[75] found toxicity to increase in >10 μm size range. In our study, we used a larger size range (around 250−1000 μm) than previous work (0.05−6 μm). Although several studies have reported polymeric composition-, size-, and total surface area-dependent effects on nematode species,[32,72,75] they used edible sizes of plastic particles. Since the edible size of microplastic by nematode species is ≤3.4 μm,[74,75] we here avoided that the nematodes fed on microplastics and followed just the influence of the potential leachates on the number of nematodes offspring. PP microplastics had a size-dependent effect, with toxicity only apparent for its smaller size range (<250 μm). We found that the additives highly contributed to the toxicity of microplastics (Figures 2 and 3), and size-dependent effects may be related to the amount of extractable chemicals. Extractable chemicals from microplastics are highly linked with various factors such as polymeric compositions and surface areas,[76] and smaller size ranges of microplastics (higher surface area) can be more chemically reactive.[77] Many variables should be considered for broader generalization since other compositions (HDPE, PET, and PS) did not show size-dependent effects. Regarding the concentration, our results showed that most of the microplastics had toxic effects after 24 h when present in higher concentrations in the soil (Figure 1). HDPE, PET, and PS presented concentration−effect toxicity since only the higher concentrations presented toxic effects on nematode offspring numbers.

Production of Extractable Additive Solutions and Their Effects. Toxicity of microplastics is often associated

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with the pollutants they sorb during exposure to the environment and the chemicals used as additives leached during the useful life and after being discarded.[78] Regarding the additives, they are moving through the bulk of the microplastic particle until they eventually reach the surface, where they might stay or migrate to the surrounding medium.[79] Our work is focused on chemicals leaching and on the concept of K_D to better understand the ecotoxicity of microplastics. The K_D is the partition coefficient of a chemical between two immiscible media; in this study, it is between the surface of the microplastic and the aqueous environment within the soil.[80] When the K_D is high, this means that additives will interact more with the apolar part, even though a small portion will migrate into the aqueous medium (Figure S4A). When the K_D is low, this means that most of the chemicals will be released into the aqueous surrounding matrix, even if a small portion still adheres to the surface of the microplastics (Figure S4B). Finally, the real picture for plastics typically means the presence of a mix of additives,[81] with a range of K_D (Figure S4C). In such a mixture, it is likely to have major fraction of the chemicals with higher K_D mostly on the surface of the microplastics and the major part of the chemicals with lower K_D in the aqueous environment.[82] Since the microplastics used in this study had no history of exposure to the environment—thus no sorption of pollutants—and the sizes used were not small enough to be ingested by nematodes, the most likely explanation of toxic effects, expressed as a reduction in nematode offspring, is chemicals leached from the microplastics to the soil. To evaluate this hypothesis, we used an extract produced under very mild conditions for the migration of an apolar additive: 24 h of contact with water for the leaching and then using this solution for the toxicity test.

The outcomes of leaching tests depend heavily on the methodology,[47] and several experiments have been conducted to simulate various leaching environments under laboratory conditions such as shaking,[44,45] static maintenance,[43,47] and the standard leaching method.[46] These approaches are based on the concept of leaving microplastics afloat because this is likely close to natural exposure conditions in an aquatic environment.[47] However, this exposure scenario is not fully applicable to the soil environment, and a direct application of standard leaching methods, including the soil column test,[83,84] batch test using the liquid-to-solid ratio,[85,86] and upflow percolation test,[87] is difficult due to a wide variation of plastic characteristics. Also, the standard leaching tests have focused on traditional pollutants such as metals and organic chemicals, and these materials have been well characterized in terms of basic information on which factors control leachability.[88] Since we have no such knowledge about microplastic in soil, we should be cautious about determining experimental procedures. In this study, we assessed two different methods for the chemicals leaching to the water: (1) floating in a liquid to emulate the conditions in aquatic bodies in nature and (2) using glass beads to keep microplastics immersed in water. While there was no effect using the 24 h extractable solution obtained by floating microplastics in water (Figure 2A), the number of nematode offspring significantly decreases when using the 24 h extractable solution prepared using glass beads (Figure 2B). The more efficient migration was likely due to the better interaction with water since the microplastics were in complete contact with water, while the floating microplastics were only partially in contact with water, with a lower interface area.

Effects of Additive-Extracted Microplastics. After determining the protocol for more efficient migration of the additives, using glass beads, we tested whether the additives were indeed the toxicity source, trying to remove them from the microplastic particles. Since the additives were successfully removed even with water, we tested the extraction with ethanol, a polar solvent but less polar than water. The higher the ethanol content, the more effective the migration of organic chemicals from plastics to the solution. Although the K_D value depends on the properties of target chemical migrants and plastics,[89−92] we believe that, as a general rule, ethanol can promote the migration of apolar additives because it is less polar than water. For example, $K_{LDPE/95\%\ ethanol}$ at 60 °C is 775 times lower than that in 50% ethanol at the same temperature,[79] and $K_{95\%\ ethanol/PET}$ at 20 °C is 3−4 times higher than that in water.[92] After one extraction, we observed that the significant effects of HDPE and PET fragments remained (Figure 3A), but all of the other microplastics no longer had toxic effects. To confirm the effect, we extracted once more (Figure 3B), and the result was no toxic effect of any microplastics tested irrespective of the concentrations and shapes. We concluded, therefore, that the toxic effects of microplastic are mainly caused by the 24 h extractable additives from the microplastics.

Untargeted Chemical Screening of the Extractable Additive Solutions. Untargeted LC−MS analysis was performed to provide a chemical explanation for the toxicity trend observed in this study. From the soil toxicity test, PAN fibers displayed the highest toxicity among different microplastic types. The experiment on the effects of extractable additive solutions also showed differential toxicity among different PP fragment sizes. However, PAN fibers exhibited no toxicity just after a single ethanol washing to remove extractable additives (Figure 3). Our LC−MS data for ethanol-extractable additives of PAN fibers also reveal the highest overall intensity in the base peak chromatogram. This result implies that PAN fibers released higher amounts of potentially toxic additives into the solution when extracted with ethanol, which in turn caused the higher toxicity to nematodes.

Although 13 features that showed a significant change in their peak intensities compared to the other microplastics could be tentatively annotated from ethanol-extractable additives of PAN fibers, their chemical identities are ambiguous because of the limitations of untargeted screening of unknowns. The use of a mass tolerance of 5 ppm in this study to search for the chemical formula of each feature resulted in multiple chemical composition candidates. Even if the elemental composition could be narrowed down to a single chemical formula, its chemical structure still could not be uniquely determined due to the possibility of the existence of various isomers. Recently, Zimmermann et al.[93] characterized the methanol extracts of biobased plastics using nontargeted LC−MS/MS screening. Although they could tentatively identify about 94% of the chemical features that were highly abundant across the samples, they failed to find the chemical use or origin on most of the identified compounds in PubChem, which was attributed to the absence of the information regarding the chemicals utilized in the polymer industry in general chemical databases. These current limitations in untargeted chemical analysis highlight the importance of further studies to improve databases, leading to the enhanced confidence of unknown environmental

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compounds. Additional use of conventional spectroscopic techniques such as nuclear magnetic resonance along with MS or a study combining untargeted screening with targeted analysis of suspected compounds could be an option to address the challenges associated with toxicological analyses.

Simulation of Wet−Dry Cycles in the Soil Environment. The diffusion of chemicals through the bulk of the plastic proceeds until they reach the surface and migrate to the other medium in a proportion regulated by the K_D. The kinetics of the diffusion influences the amount of chemicals on the surface and thus the migration to the environment. To determine the duration of the whole process of diffusion and migration, we tested the time needed to produce toxic effects on the nematodes. The desorption of hydrophobic organic pollutants from plastics is generally slow, and the leaching rate of chemical additives from plastics into water depends on time.[94−96] For example, the desorption half-lives of polychlorinated biphenyls from PE pellets are estimated to be 14 days to 210 years,[94] and the leaching rate of brominated diphenyl ether-209 from the HDPE plate is calculated as 2.1×10^5 ng/(m^2 days).[97] Chemicals keep leaching from microplastics until depletion.[96] When the microplastics are present in a soil system with low diffusivity or in a closed system (like a laboratory experiment), we can expect an increasing concentration of the chemicals leached. In this study, we expected that the toxic effects of LDPE films and PAN fibers can be intensified by repeating a wet−dry cycle in soil. Since the test duration for extractable additives was only 24 h, according to soil toxicity test conditions, there is a high possibility of toxicity increase when microplastics are maintained in simulated soil conditions for more extended periods. Our expectation was correct, and we found that these effects plateaued with a similar decreasing level until 18 days (Figure 4). Extracted microplastics showed a relatively slower increasing trend of toxic effects compared to nontreated ones (Figure S3). Our result indicates that the extractable additives from plastics can be more harmful when they are maintained in soil environments for a longer period than those used in typical testing protocols,[44,47] and toxic effects can occur at a relatively low concentration like 0.01% (100 mg/kg) for LDPE films and 0.001% (10 mg/kg) for PAN fibers. Since 8−67 500 mg/kg microplastics can be detected in the soil environment,[12−14] nematode populations would be expected to be affected given the microplastic concentrations we tested here.

We conducted a simple ecotoxicological protocol using the concept of diffusion and migration of chemical additives from microplastics. Although our study was performed on a small scale taking a more phenomenological approach, our ecotoxicological tests provide clear evidence that microplastic toxicity in the soil is linked with their characteristics and extractable additives. This study is the first to estimate microplastic levels inducing toxic effects on nematodes in the soil system, uncovering the crucial role of extractable additives. Our results strongly suggest that future tests must consider microplastic additives as a key explanatory variable.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c04641.

Method for untargeted LC−MS of the extractable additive solution, FTIR spectra of each target MP, schematic diagram of extractable additive solution preparation, result for LDPE films and PAN fibers after wet−dry cycles, list of target plastic materials, list of previous studies reporting microplastic toxicity on the nematode *C. elegans*, and scheme for the relative abundance of chemicals (PDF)

List of the first candidates at PubChem for the features that passed the manual inspection for the ethanol-extractable additive of PAN fibers (XLSX)

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Notes

The authors declare no competing financial interest.

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