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Environmental Chemistry

Comminution method and oxidative aging modulate the impact of tyre wear microplastics on the reproduction of the nematode *C. elegans*

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Environmental context. Each year, tonnes of tyre wear particles enter the terrestrial environment and undergo natural aging. This study investigates the effect of artificial weathering, particle concentration and preparation method on the reproduction of *Caenorhabditis elegans*, a soil- and water-dwelling nematode, using the standardised toxicology assay ISO 10872.

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ABSTRACT

Rationale. Tyre abrasion is one of the largest sources of microplastic (MP) in aquatic and terrestrial environments. Despite this fact, research on tyre wear particles (TWP) and its effects on soil ecosystems is scarce, especially regarding natural weathering processes. This leaves a large knowledge gap on the interactions of TWP with soil biota. **Methodology.** Cryo-milled tyre tread (CMTT) and diamond-ground tyre tread (DGTT) were artificially weathered through dry ozone treatment and heat exposure. Particles were analysed by scanning electron microscopy, particle size distribution and shape characterisation. TWP leachate composition was examined using liquid chromatography–mass spectrometry. The impact on the reproduction of the nematode *Caenorhabditis elegans*, a widely used toxicological model, was tested for leachate concentration, exposure duration and TWP aging status, using offspring per adult as the endpoint. **Results.** The comminution method significantly influenced TWP particle size distribution, with diamond grinding yielding smaller particles and a more structured surface morphology than cryo-milling. Aging with ozone (180 min) and heat (20 min at 100°C) reduced DGTT particle sizes by 27–58%, but not in CMTT. Additionally, aging increased carboxylic functional groups and led to a brittle structure in both TWP types. Leachate composition varied with comminution method and aging. Benzothiazole, *N*-Cyclo-*N*-phenylurea and aniline were more abundant in pristine and aged CMTT and aged DGTT. Diphenylguanidine had the highest concentration in all leachates. Aged TWP leachates, regardless of comminution, had significant toxic effects on *C. elegans*. Leachate from pristine CMTT was more toxic than from pristine DGTT. Nematode offspring correlated negatively with ozone exposure duration in aged DGTT. Tests with aged CMTT leachate showed even short-term exposure reduced offspring numbers. **Discussion.** The results underscore the importance of oxidative and mechanical weathering in TWP toxicity and challenge the use of pristine particles in toxicological assays for risk assessment in the natural environment.

Keywords: antioxidants, cryo-mill, diamond grinder, heat, microplastics, ozone, pollution, soil fauna, terrestrial ecosystem, tyre abrasion, vulcanisation accelerator.

Introduction

Tyre abrasion is the greatest source of microplastic (MP) pollution (Sommer *et al.* 2018), with 30–50% in the environment derived thereof (Baensch-Baltruschat *et al.* 2020). On roads connected to separate sewer systems and roads without connection to wastewater

Collection: Tyre road wear particles – chemistry and impacts

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treatment facilities, tyre wear particles (TWPs) become part of the runoff, leading to their untreated discharge into the aquatic and terrestrial environment (Polukarova *et al.* 2025). It is known that runoff from combined sewer systems can be purified from MPs through treatment with activated sludge (Lau *et al.* 2024); however, activated sludge is applied to agricultural fields as fertiliser, which exacerbate the problem on land (Baensch-Baltruschat *et al.* 2021).

The extent of tyre abrasion varies between countries: the global average is estimated to be 810 g capita^{-1} year^{-1}, with the lowest values occurring in India (230 g) and the highest in the USA (4700 g) (Kole *et al.* 2017). In Germany, each person produces an estimated amount of ~1 kg of tyre abrasion per year (Venghaus and Barjenbruch 2017; Bertling *et al.* 2018).

Due to the specific usage and composition of car tyres (Lebreton and Tuma 2006), their abrasion is not comparable with other plastic types. Also, there is a wide range of tyres, e.g. depending on season (summer, winter) or vehicle (car, lorry), all with different properties. Tyres mainly consist of polymers as the base material (40–50%), filler (30–35%), typically black carbon, silica and chalk. Further components are softeners (15%) like oil and resin, vulcanisation agents (2–5%) e.g. sulfur and zinc oxide, additives (5–10%) as preservatives, antioxidants, vulcanisation accelerators, desiccants, plasticisers and processing aids (Sommer *et al.* 2018). Except for some flame retardants, none of these additives are chemically bound into the polymer structure and thus can leach out into the surrounding environment (Campanale *et al.* 2020).

All these components can be altered during tyre usage and weathering processes in the environment, due to oxidation, heat, ultraviolet (UV), biological or physical degradation (Wagner *et al.* 2022; Weyrauch *et al.* 2023). It is unclear whether these transformation procedures lead to more or less toxic leaching compounds. A recent study revealed that the application of artificial sunlight, elevated temperature and mechanical stress, and also the method used for TWP generation changes the amount of leachables from TWP (Weyrauch *et al.* 2023). TWP in soil can alter key pathways of nitrogen and carbon metabolism, plant growth and stress response, seed germination and microbial diversity (Zeb *et al.* 2024). Leaching chemicals and particles were found to accumulate in plant tissue, underpinning its relevance for food safety (Ding *et al.* 2023). Moreover, aged TWP can cause oxidative damage and behavioural changes in various terrestrial species (Chen *et al.* 2024; Torreggiani *et al.* 2024).

Soil water serves as habitat for the semi-aquatic microfauna such as nematodes (Yeates *et al.* 1993; Ruess 2024). With estimated 0.3 × 10^9 tonnes (Mg) of biomass in surface soils, nematodes are the most abundant multicellular organisms in terrestrial ecosystems (van den Hoogen *et al.* 2019). They play an important role in several soil processes e.g. decomposition, energy flow and nutrient cycling (Nieminen 2008; Neher 2010). Due to their presence in functional groups at all tropic levels, nematodes fulfill diverse ecological roles and hence have a key position in the food web (Yeates 2010; Majdi and Traunspurger 2015). The nematode *Caenorhabditis elegans* is a popular model organism in life sciences (Hunt 2017). In ecotoxicological tests it is a sensitive indicator with stronger response to lower concentration of drugs and MPs than other organisms (Jewett *et al.* 2022).

Despite the increasing research efforts on TWP toxicity in soil biota, comparing studies remains difficult due to a lack of standardised samples and varying artificial weathering procedures, such as UV, ozone, heat and Fenton reaction, hindering comparison between studies (Ortiz *et al.* 2022; Ding *et al.* 2023). Different particle generation methods, such as cryo-milling and abrasion, produce particles that vary in size and shape, and these differences additionally affect toxicity tests (Kreider *et al.* 2010; Choi *et al.* 2020).

In this study the standardised toxicological tests with *C. elegans* for soil and liquid samples, defined by BS ISO 10872:2010 'Water quality – determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of *Caenorhabditis elegans* (Nematoda)' (International Organization for Standardization 2010), were employed. Cryo-milled tyre tread (CMTT) and diamond-ground tyre tread (DGTT) particles, gradually aged by dry ozone aeration and heat application, were used as test materials for leachate production. Different artificial weathering durations, leachate concentrations and exposure times were applied to determine effects on the reproduction of *C. elegans*. It was hypothesised that: (i) cryo-milled TWP are larger than diamond-ground ones, which results in a smaller relative surface area, limiting chemical leaching, and in turn reduced toxicity of CMTT leachates compared to DGTT leachates. (ii) The duration of exposure to TWP leachate affects the reproduction of *C. elegans*, with shorter exposure times reducing harmful effects. (iii) Artificial weathering duration and the related degradation status of TWP increases the toxicity for nematodes.

Materials and methods

Tyre wear particles (TWP)

The tyre material was provided by Continental Reifen Deutschland GmbH. The car tyre samples were provided as diamond-ground particles as well as tyre tread pellets with 5-cm diameter and 1-cm thickness. Two methods of comminution were tested: for cryo-milling the pellets were sliced on a glass cutting board into 0.5- × 0.5-cm pieces and then ground using a Retsch Mixer Mill MM 400 under regular cooling with liquid nitrogen. The 50-mL milling containers were filled with 7.5 g of the tyre pieces. In the first round the material was ground four times with a 20-mm grinding ball for 3 min at 30 Hz, with 1 min of intermediate cooling using liquid nitrogen. The ball was then replaced by four 12-mm grinding balls. The container was shaken two times for 3 min, with 1 min of intermediate cooling in liquid

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nitrogen. The grinding balls were then replaced by six 7-mm balls. The sample was ground six times for 2 min, with 1 min of intermediate cooling between each iteration. The resulting cryo-milled tyre tread thereafter is referred to as CMTT. To obtain DGTT particles, a tyre was clamped and the tread was ground using a diamond grinding wheel. This procedure was conducted by the tyre company Continental. Information about the temperature and rpm of the grinding wheel were not provided due to corporate concerns. Particles produced with this method are referred to as DGTT.

For the scanning electron microscopy (SEM) images, CMTT and DGTT particles were mounted on an aluminium stub using conductive carbon tape. According to the scanning procedure, both samples were coated with gold and analysed using SEM. Changes after ozone treatment were not visible on the surface of the ground particles, so images of larger particles were taken to observe surface effects. Therefore, 0.5- × 0.5-cm TWP particles were sliced in the middle. It was assumed that the interface of the resulting pieces was comparable. Subsequently, one piece was aged, the other not. Following the scanning procedure, both particles were coated with gold and analysed using SEM. LEO 1430 combined with the operating software *SmartSEM* (ver. 6.00, Carl Zeiss, Oberkochen, Germany).

The particle size distribution of pristine CMTT and DGTT was measured with particle size and shape analyser QICPIC using a spectrum between 4.2 and 8665 μm. The QICPIC was equipped with the dry dispersion unit RODOS and the ASPIROS micro dosing unit (Sympatec GmbH, Clausthal-Zellerfeld, Germany).

The surface changes of pristine and aged DGTT were analysed using X-ray photoelectron spectroscopy (XPS). The measurements were conducted using a spectrometer (SAGE 150, Specs, Berlin, Germany) with non-monochromatic AlKα radiation (10 kV, 20 mA) at a pressure of ~5×10^{-7} Pa in the analysis chamber. The angle between the X-ray source axis and the analyser lenses was set at 54.9°, and the analyser was mounted at an 18° angle to the surface normal. XPS spectra were recorded in constant analyser energy mode. The analysed surface area measured ~1 × 3 mm, and the elemental concentrations obtained represented the top 6 nm of the surface layer. Peak fitting was carried out using CasaXPS and Specs' software, *Specslab* (ver. 1.7, SPECS Surface Nano Analysis GmbH, Berlin, Germany). The concentration of the following correlative functional groups was determined: C–C, C–O, C=O and O–C=O.

Artificial weathering procedure

Laboratory procedures were used to replicate weathering factors found in the environment. The mechanical weathering was simulated by comminution (see 'Tyre wear particles (TWP)' subsection of the Materials and methods). Additionally, an artificial weathering procedure involving ozone aeration was applied. This simulates years of weathering within couple of hours, where chemical compounds cannot evaporate (due to rising temperature) or leach (into aqueous medium). Each gram of tyre particles was aged with ~1.7 g of ozone generated by an Ozone Air Generator OAW03 (Kinwodon, product number: 3LE0911-CF-5274, China) for 30, 60, 120 or 180 min, which is equivalent of a weathering of 5, 10, 20 and 30 months respectively (see the 'Calculation S1. Estimation of the natural aging time corresponding to the artificial ozone weathering' section in the Supplementary material). For that, 16.5 g of the tyre powder was placed into a 1.3-L (12- × 15-cm) stainless steel container, which was continuously turning with 3 rotations min^{-1}. Approximately 2 g of powder was taken out after 30, 60, 120 and 180 min. All samples were then heated for 20 min in glass batches with closed lids at 100°C in a water bath.

Leachate preparation

The leachates were prepared according to the Swedish standard 12457:2003 (Standardiserings kommissionen i Sverige 2003). Some modifications were made: according to the longevity of tyre abrasion in soil the leaching time was increased from 24 to 192 h. To ensure the survival of the test organism *C. elegans* in the solution, the tyre particles were added into M9 buffer. This standard medium for nematode culture consists of sodium chloride (NaCl) (5 g), 1 mol L^{-1} of magnesium sulfate ($MgSO_4$) (1 mL), potassium dihydrogen phosphate (KH_2PO_4) (3 g), disodium phosphate (Na_2HPO_4) (6 g) in a volume of 1 L of double distilled water (ddH_2O) (Xiao *et al.* 2023).

The leachate concentrate was prepared by incubating 2 g of tyre powder in 10 mL of M9 buffer. M9 buffer, without tyre powder served as control. After 8 days on a full rotation shaker with 24 rotations min^{-1} at room temperature, the liquid was sterile filtered through 0.2-μm pore size cellulose-acetate filter (Sartorius AG, Göttingen, Germany) and stored in glass vials at 3°C.

Leachate composition

The composition and concentration of leachables were investigated with ultra-performance liquid chromatography–high-resolution mass spectrometry (UPLC–HRMS) analysis. Leachates were examined with an ACQUITY UPLC system coupled with a XEVO G2S Q-TOF-MS (Waters GmbH, Eschborn, Germany). The details of the method are reported by Weyrauch *et al.* (2023).

Mass spectrometric data were analysed using the TargetLynx module within *MassLynx* software (ver. 4.2 (change note SCN966), Waters GmbH, Eschborn, Germany). The abbreviations of the target compounds, CAS-number, retention time, exact mass, limits of quantification and the supplier are reported in Supplementary Table S1. Stock solutions of standards were prepared in acetonitrile and diluted into MilliQ water directly before analysis. The suspect screening was done based on transformation products (TPs) observed by Seiwert *et al.* (2022) and Foscari *et al.* (2024). The detected and thus selected TPs are listed in Supplementary Table S2.

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Identification was based on exact mass with a mass accuracy of 5 ppm, retention time and peak shape comparison with standard compounds. The limit of quantification (LOQ) was defined as a tenfold signal-to-noise ratio and obtained from a dilution series of prepared standard mix. Quantification was based on a calibration curve from a dilution series of a standard mix ranging from 0.03 to 30 μg L^{-1}. Leachates were measured at 1:100 and 1:10,000 dilution to fit the calibration curve's effective range. For suspect analysis without available standard compounds, peak areas are reported.

Test organisms

The nematode *C. elegans* strain N2 was mass cultured on nematode growth medium (NGM) plates and fed with the bacterium *E. coli* OP50 at 20°C and in darkness. *C. elegans* was acquired from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN, USA).

Food availability and ingestion play a major role in toxicological tests. The TWP leachate in the sample could affect the *E. coli* growth, having an indirect negative effect by limiting the food source. A study on the rotifer *Brachionus calyciflorus* revealed that higher food supply can suppress negative effects of polyethylene MP (Xue *et al.* 2021). Therefore, a pretest with the food bacteria was performed: *E. coli* OP50 was cultured in Luria-Bertani (LB) medium prepared with 0.075 g TWP mL^{-1} leachate under constant shaking at 200 rpm and 37°C. The pretest showed no changes in bacterial growth when leachates were mixed in the LB medium.

Experimental design

Test assays

All experiments were conducted according to BS ISO 10872:2010. For this, 500 μL of the leachate was mixed with 500 μL of ~1000 formazine absorption units (FAU) of *E. coli* food stock in Cellstar 24 Well Cell Culture Plates (Greiner Bio-One International GmbH, Austria). Nematodes were rinsed off the growth plates using M9 buffer and then filtered through 10-μm polyester filters (Franz Eckert GmbH, Germany). Ten *C. elegans* first stage juveniles were hand picked into each well. The plates were then covered with the lid and parafilm, and stored at 20°C in the dark for 96 h. Afterward the nematodes were dyed with 0.3 g L^{-1} of Rose Bengal, heated with closed lids in a water bath at 80°C. Individuals were transferred to agar plates (NGM), dried and stored at 3°C until analysis. As the endpoint of toxicity, the number of offspring was determined under a microscope.

According to ISO 10872:2010 several criteria must be fulfilled to ensure quality of the test assay. In the control samples of all test series, recovery rates of exposed test organisms were at least 80%, the mean percentage of males was 10% or lower, females without eggs were not observed and mean reproduction in the control treatment was at least 30 offspring per adult. Thus, all validity criteria were met. However, individual differences among organisms can result in notable variation in offspring numbers.

Leachate types – comminution method, aging status and TWP concentration

In comparison to aquatic ecosystems, the amount of water available for chemical leaching in soil is limited and depends on the soil's water-holding capacity (WHC). Considering a WHC of 0.32 mL g^{-1} for sandy soil (Kim *et al.* 2020), the applied leachate concentrations of 0.1, 0.05 and 0.025 g TWP mL^{-1} M9 buffer (or soil liquid) are equivalent to 3.2, 1.6 and 0.8% of TWP pollution in dry soil (see the 'Calculation S2. Estimation of TWP contamination in dry soil based on applied leachate concentrations' section in the Supplementary material). This is in the range of environmental TWP levels, which vary between 0.2 and 2.6% in contaminated soils (Rødland *et al.* 2023).

The three exposure levels (0.1, 0.05 and 0.025 g TWP mL^{-1} M9 buffer) were tested in the experiments. A stock solution with 2 g of TWP in 10 mL of M9 buffer was prepared, filtrated and the respective concentrations were obtained by diluting accordingly. The control samples consisted of M9 buffer only. Three experimental runs were conducted using different leachate types: pristine CMTT, aged CMTT (180 min of ozone, 20 min at 100°C), pristine DGTT and aged DGTT (180 min of ozone, 20 min at 100°C). Each experimental run included both control and leachate treatments, with four replicates each. TWP concentrations were varied in every run, resulting in a total of 20 samples per run.

Leachate exposure time

The exposure test assays were performed in 24-well plates. At the beginning of the experiment, 32 wells were prepared by mixing 250 μL of food stock with 250 μL of M9 buffer. Ten first stage juvenile *C. elegans* were added to each well. Subsequently, an additional 250 μL of food stock and either leachate (CMTT treated with 180 min of ozone and 20 min at 100°C) or M9 buffer (for control treatments) was added for exposure durations of 96, 72, 48 and 24 h. For each day four replicates and four control samples were carried out. The plates were covered with the lid and parafilm and stored at 20°C in the dark for 96 h. Since control samples across all time points showed no significant differences, they were pooled for statistical analysis.

In all batches, the leachate was adjusted to a final concentration of 0.05 g mL^{-1} through dilution of the initially prepared leachate concentrate.

Artificial weathering of TWP

For the experiments on the effects of TWP aging on *C. elegans*, leachate concentrates (DGTT treated with 30, 60, 120 or 180 min of ozone and 20 min at 100°C) were diluted to a TWP concentration of 0.05 g mL^{-1}. The design was according to BS ISO 10872:2010 as described in 'Test assays' subsection of the Materials and methods

Fig. 1 provides a methodological overview illustrating the experimental design of all toxicological tests conducted.

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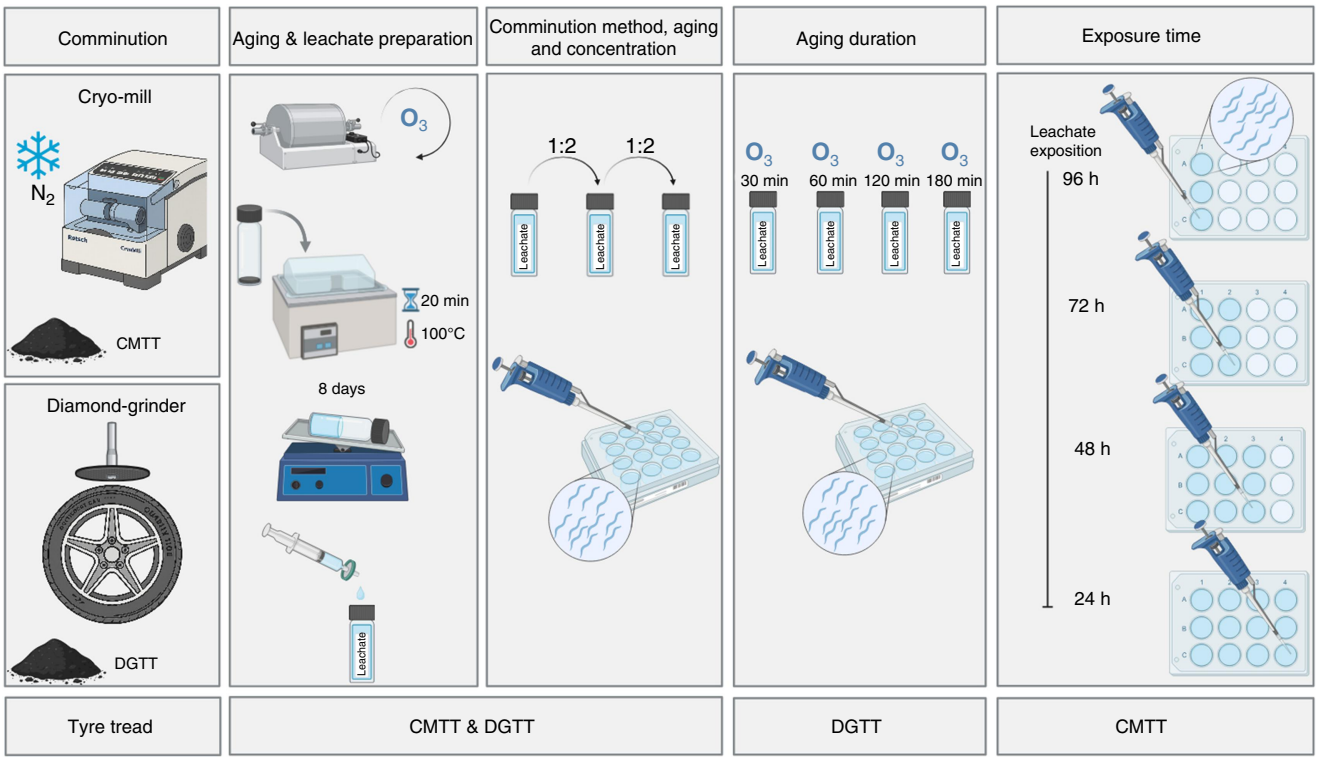

Fig. 1. Tyre tread was ground using a diamond grinder and a cryo-mill. Tyre tread particles produced by the diamond grinder are referred to as DGTT, whereas those originating from the cryo-mill are called CMTT. Both particle types were artificially aged using 1.7 g ozone h^{-1} aeration (for 30, 60, 120 or 180 min) and heat (20 min at 100 °C). After 8 days of shaking, the leachate concentrates were filtered and used in diluted form for toxicological assays with *Caenorhabditis elegans*. Aged and pristine CMTT and DGTT were tested at leachate concentrations of 0.1, 0.05 and 0.025 g mL^{-1} M9 buffer. The effect of artificial weathering was tested using 0.05 g DGTT mL^{-1} leachates. The effect of leachate exposure duration was tested using 0.05 g aged CMTT mL^{-1} leachate.

Statistics

Statistical analyses were performed in *R* (ver. 4.1.3, R Foundation for Statistical Computing, Vienna, Austria, see https://www.r-project.org/) using the packages *FSA* (ver. 0.10.0, see https://CRAN.R-project.org/package=FSA), *dunn.test* (ver. 1.3.6, see https://CRAN.R-project.org/package=dunn.test) and *rstatix* (ver. 0.7.3, see https://CRAN.R-project.org/package=rstatix). Data visualisation and manipulation were conducted with *ggplot2* (ver. 4.0.1, see https://CRAN.R-project.org/package=ggplot2; Wickham 2016) and *dplyr* (ver. 1.1.4, see https://CRAN.R-project.org/package=dplyr). For the statistical analysis of toxicity tests the influence of TWP concentration, comminution method, aging status and leachate exposure duration on the number of progenies was tested. Toxicological test data on leachate types were categorised according to comminution method, aging status and TWP concentration to evaluate the influence of these variables. Based on the results of the Shapiro–Wilk test, data were not normally distributed; therefore, a rank variance analysis was performed using the Kruskal–Wallis test, followed by the Dunn–Bonferroni *post hoc* test. The correlation between ozone application and toxicity as well as the concentration of functional groups and ozone application duration of TWP, was tested with the Spearman-correlation test. All tests were quantified with a significance level of $P < 0.05$.

Results

Morphological characteristics of TWP particles

The CMTT and DGTT methods to produce TWP resulted in differences in shape and surface structure of the particles as shown by the SEM images (Fig. 2). Particles produced by cryo-mill are clearly separated from each other, whereas particles obtained from the diamond grinder are agglomerates of smaller particles with a porous surface. Differences in the particle size were also observed: when comparing fine and coarse fractions, particles produced by the cryo-mill are larger, with D10 (fine fraction) and D90 (coarse fraction) values of 218 and 756 µm respectively, whereas those produced by the diamond grinder show values of 90 and 636 µm (Fig. 2). The particle size distribution was determined for pristine particles of CMTT as 218, 459 and 699 µm, corresponding to D10, D50 (median size) and D90 respectively. For DGTT, the values were 124, 281 and 636 µm at the same distribution percentages.

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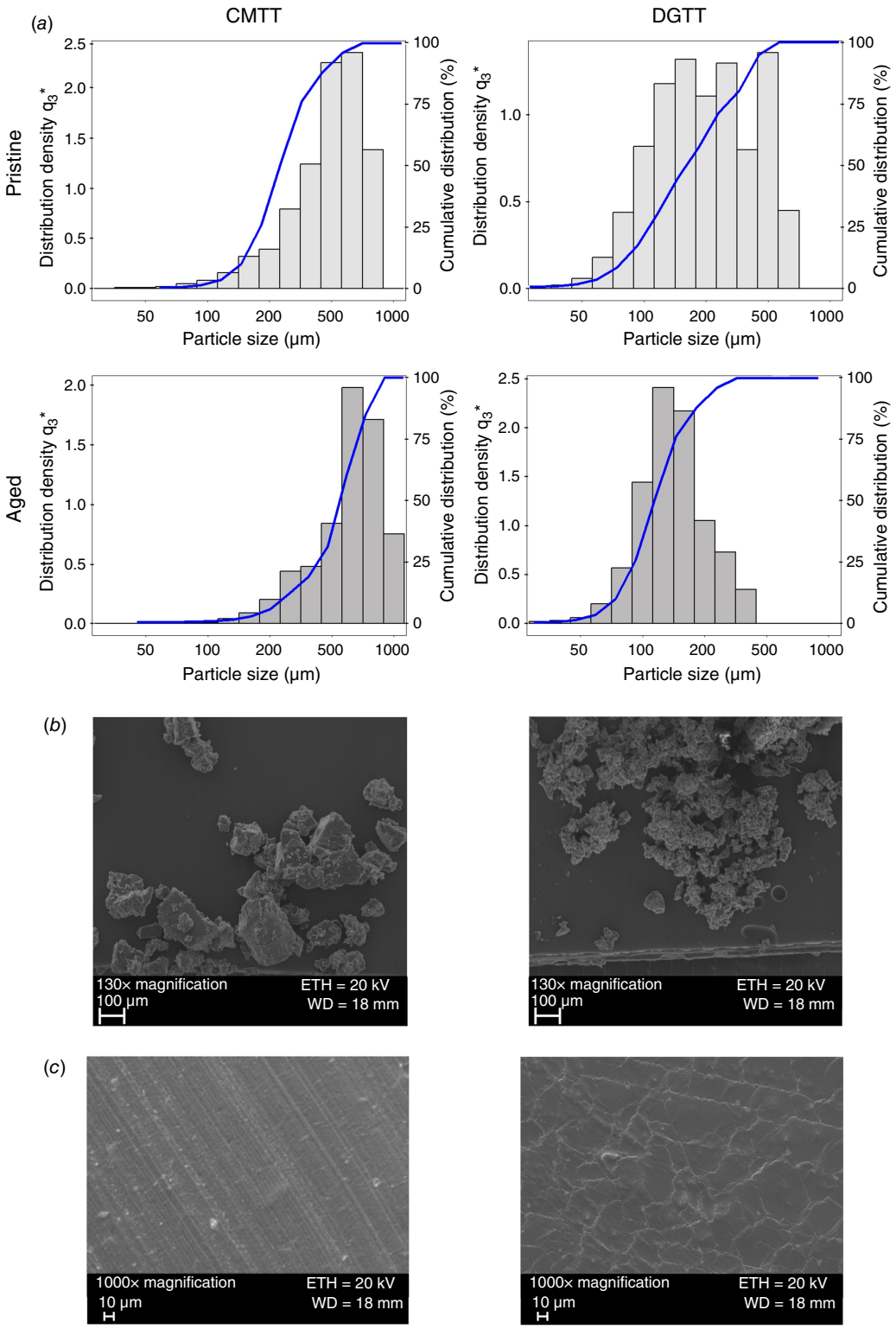

Fig. 2. (*a*) Particle size distribution of cryo-milled tyre tread (CMTT) and diamond-ground tyre tread (DGTT). *Y*-axis shows the distribution density (q_3*) and the cumulative distribution (%) of the particle sizes (µm) is given on the *x*-axis. Light grey, pristine microplastic; dark grey, aged microplastic; blue line, cumulative density. (*b*) Scanning electron microscopy (SEM) images of particles originated from cryo-mill (left side) and diamond grinder (right side) magnified 130 times. (*c*) The SEM image shows two sides of a sliced tyre tread particle. The surface on the right underwent dry ozone and heat treatment, whereas the surface on the left is pristine. Both images were taken at 1000 times magnification.

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The particle size distribution not only varied due to comminution methods, it also decreased with artificial weathering: at percentages of 10, 50 and 90%, aged DGTT contained particles smaller than 90, 147 and 266 μm respectively (Fig. 2). This corresponds to a size reduction of 27 to 58%. A similar decrease in particle size was not observed for the CMTT particles. Here, the size of pristine particles differed from the aged particles by 4–8% only. As a result, the differences in size distribution between the two TWP increased, with the particle sizes of aged CMTT powder being two to three times larger than those of aged DGTT powder.

Artificial weathering also affected the aspect ratio and sphericity of the DGTT powder (Fig. 2). The smallest 10% of the aged DGTT particles are 27% smaller and 42% more spherical than the pristine ones (Supplementary Table S3). In terms of cumulative density, the middle 50% of the aged particles are 48% smaller and 25% more spherical compared to pristine DGTT. Similar changes in size and structure were not observed for the CMTT powder.

The SEM images in Fig. 2*c* show the brittle surface changes in DGTT after ozone (1.7 g h^{-1}) and heat (20 min at 100°C) treatment.

Functional groups on the TWP surface

The results of the XPS measurement are presented in Table 1. The strongest effect of artificial weathering was observed in the formation of O–C=O groups as final oxidative state of the material. Their concentration increased more than threefold from 1.8% in pristine DGTT to 6.3% in 180-min ozone-aged samples. In 30, 60 and 120-min aged DGTT, O–C=O groups showed increases of 92, 91 and 113% respectively, with a moderate positive correlation confirmed by the Spearman test ($\rho = 0.55$, $P < 0.01$).

Comparably, C=O groups increased throughout all artificial weathering durations, indicating progressive oxidation. Concentrations were 7.8, 3.2, 7.9 and 7.5% for 30, 60, 120 and 180-min aged DGTT respectively, but only 3.7% in the pristine material (Table 1), yet the weak positive correlation by the Spearman test was not significant ($\rho = 0.32$).

For C–O groups the proportions of 5.2, 6.1, 5.7 and 8.4% after 30, 60, 120 and 180 min ozone treatment differences to pristine DGTT (5.1%) was less pronounced.

The C–C bonds decreased with increasing artificial weathering, likely as a direct response to the increase in oxidative functional groups, rather than an independent trend. C–C concentrations in DGTT ranged between 78 and 87%, reflecting a reduction of 13% compared to pristine material (89%, Table 1).

The abundance of O–C=O, C=O, C–O and C–C in pristine CMTT was measured at 1.2, 4.3, 1.7 and 92.9% respectively. In aged CMTT, the concentrations of these groups were altered to 4.2, 8.4, 2.0 and 85.5% respectively.

Table 1. Functional groups on tyre wear particle surface.

	CC			CO			C=O			O–C=O		
Ozone application (min)	Area (%)	s.d.	Change (%)	Area (%)	s.d.	Change (%)	Area (%)	s.d.	Change (%)	Area (%)	s.d.	Change (%)
DGTT												
0	89.43	4.1		5.09	1.4		3.71	1.7		1.77	1.3	
30	83.53	9.1	-7	5.22	2.4	3	7.85	4.7	112	3.41	2.4	92
60	87.36	3.5	-2	6.11	1.2	20	3.16	0.9	-15	3.38	3.4	91
120	82.63	6.0	-8	5.74	2.4	13	7.86	2.9	112	3.77	1.8	113
180	77.87	23.3	-13	8.38	14.9	65	7.45	7.0	101	6.31	4.4	256
CMTT												
0	92.85	5.9		1.7	1.5		4.26	5.3		1.19	2.0	
180	85.48	1.9	-8	2.01	3.5	18	8.35	2.5	96	4.16	0.9	251

Peak areas (%) determined using X-ray photoelectron spectroscopy (XPS) for different chemical groups on the tyre wear particles (TWP) surface ($n = 4$). The TWP derived by a diamond grinder (DGTT) or cyro-mill (CMTT) were subjected to different aging times with ozone aging combined with consistent heat application. The measured groups include carbon-carbon bonds (CC), ether compounds (CO), carbonyl groups (C=O) and carboxyl groups (O–C=O). Presented are the area (%) of the main peak, the standard deviation (s.d.) and the change (%) of concentration compared to the pristine material.

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TWP leachate composition

A total of 25 tyre related chemicals were detected and quantified in the TWP leachates (Supplementary Table S1 and Fig. S1). These chemicals consisted of tyre additives (mainly antioxidants, vulcanisation accelerators and curing agents), production residues and degradation products. The highest concentrations were determined, for diphenylguanidine (DPG) with 52–77 mg L^{-1} with higher concentrations for pristine DGTT (77 mg L^{-1}) than for pristine CMTT leachate (52 mg L^{-1}). The N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine (6-PPD) concentration showed higher concentration in leachate from pristine CMTT (2.6 μg L^{-1}) compared to leachate from pristine DGTT (1.2 μg L^{-1}). 6PPD-quinone (6-PPDQ) was below the quantification limit of 0.03 μg L^{-1} even after ozonation and heat application in both CMTT and DGTT leachates. 2,5-*bis*(phenylamino)-2,5-cyclohexadiene-1,4-dione (DPPD-Q) was measured in comparable concentrations in both leachates from pristine materials (4.8–6 μg L^{-1}) and decreased after weathering with ozone and heat. Of the 25 targeted chemical, the total amount of the detected chemicals is slightly higher in leachates from pristine DGTT (64 mg L^{-1}) compared to leachates from pristine CMTT (84 mg L^{-1}) and increases after artificial weathering in both materials to 82 and 104 mg L^{-1} respectively.

For six compounds, the concentration in leachate differed according to the comminution method used for the TWP production (Supplementary Table S1 and Fig. S2). Concentration of dicyclohexylamine (DCH), *N*-dicyclohexylmethylamine (DCHM) and hexamethoxymethylmelamine (HMMM) were an order of magnitude higher in pristine DGTT leachate than in pristine CMTT leachate, whereas aniline, benzothiazole (BT) and tributylamine (TBA) were enhanced in pristine CMTT. For further details please see Supplementary Tables S1 and S2, Fig. S1 and S2.

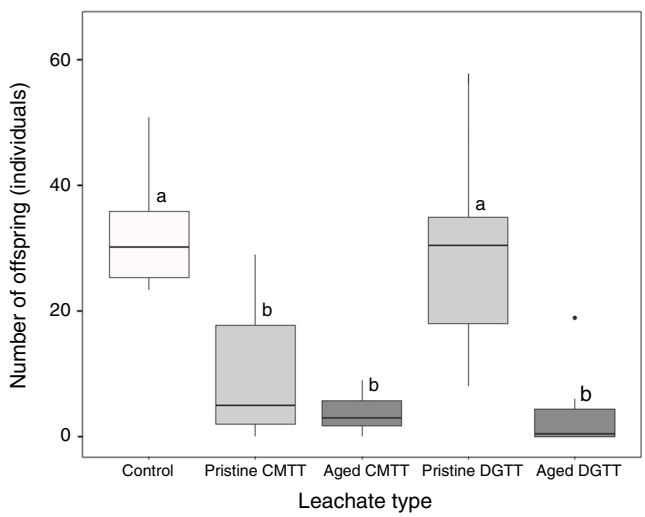

Fig. 3. Nematode offspring (box-plot of the number of individuals) per adult specimen of *Caenorhabditis elegans* after exposure to leachates from pristine and aged tyre wear particles (TWP). TWPs were derived by cryo-milling (CMTT) or by diamond-grinding (DGTT). Nematode control groups were exposed to M9 buffer (control). Bars with the same lowercase letters (a or b) are not significantly different according to the Bonferroni test at $P < 0.05$.

Toxicity of different leachate types in *C. elegans*

The effects of TWP leachate type on the number of juveniles per adult nematode was highly significant (Kruskal–Wallis: $P < 0.0001$; Fig. 3). The negative effect on nematode offspring was particularly evident after TWP weathering, where irrespective of leachate concentrations the number of juveniles per adult nematode in the CMTT leachate assays decreased to an average of 4 ± 3 ($P < 0.0001$) and to 2 ± 2 ($P < 0.0001$) in the DGTT leachate tests compared to the control with 30 ± 7 juveniles. Also, pristine CMTT leachate caused negative effects on nematode reproduction. With an average of 5 ± 5 juveniles, the offspring was significantly reduced compared to the control ($P < 0.01$; Fig. 3).

With an average of 29 ± 15 juveniles pristine DGTT leachate did not lead to a significant change in reproduction. This contrasts the strong negative effect of aged DGTT leachates reducing offspring by 93% compared to its pristine condition ($P < 0.001$; Fig. 3). Overall, the impact of pristine CMTT and DGTT leachates on nematode reproduction were significantly different: leachates from pristine tyre tread had a negative effect on progenies, when originated from cryo-mill ($P < 0.01$), but not when comminuted with a diamond grinding machine. Nematodes exposed to pristine CMTT leachate at concentrations of 0.05 g TWP mL^{-1} produced 76% less offspring than nematodes exposed to pristine DGTT leachate.

Effects of TWP comminution method, artificial weathering and concentration

The main effects of the different TWP conditions on nematode offspring are summarised in Fig. 4. With regard to type of comminution, CMTT significantly reduced the number of offspring to 4 ± 4 individuals compared to the controls with 30 ± 7 individuals ($P < 0.001$; Fig. 4*a*). Irrespective of concentration and weathering, with exposure to leachates originating from DGTT nematodes produced an average of 16 ± 18 progenies, which did not differ significantly to control or CMTT treatments.

As shown in Fig. 4*b*, the aging status of TWP had an overall negative effect on the reproduction of nematodes ($P < 0.001$). The number of offspring decreased significantly when adults were exposed to aged tyre tread powder in comparison to control ($P < 0.001$) and pristine TWP treatments ($P < 0.001$). Although the difference in progenies between control groups (3 ± 3 juveniles) and pristine TWP (19 ± 17) treatments were not significantly different, leachates from aged TWP samples caused a 90 and 84% decline in reproduction respectively.

The average offspring per adult *C. elegans* accounts for 6 ± 8, 12 ± 17 and 14 ± 16 at leachate concentrations of

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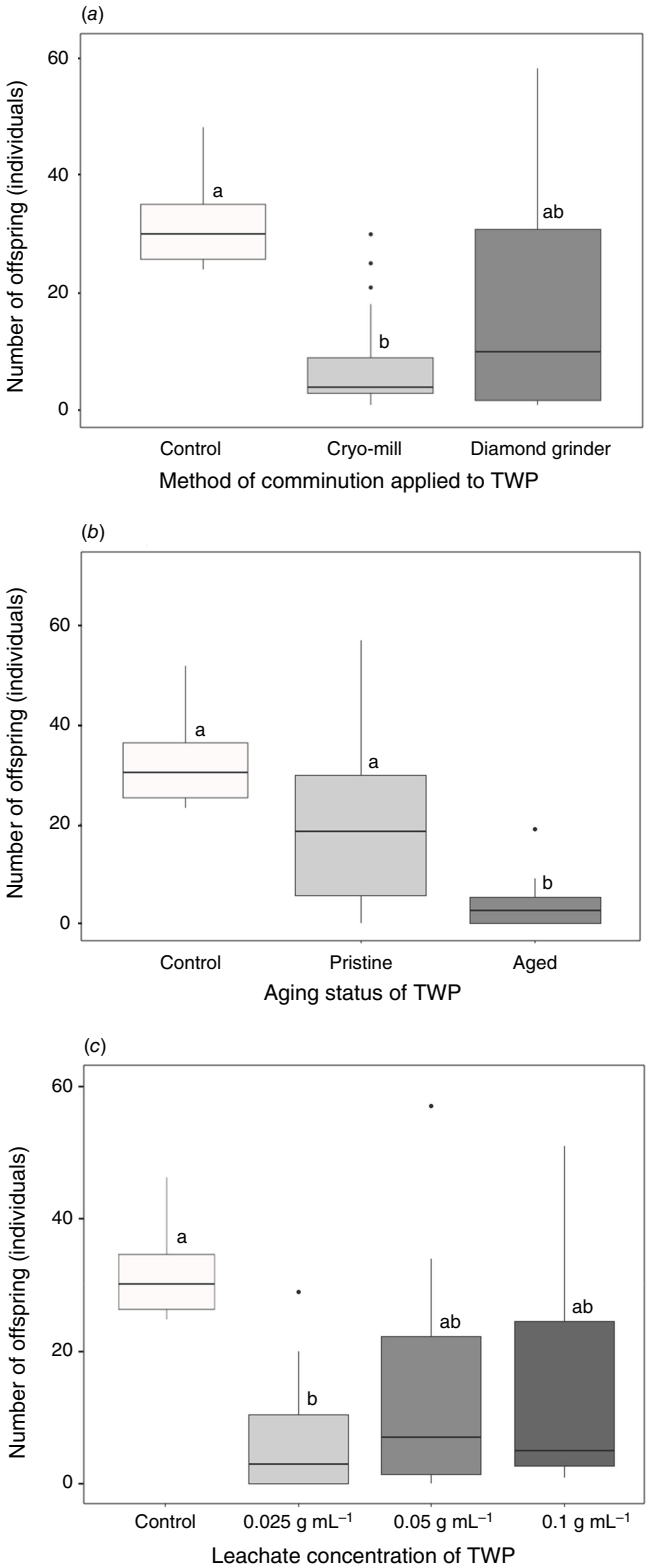

Fig. 4. (*Caption on next column*)

Fig. 4. Offspring per adult specimen of *Caenorhabditis elegans* (box-plot of the number of individuals) after exposure to leachates from tyre wear particles (TWP). The results were categorised by: (*a*) Comminution method (cryo-mill or diamond-grinder); (*b*) Pristine or aged TWP, and (*c*) Leachate concentrations of 0, 0.025, 0.05 and 0.1 g mL^{-1} M9 buffer. Nematode control groups were exposed to M9 buffer. Bars with the same letters (a, b or ab) are not significantly different according to the Bonferroni test at $P < 0.05$.

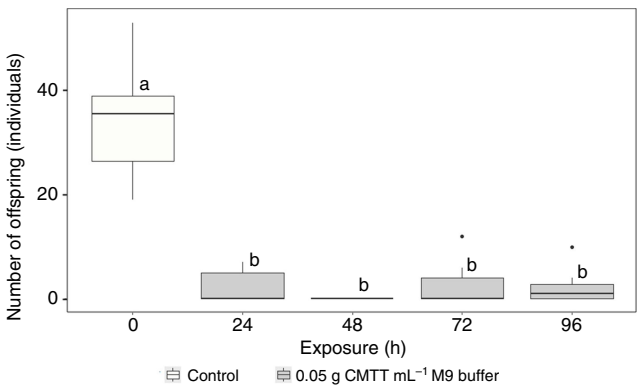

Fig. 5. Number of offspring per adult specimen of *Caenorhabditis elegans* (box-plot of the number of individuals) after varying exposure time (24, 48, 72, 96 h) to tyre wear particles (TWP) with leachate at a concentration of 0.05 g mL^{-1}. Nematode control groups were exposed to M9 buffer. Bars with the same letters (a or b) are not significantly different according to the Bonferroni test at $P < 0.05$. CMTT, cryo-milled tyre tread.

0.025, 0.05 and 0.1 g mL^{-1} TWP respectively (Fig. 4*c*). Compared to the control with 30 ± 7 of offspring, this reduction was significant for the lowest leachate concentration ($P < 0.001$). However, at higher concentrations, in the groups with 0.05 and 0.1 g mL^{-1} leachate concentrations, the variability in the data was too great to confirm a statistically significant effect, as the observed results differed considerably between replicates.

Toxicity tests on exposure time and artificial weathering duration of DGTT

When testing the effect of aged CMTT leachate exposure durations (24, 48, 72, 96 h), all treatments reduced the number of offspring (Fig. 5). The nematodes exposed to leachates had on average 93% less offspring, than nematodes in the control samples ($P < 0.0001$).

The impact of the TWP derived by diamond grinding (DGTT) on nematode reproduction was investigated in

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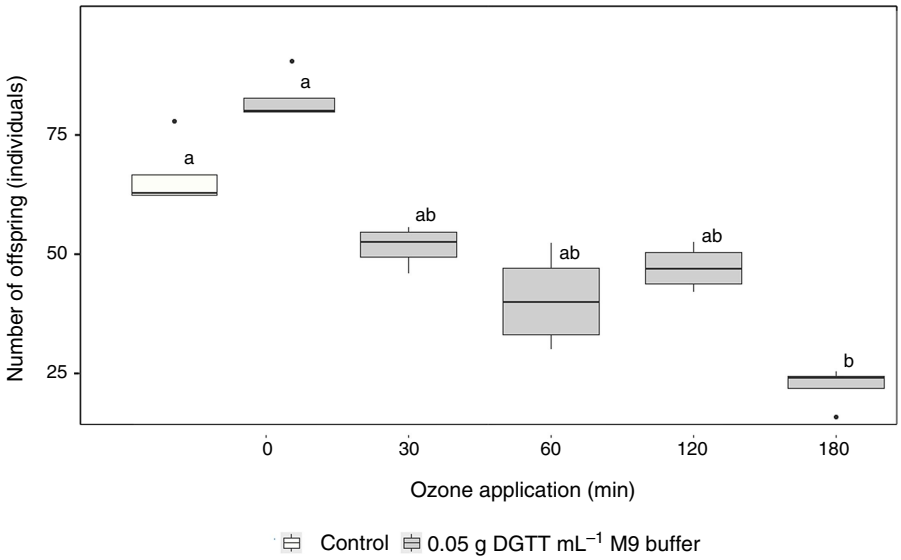

Fig. 6. Nematode offspring per adult specimen of *Caenorhabditis elegans* (box plot of the number of individuals) after exposure to leachates from pristine and aged tyre wear particles (TWP) at a concentration of 0.05 g mL^{-1}. TWP derived by diamond grinding (DGTT) was heated and additionally exposed to varying durations (30, 60, 120, 180 min) of dry ozone treatment. Nematode control groups were exposed to M9 buffer. Bars with the same letters (a, b or ab) are not significantly different according to the Bonferroni test at $P < 0.05$.

more detail regarding the artificial weathering duration. The increased ozone treatment of tyre tread particles (heat application kept constant throughout all samples) negatively affected the reproduction of *C. elegans*. The Kruskal–Wallis test indicated a negative effect on nematode's offspring ($P < 0.001$), reflecting the consistent decline of ozone aged DGTT compared to unaged DGTT and controls (Fig. 6). However, the Bonferroni *post hoc* test revealed significant differences only for the longest ozone treatment with 180 min, causing 67% less offspring compared to controls ($P < 0.01$). This decline in nematode reproduction was also observed in comparison to the pristine DGTT treatment ($P < 0.01$, Fig. 6). Leachates from DGTT aged at earlier time points resulted in only 21% (30 min), 39% (60 min) and 29% (120 min) in offspring compared to the control group with 66 ± 8 offspring, which was not significantly different. However, Spearman's rank correlation coefficient indicates significant negative interactions between ozone treatment duration and number of offspring ($\rho = -0.87$, $P < 0.001$).

Discussion

TWP morphological characteristics

This study compares the two commonly used techniques to produce MP particles for research, cryo-mill (Kreider *et al.* 2010; Weyrauch *et al.* 2023) and rotating abradants (e.g. diamond grinding) – which are also typical procedures for the production of TWP (Ren *et al.* 2020; Grigoriadi *et al.* 2023). The results revealed distinct differences in shape and particle size between these two comminution methods. Particles from the diamond grinder were smaller in size and had an irregular and porous shape, whereas particles from cryo-mill were large, with cubist forms and less surface porosity. Firstly, the mode of pressure and rotation movement during grinding can explain these findings. The ball cryo-mill applies rhythmic and even impact force, whereas with a diamond grinder are contact pressure and grinding force dominate. Secondly, temperature may have an effect: diamond grinding generates high temperatures, resulting in elastic material transformed into small particles, whereas cryo-milling works at lower temperatures, and brittle material crumbles apart.

These differences in particle size distribution and shape also affected the impact of artificial weathering on TWP. Whereas particles of DGTT shifted to smaller sizes, those of CMTT showed no changes after simulated weathering. Likely, the multifaceted structure of DGTT, with small corners, pores and edges, is more accessible for ozone and therefore prone to further embrittlement, resulting in an overall increase in surface size. The latter is crucial for leaching and TWP toxicity (Wang X. *et al.* 2020) and also relevant for ingestion by the soil fauna.

TWP functional groups

All oxygen containing functional groups for CMTT and DGTT particles increased through ozone weathering, especially carboxylic groups with longer exposure duration. This is due to carboxylation, that occurs by augmentation of C–O and C=O groups with additional oxygen, which are further transformed into O–C=O groups (Kye *et al.* 2024). This process can be amplified by anti-oxidative ingredients in the tyre material, binding ozone by double bonds (Seiwert *et al.* 2022). Such antioxidants were also present in the tyre samples. Since both TWP originate from the same material, no differences were expected. This correlates with the increased oxidative TPs in the leachate observed after ozone treatment in DGTT.

Functional groups change the properties of the material in two ways, one is the decrease of hydrophobicity, the

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other is the enhanced reactiveness (Shi *et al.* 2024). Aged particles were observed to disperse in the solution, whereas the pristine particles exhibited hydrophobic behaviour, causing them to cling to the glass vial. This hydrophilic characteristic of aged particles can play an important role in leaching procedures, as the contact to water increases and promotes the leaching of the substances (Li *et al.* 2024). As TWP in the environment are regularly prone to weathering by ozone and heat (Wagner *et al.* 2022), this has an impact on the environmental problems caused by these MPs.

Leachate composition

The leachate composition was analysed as one replicate. The tyre leachates contained a complex composition of many organic compounds, of which 25 different target chemicals were quantified. The concentration of the 4-HDPA and 6-PPD was low compared to other studies of both CMTT and DGTT (Unice *et al.* 2015; Weyrauch *et al.* 2023). 4-HDPA is the main hydrolysis product of 6-PPD, which is formed with half-lives of 4 h and is expected to be present in the leachate. This difference could be due to low recovery of these antioxidants after the filtration process used in the test assays. This highlights the fact, that toxicity tests with the soil fauna need to be performed in soil environment to achieve realistic, environmentally relevant results.

The total amount of leached chemicals does not directly correlate with the observed toxicity. The highest concentrations of chemicals were found in aged DGTT leachate. Pristine DGTT and aged CMTT exhibited similar total concentrations of leached compounds. However, although aged CMTT leachate was found to be toxic to *C. elegans*, pristine DGTT leachate had no negative effect on number of offspring. The lowest chemical concentrations were observed in pristine CMTT leachate. Yet, it still led to a reduction in *C. elegans* offspring. The differences in total chemical concentration between aged DGTT and aged CMTT, pristine DGTT and aged DGTT, and pristine CMTT and pristine DGTT cannot be clearly attributed to a single factor. The differences between pristine and aged DGTT could arise from artificial weathering or from the reduction in particle size due to weathering. Aged DGTT and aged CMTT differ in comminution method and particle size, as CMTT particles are larger. The same applies to the comparison between pristine DGTT and pristine CMTT. The higher total amount of leached chemicals could result from the smaller particle size of DGTT or from the comminution method. For the latter, not only the comminution conditions but also whether the tread was abraded from the whole tyre or cut out for further processing may play a role. The higher total chemical concentration in aged CMTT leachate compared to pristine CMTT can clearly be attributed to the artificial weathering process. Since no particle size changes were observed after artificial weathering and both samples were subjected to the same comminution method, the increase in leached substances can be linked to the weathering factor. The substance with the highest concentrations in CMTT leachate was TBA, a polymerisation catalyst (Nomeir *et al.* 2019). With low melting points of −70°C (ChemInfo 2025), it was likely preserved by low milling temperatures. DGTT leachates contained high levels of DCHM, DCH and HMMM. In the comminution process of DGTT the tyre was fixated and the tread was abraded, whereas CMTT were prepared from pellets that are purely from the tread material. All three chemicals are used for the textile backbone of the tyre and can migrate from there into the tyre rubber matrix, which further is favoured by higher temperatures during the DGTT preparation. This migration cannot be traced with cryo-milled particles, since the method requires cutting down the material into smaller pieces for further processing. By contrast, the use of a diamond grinder mimics the abrasion of the tyre tread from the whole tyre, as observed in the daily use of tyres. This is a major drawback of the cryo-milling. Future studies on TWP toxicity should take into account, that leachate composition can vary considerably due to the initial tyre material used for particle production, and that this may additionally be modulated by the methods of comminution.

The analysis highlights two substances of the target compounds the model organisms *C. elegans* was exposed to: aniline and BT, both with high concentrations in pristine CMTT, aged CMTT and aged DGTT leachates, but low levels in pristine DGTT leachates, which is in alignment with the observed effects. Both compounds are expected to be formed by artificial weathering with ozone and heat. Aniline is easily transformed by ozone, where hydroxyaniline is one possible TP (Tekle-Röttering *et al.* 2016), which was detected enhanced in aged DGTT. Tyres can contain *N*-cyclohexylbenzothiazol-2-sulfenamide (CBS), which decomposes at 145°C (Institut für Arbeitsschutz der Deutschen Gesetzlichen Unfallversicherung 2024) as vulcanisation accelerator precursors. As a result, BT, 2-hydroxybenzothiazole (OH-BT), methylbenzothiazole (MBT), benzothiazole sulfonic acid (BTSA) and DCH are formed (Unice *et al.* 2015). BT and OH-BT are formed from MBT by ozonolysis (Derco *et al.* 2014). In absence of radical scavengers BT is reduced by ozone further (Valdés *et al.* 2004). All these components were detected in the leachates of aged TWP (Supplementary Table S1). To date, no studies have been identified addressing the toxicity of aniline and BT on *C. elegans*. However, a recent study by Zhu *et al.* (2025) identified nematicidal effects of BT on the nematode *Meloidogyne incognita*. Further toxicity tests with aniline and BT are needed to analyse potential effects of both substances on *C. elegans*. In our study, aniline and BT showed concentrations that may contribute to the observed effects. We measured high BT concentrations of 1366, 720, 293 and 3570 μg L^{-1}, and aniline concentrations of 4373, 4323, 351 and 3131 μg L^{-1} in pristine CMTT, aged CMTT, pristine DGTT and aged DGTT leachates respectively. The lowest concentrations for both substances were found in

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pristine DGTT leachate, which was also observed to have no effect on the reproduction of *C. elegans*. By contrast, the other leachates significantly reduced the number of offspring. Although the concentrations vary considerably, we assume that a lethal level of the substances may play a role. Although the leachates contain a wide range of substances, only aniline and BT exhibited concentrations that might explain the observed toxicity trend, making extended investigation worthwhile. Nevertheless, given the complexity of the leachate composition, the influence of non-target chemicals cannot be excluded.

The higher concentrations of 6-PPD and 4-HDPA in pristine CMTT leachate compared to pristine DGTT were expected as the milling were done under nitrogen cooling that should preserve the antioxidant. However, a more detailed analysis of the oxidative TPs of DPG, 6-PPD and MBT showed that the CMTT particles undergo rapid weathering during storage. We observed increased peak areas for the oxidised TPs compared to the parents (Supplementary Table S2). Thus, already before the artificial weathering procedure the particle surface contained known oxidative TPs, that decreased after the weathering, probably due to further transformations. Interestingly in DGTT leachate the oxidative TPs generally increased as expected by ozone treatment, but a decrease was observed for DPPD-quinone and 6-PPDQ was below the limit of quantification for all measured leachates. These observations show that the leachate composition is highly dynamic, changed by weathering processes and the milling method. The toxicological effects cannot be conclusively assigned to specific compounds; however, both substances suspected of being linked to the observed effects are TPs, which highlights the need to consider the entire lifespan of chemicals transported into the environment in TWP.

Comminution method, aging status and leachate concentration effects to *C. elegans*

TWP leachates affected the reproduction of *C. elegans*, which was assessed by the number of offspring. The direct impacts of TWP particulates by ingestion or physical interaction can be omitted, as particles were filtered out from the leachate solutions. Although the reproduction of nematodes after exposure to the leachate of pristine CMTT was significantly reduced, the number of progenies after exposure to leachate of pristine DGTT did not differ from controls. This indicates that the method of comminution has a strong influence on the outcomes of toxicological experiments for *C. elegans* in this study. Further, the results contradict the hypothesis that the leachate from larger and less porous CMTT has lower toxicity compared to the DGTT. By contrast, Weyrauch *et al.* (2023) report higher leachable concentrations in CMTT, than in particles collected at outdoor driving test stations, the so-called road wear particles. This points to the temperatures during the comminution procedure as an exploratory factor. Heat during the comminution procedure (DGTT) could lead to the evaporation of chemicals in the tyre, whereas low temperatures (CMTT) may preserve chemicals. These observations have implications for the toxicity of TWP in the environment, since heat is as one of the common weathering processes of tyre tread on overheated asphalt paths (Wagner *et al.* 2022). However, the chemical analysis of leachates implies that oxidative transformation occurred already in the pristine CMTT. Thus, the comminution and weathering are complex processes, in which the chemical composition is difficult to predict.

The number of offspring of nematodes significantly decreased, when DGTT was aged with ozone and heat, indicating that leachate from aged particles have a higher toxicity. Similar findings were made with UV-aged TWP, causing severe oxidative damages on the earthworm *Eisenia fetida* compared to pristine material (Chen *et al.* 2022). This difference may derive from the following aspects: one is that embrittlement of particles increases their surface. However, this seems not to be a major factor, since the enlarged surface of DGTT did not alter its toxicity. Moreover, the degradation of chemicals can affect their toxicity (Lake *et al.* 1977). And finally, the decrease in hydrophobicity caused by various oxygen containing functional groups formed during the artificial weathering. The latter ensures a better contact surface with the surrounding medium, and therefore higher amounts of leachables (Wang X. *et al.* 2020).

Further, it should be taken into account that degradation products of certain components can differ in toxicity compared to the initial chemical. For example, DPG is used as vulcanisation accelerator in tyre wear production and its primary degradation product aniline can be twice as toxic to mice (French Agency for Food, Environmental and Occupational Health Safety 2020). This consideration highlights the urge to combine toxicological tests with analytical studies on degradation processes of tyre tread in nature for a realistic picture on TWP interactions with biota in terrestrial ecosystems.

The experiments with varying leachate concentrations did not result in the expected reduction in offspring with increasing concentration. In fact, the opposite was observed. Nematodes exposed to the lowest TWP leachate concentration produced less progenies, than individuals in high TWP leachate concentrations. Biological differences between individual *C. elegans*, slight inconsistencies in exposure conditions, or stochastic effects in reproduction can lead to variability in data.

Besides the high variability of the organisms, another explanation may be that at high concentrations, the pathways or mechanisms of action become saturated, preventing the substances from affecting the organism beyond a certain point (Wang 2022). Additionally, a feedback reaction could be triggered only at high concentrations of the compounds in leachate, whereas low concentrations can harm the organism without inducing this response (Wang 2022).

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Overall, the results highlight the critical role of the comminution method in determining the toxicological impact of tyre wear particles. Although both CMTT and DGTT were produced from the same tyre material and tread layer, the different conditions during the comminution procedure not only influenced the chemical composition of both, but also size, shape and even the toxicity of the particles. Understanding how comminution processes influence the properties, chemical composition and leachability of TWP is essential for assessing their environmental and biological interactions.

Exposure time

Chemicals leaching into soil spread slowly, can be retained, washed away into lower layers and groundwater or accumulate in certain soil horizons (Madrid *et al.* 2007; Keesstra *et al.* 2012; Li *et al.* 2022). The speed of movement of organisms affects their distribution within soil compartments, potentially influencing their exposure levels. All these factors can lead to changing exposure durations of soil biota to contaminants, altering the impact of TWP leachates on soil biota. Moreover, the development stage e.g. in the life cycle of *C. elegans*, affects the sensitivity towards toxicity of substances (Wu *et al.* 2012). Generally, with a shorter chemical exposure duration, less negative effects can be expected, which was shown for *C. elegans* for ferric iron nanoparticles (Fe_2O_3-NP) and bisphenol A (Wu *et al.* 2012; Ruszkiewicz *et al.* 2023). Depending on the life stage of the nematode two possibilities can be stated: one is, that early stage nematodes adapt to toxic conditions, thereby increasing their survival rate. By contrast, adult worms can be more resistant, since fully developed and less prone to contaminants (Li *et al.* 2014). However, in the present study, the life stage had no influence on the reaction of *C. elegans*, as all exposure durations to TWP leachate showed a significant decline in number of offspring. A study carried out by Kim *et al.* (2021) in soil revealed stronger adverse effects on the survival rate of *C. elegans* at lower TWP concentrations during long-term exposure (10 days) compared to short-term exposure (2 days). However, due to differences in experimental design (i.e. the use of soil as a medium, varying endpoints and TWP concentrations) direct comparability is limited. Although this study found adverse effects across all exposure durations, it is still possible that exposure time may play a role in soil environment. In water, substances are diffused and immediately available, as they are not bound to any matrix (Keesstra *et al.* 2012). By contrast, in soil, chemical mobility can be restricted by the soil matrix, which may reduce bioavailability. Nematodes inhabit pore spaces, they only have access to the water within these pores and require time to bioaccumulate the substances.

Artificial weathering

It has been hypothesised that plastic pollution may pose its greatest environmental risk during the final stages of its lifespan, when degradation is complete and contaminants are released into the environment through leaching (Rillig *et al.* 2021). However, contaminants may also be washed out in the early phases of degradation. This is supported by the present study, yet only for TWP leachate after heat treatment combined with 180 min of ozone treatment. Additionally, a significant negative correlation was observed between ozone application of TWP and the production of offspring by *C. elegans*, which underpins the importance of weathering as a factor of toxicity. Although the data generally support a negative correlation, significant differences in toxic effects in relation to pristine TWP is observed solely for 180 min of ozone- and 20 min of heat-aged TWP. Ozone treatments less than 180 min show slightly decreasing offspring numbers but no significant differences to pristine TWP. In the context of environmental pollution, released particles may have a degradation threshold beyond which they become toxic or fully develop their toxic potential. Since weathering and leaching are not separated procedures in the environment, but occur simultaneously, their combined effects must be considered.

Weathering is caused by various environmental factors, which may alter the morphology of TWP in different ways. Most of the MP studies use UV weathering to generate aged particles (Hankett *et al.* 2013; Wang Q. *et al.* 2020; Ouyang *et al.* 2022). However, it is experimentally difficult to age all particles evenly. Another challenge is possible evaporation of compounds into the environment due to high temperatures, resulting in an underestimation of chemicals experiments. Overall, the dry ozone treatment used in this study is a time efficient and easy way to generate aged particles in a closed rotating chamber, ensuring a reasonably even artificial weathering of all particles and preventing chemical loss by evaporation. Additionally, since no liquid medium is used during the weathering process, released substances do not transfer into any surrounding matrix and are therefore not discarded with the medium, preserving the chemical integrity of the aged particles.

Conclusion

This study systematically assessed the impact of comminution methods and artificial weathering on the toxicity of TWP for soil nematode and model organism *C. elegans*. It revealed that comminution method had a strong impact on the outcome of test assays. Whereas diamond grinding is able to simulate road abrasion on the entire tyre, cryomilling requires that only sections of the tyre are cut out, which was found to make a difference in the composition of leachates. Diamond-ground material, with its elongated and porous structure, likely better represent environmental relevant TWP. Artificial weathering significantly altered particle properties, e.g. exposure to ozone introduced reactive oxygen-containing groups. These enhance the likelihood of incorporating surrounding pollutants into the particles, while simultaneously

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decreasing their hydrophobicity. Thereby, weathering can facilitate a more effective release of toxic substances into the environment.

The present findings further question the significance of studies using pristine particles, as TWP exists only in aged forms in the environment. Standardised artificial weathering procedures are urgently needed to obtain relevant and valid results. The dry ozone treatment tested here holds promise for efficient weathering, but requires comparison to natural and other artificially aged TWP. The observed differences in the leached chemicals further highlight the importance to implement diverse weathering factors such as heat, ozone and mechanical abrasion.

Finally, the complexity of the soil substrate, which includes organic matter, metals, various particle sizes and types, poses a challenge for test assays. Depending on functional groups, substances leaching out from TWP can interact differently with these soil components and binding may decrease toxicity. Moreover, particle size and shape can affect the filter function of soil, and pose a risk to the groundwater. Overall, this calls for more TWP studies performed under realistic soil conditions.

Supplementary material

Supplementary material is available online.

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

Conflicts of interest. The authors declare that they have no conflicts of interest.

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