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Deep dive into the chronic toxicity of tyre particle mixtures and their leachates

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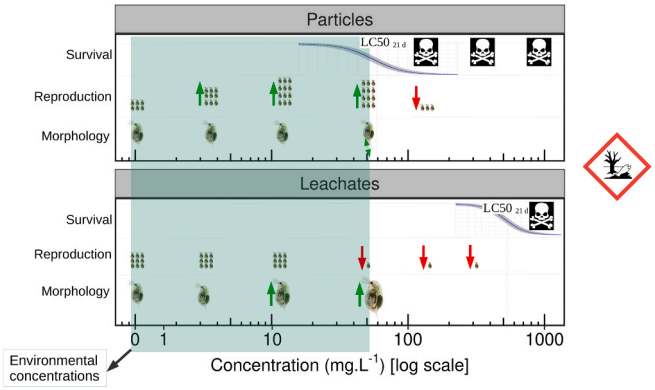
HIGHLIGHTS

- Tyre-tread microparticles are a class of plastic pollutants of high concern.
- Tyre-tread particle mixtures were prepared from widely used brands of tyres.
- Several organic chemicals were identified by LC-QToF and quantified by LC-MS-MS.
- Both tyre microparticles and their leachates induced toxicity responses on life-history traits.
- Tyre particles were more toxic than leachates (LC50 $_{21d}$ of 60 *vs* 542 mg.L^{-1}).

GRAPHICAL ABSTRACT



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ABSTRACT

Particles from the tread of vehicle tyres are a global pollutant, which are emitted into the environment at an approximate rate of 1.4 kg.year^{-1} for an average passenger-car. In this study, popular tyre brands were used to generate a tyre tread microparticle mixture. The chronic toxicity of both particles and chemical leachates were compared on a planktonic test species (*Daphnia magna)*. Over 21 days of exposure, pristine tyre tread microparticles were more toxic (LC50 60 mg.L^{-1}) than chemical lechates alone (LC50 542 mg.L^{-1}). Microparticles and

Abbreviations: 2-ABT, 2-aminobenzothiazole; BTH, Benzothiazole; BTR, 1 H-Benzotriazole; CPU, 1-cyclohexyl-3-phenylurea; C-DMU, 3-cyclohexyl-1,1-dimethylurea; DCU, 1,3-dicyclohexylurea; D-DPU, 1,3-diethyl-1,3-diphenylurea; DPG, 1,3-diphenylguanidine; HMMM, 2,4,6-Tris[bis(methoxymethyl)-amino]− 1,3,5-triazine, hexa(methoxymethyl)-melamine; 5-MBTR, 5-methyl-1 H-Benzotriazole; M-DCA, N,N-dicyclohexylmethylamine; 24MoBT, 2-morpholin-4-yl-Benzothiazole; 2-MTBT, 2-(methylthio)benzothiazole; NCBA, N-cyclohexyl-1,3-benzothiazol-2-amine; 2-OHBT, 2-hydroxybenzothiazole; 6PPDQ, N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine-quinone).

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leachates showed distinct effects on reproduction and morphological development at environmentally relevant concentrations, with dose-dependent uptake of particles visible in the digestive tract. Chemical characterization of leachates revealed a metal predominance of zinc, titanium, and strontium. Of the numerous organic chemicals present, at least 54 were shared across all 5 tyre brands, with many classified to be very toxic. Our results provide a critically needed information on the toxicity of tyre tread particles and the associated chemicals that leach from them to inform future mitigation measures. We conclude that tyre particles are hazardous pollutants of particular concern that are close to or possibly above chronic environmental safety limits in some locations.

1. Introduction

The use of tyres by cars, buses and other vehicles is a ubiquitous feature of modern life, served by a global tyre industry of massive scale (about 19 Mt in 2019 [49]). This figure is forecast to increase by around 3.4% year-on-year in the near future [49] and the tyre-tread particles generated during tyre use are a major environmental pollutant. Once on the roads, an important proportion will be washed into waterways. It is estimated that approximately 18% of the tyre-wear particles polluting lands eventually reach freshwater and 2% reach estuaries (Seine watershed study) [19,55,56]. Predicted environmental concentrations (PECs) of tyre particles in run-off waters average 30 $mg.L^{-1}$ and are estimated at 120 $\mu g.L^{-1}$ in surface water [50]. However, there are great disparity between locations and studies, with maximum PEC surface waters reported to range from 0.03 to 56 $mg.L^{-1}$ [57]. Motorway pond influents in UK have been quantified in the range of 0.2–29.8 $mg.L^{-1}$ and these concentrations drop down to 0.0–3.2 $mg.L^{-1}$ at the outfalls [38]. Noteworthy, these artificial habitats host a range of living species. Despite a growing body of knowledge suggesting high concentrations in the environment, the toxicological effects of tyre particles to aquatic organisms remain understudied, especially when compared to non-rubber fossil-fuel microplastics.

Two main features characterize tyre-tread particles: their elasticity (synthetic rubber) and their high number of chemical additives designed to impart car safety properties (*e.g.*, better grip on wet surfaces or mechanical resistance). An important challenge with tyres is to disentangle the toxicity of the rubber-particles (*e.g.*, physical toxicity) from the toxicity triggered by hazardous chemical additives. Both components may exert toxicological effects on wildlife, in isolation or in concert, creating a complex array of long-term pollution.

Most studies of tyre tread particles have focused on acute, short-term exposures of aquatic species to either particles or leachates (containing chemical additives leached from the particles). For example, the acute toxicity of tyre particles to the freshwater flea *Daphnia* (*D. magna*) varied widely from 0.06 to 2.41 $g.L^{-1}$ ($LC50_{48\,h}$) depending on tyre season and brand, as well as on how the particles were prepared [58]. Factors including exposure to UV light [19,31,58], duration of leaching and the size of the particles formed can all alter their effects. Tyre tread nanoparticles induced higher acute toxicity to *D. magna* than tyre microparticles, considering both exposure to particles directly and their leached chemical counterpart [9].

Long-term ecotoxicity studies of tyres are scarce, especially for leachates, despite the length of time tyre particles and their chemical burden are likely to remain in the environment. An attempt to study chronic toxicity in *D. magna* over 21 days with tyre particle leachates could not report an LC50 value due to high toxicity [16]. A 7-day study found no toxicity in daphnid species, *Ceriodaphnia dubia* when tyre tread particles were spiked into sediments [37], although *C. dubia* reproduction was significantly affected by tyre leachates with an $EC50_{9d}$ of 13 $mg.L^{-1}$ for the most toxic tyres [59]. More recently, tyre leachates were shown to alter the reproduction and population growth (for 9 days) of brackish water rotifers down to a concentration of 0.2–0.3 $g.L^{-1}$ [47]. Regarding direct exposure to tyre tread particles, the survival of *Hyalella azteca* (freshwater crustacean) was significantly impacted over 21 days of exposure at 0.58 $g.L^{-1}$, whereas reproduction and growth decreased at 0.29 and 0.145 $g.L^{-1}$, respectively [27]. In another study, chronic exposure to tyre particles reduced *H. azteca* growth even at lower concentrations (0.13 $g.L^{-1}$) [18].

To date, there is only one chronic study of tyre leachate (> 9 days) which was performed with marine zooplankton. Here a 17-day exposure to car tyre tread leachates increased the mortality of two marine copepods from exposure to 5 $g.L^{-1}$ [20]. The scarcity of chronic studies (especially for leachates to freshwater invertebrate species) hinders efforts to determine risk to aquatic species, as well as to identify the toxic molecules involved and which physiological functions are at risk. In the present study, *Daphnia magna* (water flea) was selected because it is an ecologically relevant crustacean species playing a pivotal role in ecosystem functioning. It is at the base of the food chain (primary consumer), a filter-feeder species being pelagic but can act as a coupler for pelagic-benthic habitats and food chain [48]. It is considered as a relevant sentinel species for environmental health protection [1], and it is sensitive to microplastics [34].

Here, we took a novel approach. Since each tyre was subtly different, we combined a mixture of the most widely commercially available tyres to encompass the particles most likely to be generated on a typical road. They were used to prepare a 'typical' tyre particle mix and to prepare a matching 'typical' chemical leachate. We used state-of-the-art analytical LC-MS/MS targeted and LC-QToF non-targeted analysis methods to identify many of the chemicals present and to grade them by hazard. The chronic toxicity of both particles and chemical leachates were tested using *D. magna* as a model sentinel planktonic species. Our study set out to address the following questions: How does the chronic toxicity differ between exposure to tyre particle *vs* leachates? Are life-history traits altered and if so at what concentrations? What chemical additives are involved?

2. Materials and methods

2.1. Preparation of tyre particle mixtures

All-season passenger tyres were selected for this study due to rapid sales growth in recent years and a market share of 42% in UK in 2019 [54]. New tyres were sourced from five commonly used manufacturers to ensure a diverse sample set: Nokian (Weatherproof), Bridgestone (Weather Control A005 EVO), Continental (AllSeasonContact), Vredestein (Quatrac) and Pirelli (Cinturato All-Season). They were anonymised irrespectively of this presented order (Tyre 1 to Tyre 5, see Table 2 and Fig. 8). All tyres used in the study were of the same dimensions: 205/55 R16. The tyre tread was removed at the Tun Abdul Razak Research Centre (TARRC) in United Kingdom using a Colmann GmbH & Co buffing machine. The tyre tread was removed without cutting into the undertread rubber. The resulting tyre fragments were cryoground at the University of Exeter using a Freezer Mill (®6870 Spex Sampleprep, 3 cycles, 1 min precooling, 2 min run, 2 min cooling, 15 cps rate). Then, the pristine particles from each individual brand were dry-sieved using RETSCH meshes with 800, 600 and 300 μm apertures. Finally, the particles from each tyre brand sieved through 300 μm were mixed in equal proportion (1/5, w/w), and the tyre particles retained between the 300 μm and 600 μm sieves were also mixed in equal proportion (1/5, w/w).

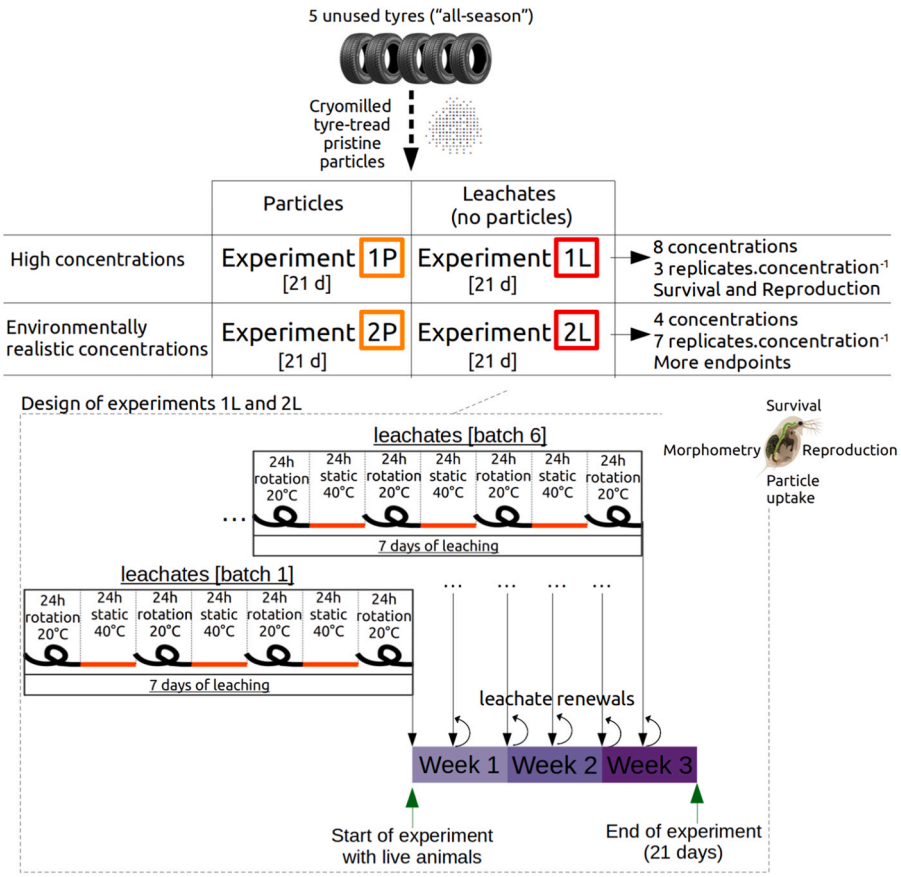


Fig. 1. Study design.

2.2. *Exposure experiments with elevated concentrations (1 P and 1 L)*

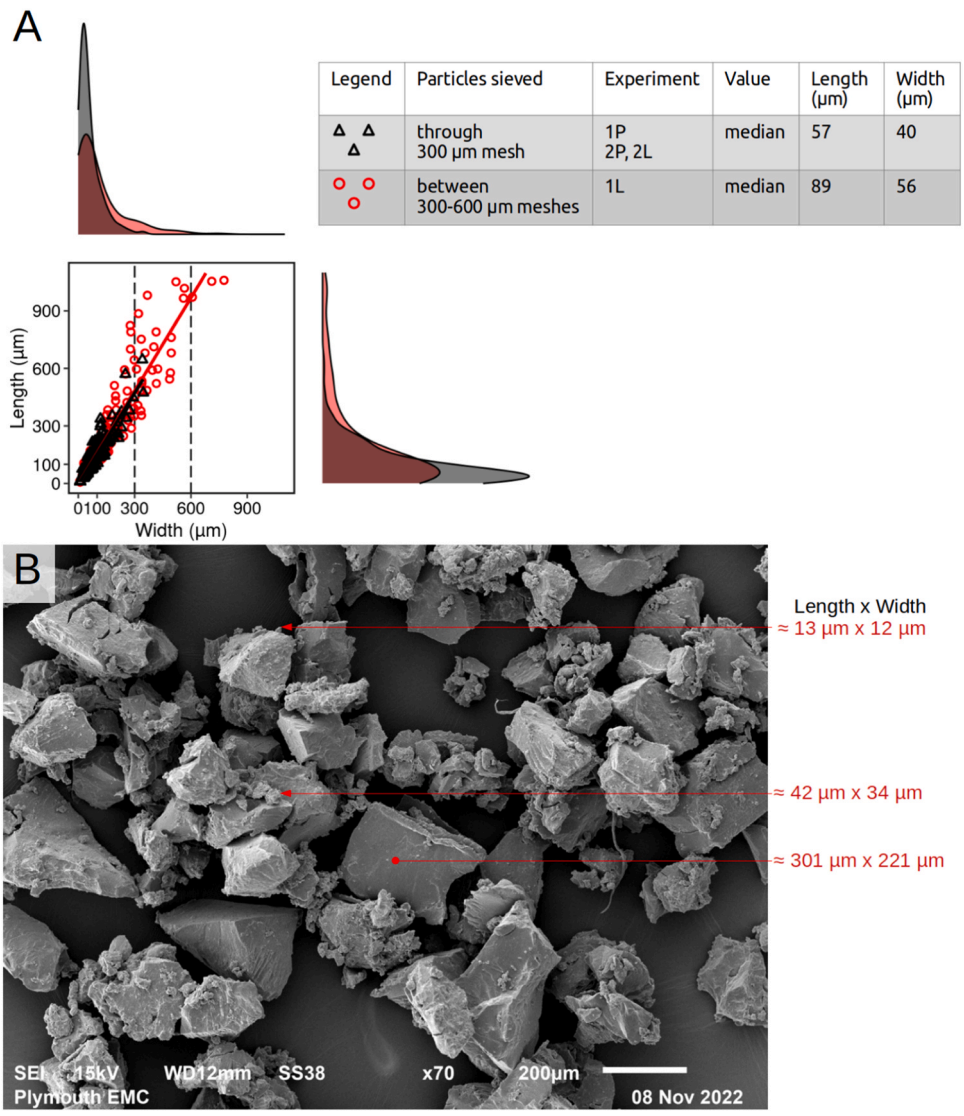
2.2.1. *Experimental features*

Two dose-response chronic toxicity tests at high concentrations were performed (experiment 1 P and 1 L, 'P' standing for particles and 'L' for leachates), one corresponding to direct pristine particle exposure, the other to tyre leachates (see study design in Fig. 1). *D. magna* ephippia (MicroBioTest inc., Gent, Belgium) were hatched in petri dishes for 75 ± 3 hrs (20.8 °C, 2500 ± 250 lx□ of continuous light). Neonates were then transferred to beakers filled with their target exposure medium. Each test had 8 concentrations, with two types of controls at 0 $mg.L^{-1}$ (see below). Each concentration was setup in beaker triplicates (except experiment 1 P having 4 beaker replicates for each of the two types of controls), with 10 neonates per beaker at t = 0, which corresponds to a total of 27–29 beakers, 270–290 neonates per test. Each test lasted for 21 days. Exposure media were renewed twice a week and so each experiment had a total of 6 renewals (*i.e.*, 6 medium batches, Fig. 1). Here, we define a batch of medium as a unique series of operations required to produce a unique solution at a specific time. A pierced silicon lid was added on top of beakers to limit evaporation. Each medium renewal was prepared one day in advance and left at 20.8 °C until the transfer of daphnids. Experiments were run in a thermo-controlled room in which temperature was recorded every hour (Hobbo loggers, see supplementary information, average 20.8 °C, sd = 0.2, Fig S1).

The water used throughout this study was iso-media (ISO 6341), being chelatant-free, which was prepared with concentrated salts sourced from MicroBiotest (Gent, Belgium). Daphnids were fed daily with 300 $\mu L.beaker^{-1}$ of a solution made of Spirulina 200 mg (Sevenhills Wholefoods, Organic Spirulina Powder, Sheffield, UK, origin China) and yeasts 100 mg (baker yeasts Allinson's, Peterborough, UK) in 100 mL of iso-media, an amount adapted from previous published studies [15,32,46]. This corresponds approximately to a daily beaker inoculum of 5.6×10^6 Spirulina algae and 5.3×10^6 yeasts. At preparation, the food was warmed to 37 ± 2 °C and then strained through 40 μm mesh (Nylon cell-strainer, Falcon, Corning Brand). The food solution was renewed at least thrice a week and stored at 5 °C. Food solution was always equilibrated to 22 ± 3 °C before feeding. The survival of daphnids was recorded at least every two days (mortality was assumed when no movement of the individual was seen for approximately 10 s). Daphnids were individually transferred to their renewal beaker medium by using cut-off wide-open pipette tips. The number of newborn neonates was recorded and they were discarded (in 25–50% ethanol). Water parameters were regularly monitored and are presented in supplementary information (pH, conductivity, dissolved oxygen, hardness, NH_4, NO_2, Fig. S30-32 and Table S21). At the end of the two tests (21 days exposure), adult daphnids were individually added in 24-well plates with 1 mL of their medium. Plates were then placed in the freezer at − 80 °C until further processing.

2.2.2. *Tyre particles exposures (experiment 1 P)*

The nominal pristine particle concentrations were set at 1.000, 0.500, 0.250, 0.125, 0.063, 0.031, 0.016 $g.L^{-1}$. The stock solution was prepared in falcon tube which, after incubation and rotation (10 rpm) for 24 h, was then serially diluted (1:2) with cut-off pipette tips in further falcon tubes containing iso-media with tween 80 at 0.005% v/v (further details in Table S2). Glass-beakers of 150 mL capacity were loaded with 95 mL of freshly prepared iso-media and then with 5 mL of their respective stock solution (at 20X). Each beaker had a final concentration of tween 80 of 0.00025% v/v. Two controls were set up: one surfactant controls (iso-media with tween 80 at 0.00025%) and one surfactant-free controls [iso-media]. Note, each intermediate stock was carefully resuspended each time during the serial dilutions. In spite of



Legend	Particles sieved	Experiment	Value	Length (µm)	Width (µm)
△ △ △	through 300 µm mesh	1P 2P, 2L	median	57	40
○ ○ ○	between 300-600 µm meshes	1L	median	89	56

Fig. 2. Size distribution and shape of tyre-tread particles. A: Curves represent draws kernel estimate (smooth version of histograms). Graph (n = 289 datapoints for each particle category for better comparison). Medians (n = 4798 for particles sieved through 300 µm mesh and n = 289 for the particles sieved between 300–600 µm meshes). B: Particles observed by scanning electron microscopy.

this, due to the particularly sticky characteristics of the tyre particles on falcon tubes and pipette tips, a fraction was lost at each dilution step. Based on particle counts of this experiment compared to the experiment 2 P which was considered as a reference with negligible loss, we estimated and assumed a constant gradual loss in the experiment 1 P of 21% (*i.e.*, 21% at each dilution). The nominal concentrations were therefore adjusted to estimated exposure concentrations of 790, 312, 123, 49, 19, 8 and 3 $mg.L^{-1}$ (see supplementary information, SI, Fig. S9 and Table S3).

2.2.3. *Leachate exposures (experiment 1 L)*

An amount of 10.00 g of particles (particle mixture retained between sieves of 300 µm and 600 µm), was added into 1 L Youtility glass bottles, to which 1000 mL of iso-media was added, leading to a solution at 10.00 g: 1 L ratio. The bottle was wrapped with aluminium foil. First it was placed in an overhead rotator (Velp Scientifica, rotax 6.8, overhead mixer) at 10 rpm for 24 h at 20.8 °C. Second, the bottle was placed in an incubator at 40 °C (static condition) for 24 h so to accelerate the release of chemical additives (*e.g.*, Halle et. al, 2020). No UV or light treatment was used in order to prevent the reactivity of chemicals and preserve their structure (see *e.g.*, [13]).

These two cycles were repeated for a total of 7 consecutive days to produce one batch of leachates (see Fig. 1). After this leaching period, the water was filtered through 0.2 µm (sterile filters, Whatman, ref. WHA10406972, Merck Life Sciences, UK). The resulting solution constituted the 100% tyre leachate (equivalent to 10 g: 1 L leachate). Other bottles with clean iso-media but without particles were also subjected to the same conditions (*i.e.*, 7 days of incubation alternating rotation at 20 °C and incubation at 40 °C, referred below as "incubated controls"). The 100% leachate solution was then serially diluted (1:2, v/v) with "incubated controls" solution, which corresponds to leachate concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.13% and 1.57% (which in turn correspond to theoretical particle equivalences of 10, 5, 2.5, 1.25, 0.62, 0.31 and 0.16 $g.L^{-1}$). Two controls were set up: "incubated iso-media controls" and "freshly-prepared iso-media controls".

2.3. *Exposure experiments with low concentrations (2 P and 2 L)*

2.3.1. *Experimental features*

Based on the results of the first trial, two refinement tests were then performed at lower concentrations which were closer to concentrations encountered in natural environments. Fewer concentrations per test

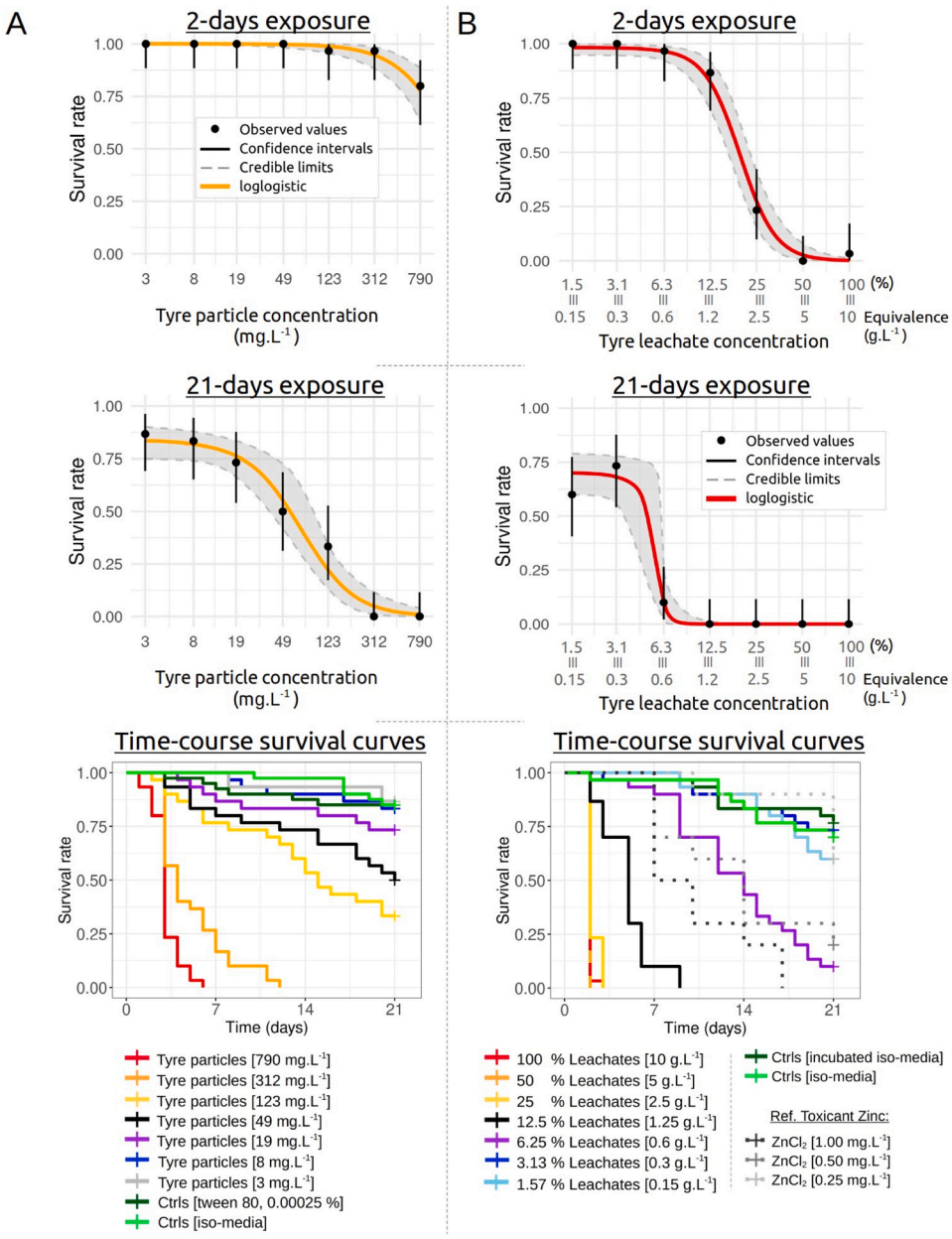


Fig. 3. Survival of daphnids in experiment 1 P and 1 L (A: Tyre particles, B: Tyre leachates).

were set up but more replicates per concentration were used, allowing better statistical conclusions and further biological endpoints to be assessed. The features of experiments 1 P and 1 L were used with some modifications. In the experiments 2 P and 2 L, four concentrations were used (including controls) with 7 beaker replicates (instead of three), each with 10 daphnids at start of the experiment (*i.e.*, 70 daphnia per concentration, 280 daphnia per test). The food solution was prepared as described previously, but an incremental inoculum was provided throughout the tests in order to accommodate better the early stages of daphnia development: 75 μL.beaker^{-1} for days 0–4, 150 μL.beaker^{-1} for days 5–8, 225 μL.beaker^{-1} for days 9–13, 300 μL.beaker^{-1} for days 14–21.

The survival was recorded every day. At day 7, few daphnids were removed from beakers (and calculations) because they were very small and could be confounded with upcoming neonates, leading to n = 65 – 70 daphnia per concentration. During the last three days, few adjustments between beakers of the same concentration were done to keep a homogeneous daphnids density of 8 ± 2 adults per beaker and so to minimise potential beaker replicate effects.

2.3.2. *Low concentration tyre particles exposures (experiment 2 P)*

Pristine tyre-tread particles stock solutions were prepared as described in the section above using the particle mixtures sieved through 300 μm mesh. The initial stock solution was prepared at 50 mg .50 mL^{-1} of iso-media (tween 80, 0.005%) in falcon tubes from which two serial dilutions of 1:4 were performed. Then, 5 mL of the stock solutions (20X) were added to their target beakers pre-loaded with 95 mL of fresh iso-media. The controls corresponded to iso-media with tween 80 surfactant at 0.00025% (same concentration as every other group). The final nominal concentrations were 50, 12.5, 3.1 and 0 mg.L^{-1}. Because particles sedimented, it could be relevant to express these concentrations as 5 mg.beaker^{-1}, 1.25 mg.beaker^{-1}, 0.31 mg.beaker^{-1} and 0 mg.beaker^{-1}. Note, the initial amount of particles used in the experiment 2 P was much lower than in the experiment 1 P. Furthermore, fewer dilutions were performed and of smaller volumes. Therefore, we consider the impact of potential particle loss along serial dilutions to be

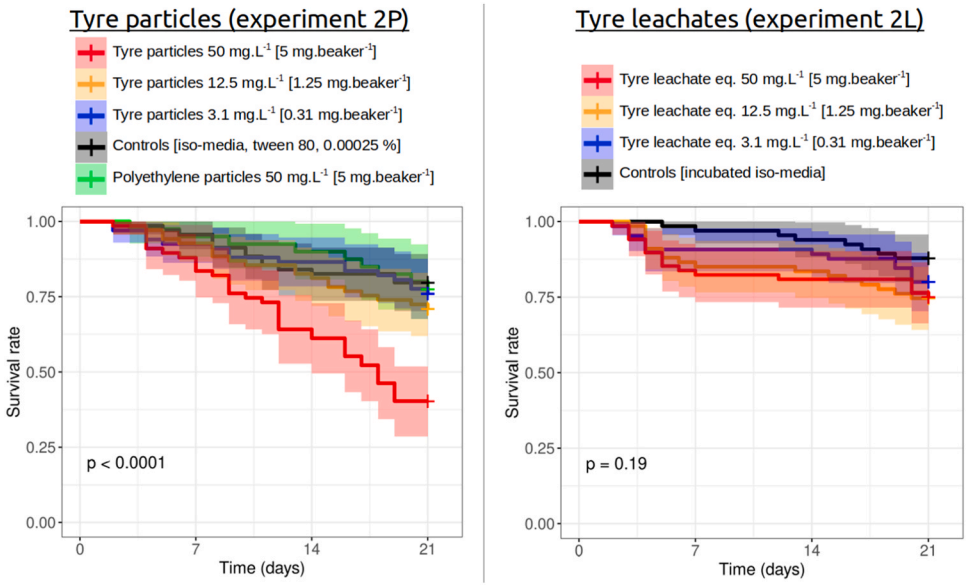


Fig. 4. Time-course survival of daphnids in experiments 2 P and 2 L. Note: Polyethylene particles exposure was run during the experiment 2 L but this group was added on the graph of experiment 2 P for better comparison with tyre particles. The shadowed ribbons surrounding Kaplan-Meier curves represent pointwise plain 95% confidence intervals calculated from the Greenwood formula. P-values on graphs are the result of log-rank tests to detect any global difference between all groups (for experiment 2 P, $p < 0.0001$ with or without the PE group). Fisher pairwise multicomparisons with Holm adjustments are presented in SI.

much lower and so assumed it negligible in experiment 2 P (Table S4).

2.3.3. Low concentration leachate exposures (experiment 2 L)

For a better comparison with the tyre particles experiment 2 P, the particles used in the experiment 2 L were of the same size fraction, *i.e.*, the ones sieved through 300 μm (contrary to the first leachate test 1 L). The leachate bottle was set at 50.0 mg: 1000 mL of iso-media. The incubation was the same as described above and in Fig. 1 (*i.e.*, 7 consecutive days, alternating rotation and temperatures). Particles were removed by filtering water through 0.2 μm, as described previously. This resulting 100% leachate solution was then serially diluted 1:4 with "incubated iso-media", leading to nominal concentrations of 100%, 25% and 6%, which in turn correspond to solutions made up from an equivalent amount of tyre particles of 50, 12.5 and 3.1 mg.L^{-1}. The controls corresponded to "incubated iso-media". Due to a technical issue, the controls and only the controls from the second renewal were exposed to "clean freshly-prepared iso-media" (medium renewal batch n° 2 reduced to 48 h exposure, *i.e.* 9.5% of test duration), whereas this same group of controls were indeed exposed to "clean incubated iso-media" for the rest of the experiment (*i.e.*, medium renewal batch n° 1, 3, 4, 5, 6).

2.4. Experiment calibration and additional controls

The experiment was calibrated with Zinc Chloride anhydrous (98%, Alfa Aesar), using a stock at 200 mg.L^{-1} (of iso-media) and stored in the fridge. Zinc chloride was chosen as a referent toxicant for testing the sensitivity of our experimental setup and because it is a known major chemical contributor of overall tyre toxicity [8,61]. Hence, direct comparison with tyre toxicity of the present study could be done. Three beakers were set up during the experiment 1 L, at 1, 0.5 and 0.25 mg.L^{-1} in iso-media, 100 mL.beaker^{-1} (1 beaker.concentration^{-1}, 10 daphnids. beaker^{-1}). In the second round of experiments, additional controls were set up, consisting in 4 beakers of 10 daphnids exposed to polyethylene particles at 50 mg.L^{-1} (Cospheric, 32–38 μm Phosphorescent Yellow-Green microspheres, Santa Barbara, USA). The rationale of these additional controls was to determine if the tyre particles were more toxic than common microplastics (polyethylene) at similar size range and at this highest tested concentration.

2.5. Morphometry (experiment 2 P and 2 L) and particle uptake (experiment 2 P)

Individual daphnids were snapshot with a stereomicroscope (Olympus SZX16) and images processed through the ImageJ software to measure the body length (from the tail base to the head extremity in the eyes axis), body width (both extremities perpendicular to the length) and the tail (from the tail base to its extremity). Individuals that appeared damaged on microscopy snapshots after thawing were excluded from analysis and the likelihood of being damaged was considered independent from the concentration (n = 0, 1, 3, 2 for body length/width of experiment 2 P, n = 1, 1, 0, 0 additionally for tails of exp. 2 P, n = 0, 3, 4, 0 for body length/width/tail of experiment 2 L, respectively for concentrations of 0, 3.1, 12.5 and 50 mg.L^{-1}). No males were identified. Every individual was considered as female (criteria from [36]). The tyre particle uptake in individual digestive track was assessed using a semi-quantitative (likert) scale with 7 levels: "*None*": not any black dot observed; "*Very low*": ≤ 3 black dots or ≤ 1 black particle, not necessarily tyre particles; "*Low*": clear tyre particle uptake and > 3 black dots or black particles with an overall dispersion $< 10\%$ of the digestive track length; "*Intermediate*": black particles covering more than 1/10 but less than 1/3 of the digestive track length; "*High*": particles covering approximately 1/3 to 2/3 of the digestive track length; "*Very high*": particles covering more than 2/3 of the digestive track length, but not very stacked; "*Full*": particles covering more than 2/3 of the digestive track length and being very stacked with each other.

2.6. Particle characterization (shape, size distribution and counts)

Grain size, morphology, and surface characteristics of the cryo-ground particle mixture sieved through a 300 μm mesh were examined using a JEOL 6610 LV secondary electron microscope. The rubber particles' surfaces were coated with a layer of gold by sputtering using an Emitech K550 gold sputter coater. Particles were also characterised with a stereomicroscope (Olympus SZX16). Leftovers from 4 batches stock solutions of the tyre particle experiment 1 P and one batch stock solution newly prepared after the experiment were characterised for each of the 8 concentrations. One stock solution newly prepared after and as in the experiment 2 P was also characterised. Further dilutions were

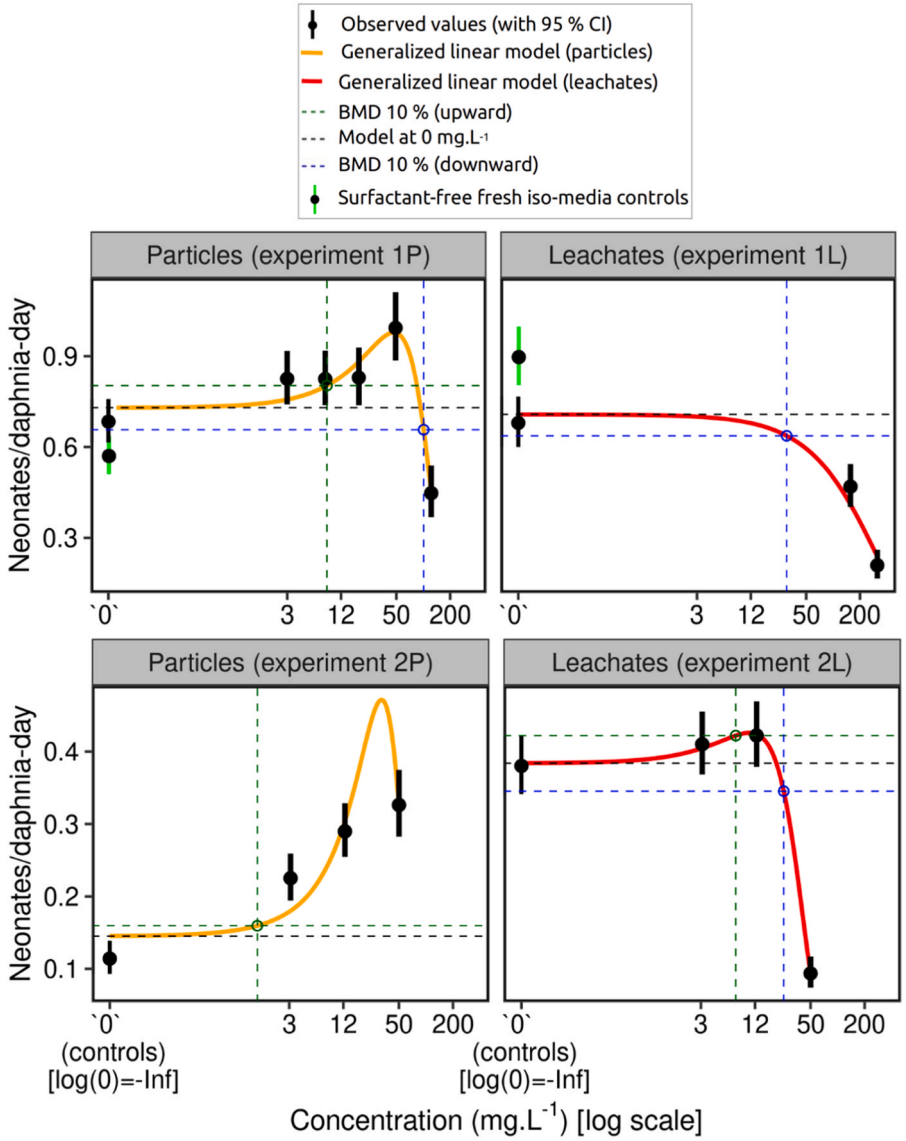


Fig. 5. *Daphnia* reproduction as a function of tyre concentration. Sample size per concentration at t = 0 were n = 30 in experiments 1 P and 1 L, and n = 65 – 70 in experiments 2 P and 2 L.

performed in falcon tubes as necessary to allow particle counting. A clear drop of 200 µL, using cut-off tips, was deposited on a well of a sterile 12-well plates, in duplicate for each concentration. The entire tyre particle content in the well was snapshot at the lowest magnification. Particles were then counted using the ImageJ software. Accounting for the dilutions and the controls background, the number of particles. L^{-1} was finally estimated (Table S3, S4). The particle sizes were measured from the snapshots explained above using ImageJ for uniplicates of three batches of experiment 1 P, when particles were big enough at this lowest magnification (so to cover the entire range of particle size distribution on the 200 µL). Particles too small to be measured at this magnification were assigned 10 µm (appearing as greyish dots) or 30 µm (appearing as black points) and confirmed to be in this size range at higher magnification. Data was background corrected (Fig. S2-S3). Medians were calculated on each of the two particle fractions (details in Table S1).

2.7. Chemical characterization of tyre leachates

2.7.1. Metals

Three leachate batches from experiment 1 L prepared at 10 g: 1 L were analysed. Each batch was screened for a total of 23 metals and averaged over 5 technical replicates per batch. Leachates produced were analysed for metal content, *via* ICP-MS analysis. Leachate samples were first acidified with 100 µL of 50% HNO_3 solution, to ensure sample stability and overall heterogeneity. A series of multi-elemental (Al, Ag, As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Mn, Mo, Pd, Pt, Pb, Rh Sb, Sn, Sr, Tl, Ti, V and Zn) calibration standards were prepared for quantification purposes (in a 5% HNO_3 solution), ensuring that the standard matrix matched that of the samples. To all samples and calibration standards, ^{115}In and ^{193}Ir were added as internal standards. Metal determination was performed using Thermo Scientific ICP-MS iCAP TQ complete with ASX-560 autosampler.

2.7.2. Organic chemicals

For analysis of organic chemicals, independent fresh leachate samples were prepared in triplicate in miniaturised similar conditions to the experiments. To that end, 50 mg of each tyre from the fraction previously dry-sieved between 300 and 600 µm sieves were added to 5 mL of MilliQ water. Samples were then alternately rotated at 13 rpm for 24 h using a Multi Bio RS-24 rotator (Biosan, Latvia), and incubated at 40 °C for 24 h in an Orbital incubator (Thermoline Scientific, Wetherill Park,

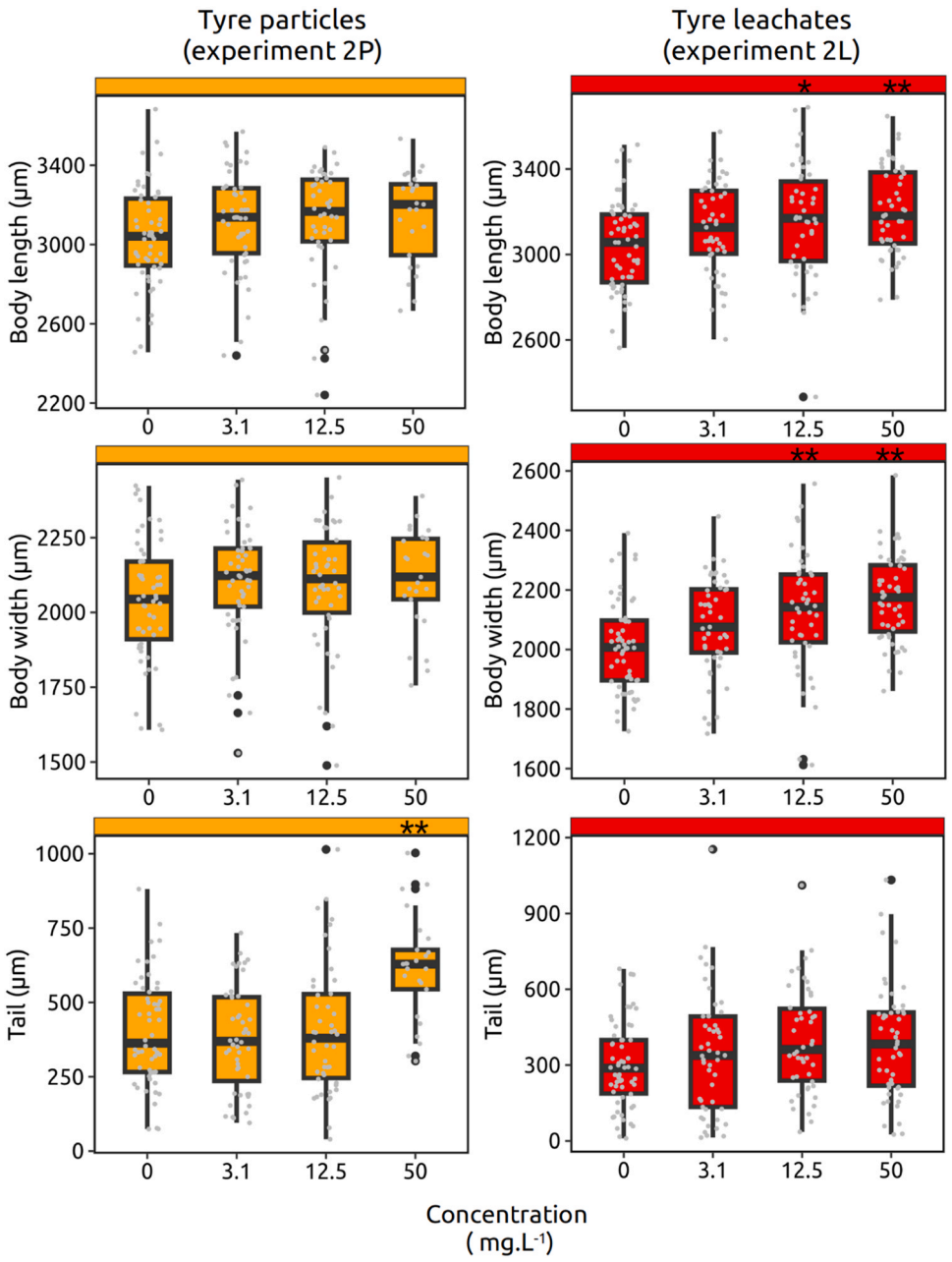


Fig. 6. Morphometry of adult daphnids at the end of chronic tests (21 d exposure). [Significance level of pairwise comparisons with controls: *$adj.p < 0.05$, * *$adj.p < 0.01$].

Australia), for a total of 7 d. After the seven day leaching process, samples were filtered through a 0.2 µm cellulose filter (Whatman ME 24/21 ST, Sigma Aldrich, Australia) to remove particles and a 1 mL aliquot of each solution transferred to a LC vial and spiked with 20 ng of d_6-5-methylbenzotriazole (d_6-5-MBTR) and d_5-atrazine as internal standards.

2.7.2.1. Target analysis. Samples were analysed for target additives (selected as per previous studies, *e.g.*, [39]) using liquid chromatography tandem mass spectrometry (LC-MS/MS; SCIEX Triple Quad™ 6500$^+$ from AB Sciex, Ontario, Canada), following previously reported methodologies [39]. Details of the 15 analytical standards of tyre additives are listed in Table S1a of Rauert et al. [39] with monitored MRM transitions and optimised MS/MS conditions listed in Table S3 of the previous manuscript [39].

2.7.2.2. Non-target analysis. For non-target and suspect screening, sample analysis was performed on a SCIEX high-performance liquid chromatograph coupled to an X500R Quadrupole Time-of-Flight (QToF) mass spectrometer (AB Sciex, Ontario, Canada). Chromatographic separation was achieved by a Kinetex EVO C18 100 Å analytical column (2.6 µm, 100 mm × 2.1 mm; Phenomenex, Lane Cove, Australia) fitted with a guard cartridge (SecurityGuard™, Phenomenex, Lancove, Australia). Mobile phases consisted of MilliQ water (A) and methanol (B), both acidified with 0.1% formic acid. The flow rate was set at 0.4 mL.min^{-1}, and 10 µL of the sample was injected onto the column. A linear gradient starting with 10% organic and gradually increasing to 99% organic over 20 min was used to elute the sample.

High-resolution mass spectrometry data were acquired in positive ionisation mode across 100 − 1100 *m/z* (MS1) and 50 − 1100 *m/z* (MS/MS) with SWATH data independent analysis mode. High-purity nitrogen was used as the nebulizer, curtain, and collision gasses. The parameters

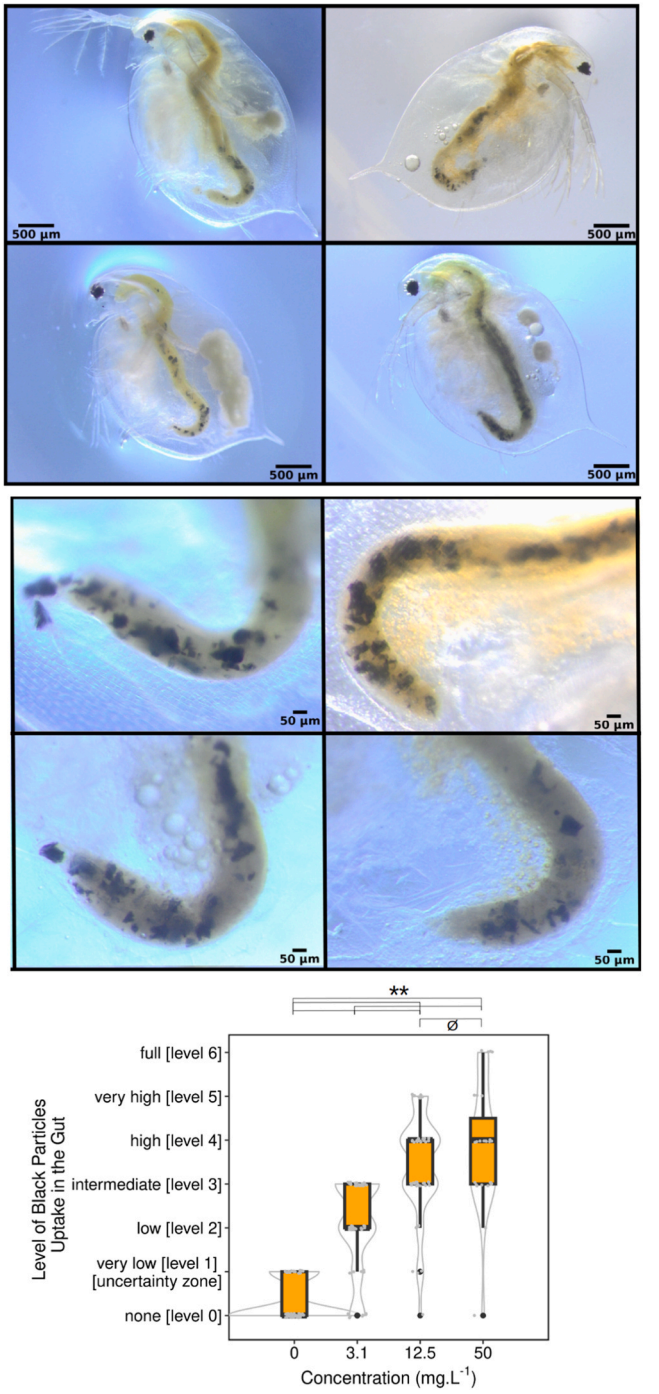


Fig. 7. Uptake of tyre particles by *Daphnia magna* (experiment 2A). For the semi-quantification of particle uptake, statistical analyses were done on ordered categories ("none" to "full") using proportional odd logistic regression models (pairwise comparisons significance levels: * = $p < 0.05$, ** = $p < 0.01$). For graphical representation (likert scale), each ordered category was assigned a numerical value (0 to 6) corresponding to its level of uptake.

of the WATH data analysis are listed in Table S29. The non-target data processing was conducted with SCIEX OS software version 2.2. The suspect screening was performed with an in-house suspect list using the SCIEX OS software. The prioritised suspects were manually confirmed with MS2 data. The methods for the quality assurance and quality control are presented in SI (Table S30).

2.8. Statistical analyses

2.8.1. Survival data

The dose-response survival at target time was analysed with the *Morse* package [6] in R by fitting log-logistic binomial models from which were extracted toxicological values (*e.g.*, $LC50_{21d}$ accounting for controls mortality) and confidence intervals. Post-predictive checks of each model were verified (Fig. S10-11). The time-course survival curves (Kaplan-Meier) were plotted using the *survival* [51] and *survminer* [26] packages in *R*. In the experiment 2 P and 2 L, the logrank test was used to detect a global difference in survival curves between concentrations. When a significant difference was detected ($p < 0.01$), pairwise comparisons of all possible combinations on the survival at day 21 were done by using Fisher's exact test for count data with Holm adjustment of *p-values* ('*fisher.multcomp*' function from the *RVAideMemoire* package [22]).

2.8.2. Reproduction data

Daphnia reproduction rate was calculated by summing the total number of neonates per concentration and then dividing by the number of individual-days (NID), as recommended in [11]. Only the period from days 7 to 21 were accounted in the NID, as they were immature to reproduce before. Concentrations for which the survival at 21 d was lower than 25% were excluded from reproduction modelling. The reproduction rate confidence intervals were calculated using the *pois.exact* function from the *epitools* package [4]. When intervals did not overlap between two concentrations, the difference was considered statistically significant. Reproduction dose-response models were fitted to each experiment. A generalized-linear-model (GLM) with Poisson error was used for each experiment, describing the logarithm of the NID as a linear function of the concentration for the experiment 1 L and as a quadratic function of the concentration for experiments 1 P, 2 P and 2 L. Note, the log(NID) was written as an "offset" (R-formula in Table S11). The benchmark doses (BMD-10%) were calculated from the fitted models as recommended by the European Food Safety Authority [12].

2.8.3. Morphometric trait

The morphometric endpoints (body length, body width, tail) were statistically assessed with simple linear models as a function of concentration taken as qualitative variable. When global model *p-value* was below 0.05, pairwise two-sided T-tests with pooled SD were performed on all possible concentration combination and *p-values* were adjusted with the Holm method. Since few linear models could be argued to have a slight deviation from their assumptions, complementary non-parametric Kruskal-Wallis (KW) tests were performed. When $p < 0.05$, they were followed by pairwise comparisons using Wilcoxon rank sum test with continuity correction and *p-values* adjustment with the Holm method.

2.8.4. Particle size distribution and particle uptake

In order to characterise better the tyre particles, their size distribution was fitted using the *fitdistrplus* package in R [10]. To determine if the particle uptake occurred in a dose-response manner, proportional odds logistic regression for ordered category outcomes (likert scale) were used as a function of concentration taken as a qualitative variable by using the *MASS* package in R [42]. Pairwise comparisons of all contrast combinations in the model were considered statistically significant when $p < 0.01$.

3. Results and discussion

3.1. Overview of results

Chronic toxicity was nearly 10-fold greater for pristine tyre particles than leachates experiments ($LC50_{21d}$ of 60 $mg.L^{-1}$ on average *vs* 542 $mg.L^{-1}$, see below). Toxicity was more complex when looking at

Table 1
Target inorganic chemical analyses of tyre leachates (ICP-MS).

Normalised concentrations per gram of tyre
(in the tyre mixture only, average of n = 3 samples, µg.g.L^{-1})

Metal	mixture	Hazard	metal	mixture	Hazard	metal	mixture	Hazard
Zn	176.58	**	Pb	0.013	**	Rh	< 0.001	*
Ti	11.15	*	V	0.007	**	Pd	< 0.001	***
Sr	10.926		As	0.006	**	Hg	< LOD	***
Al	0.352	**(*)	Sb	0.005		Ni	< LOD	***
Mn	0.260	**	Tl	0.005	***	Cu	< LOD	***
Fe	0.114	**	Cd	0.002	***	Sn	< LOD	**
Cr	0.018	**	Ag	0.002	***	Co	< LOD	**
Mo	0.013		Pt	< 0.001	***			

Limits of detection (µg.L^{-1}) of individual metals
Zn = 0.12 ; Ti= 0.17; Sr= 0.03; Al=3.21; Mn= 0.84; Fe=0.3; Cr=0.01; Mo=0.02;
Pb=0.0069; V=0.01; As=0.024; Sb=0.0046; Tl=0.01; Cd=0.0112; Ag=0.006; Pt=0.0006;
Rh=0.0001; Pd=0.0002; Hg=1;Ni=1.36;Cu=1.03; Sn=0.0125; Co=1.03

Zn = 0.12; Ti= 0.17; Sr= 0.03; Al= 3.21; Mn= 0.84; Fe= 0.3; Cr= 0.01; Mo= 0.02;
Pb= 0.0069; V= 0.01; As= 0.024; Sb= 0.0046; Tl= 0.01; Cd= 0.0112; Ag= 0.006; Pt= 0.0006;
Rh= 0.0001; Pd= 0.0002; Hg= 1;Ni= 1.36;Cu= 1.03; Sn= 0.0125; Co= 1.03
Note: Metals in leachates were quantified from an initial preparation at 10 g.L^{-1} of tyre particles and then divided by these 10 g to express concentration units as µg.g.L^{-1}. Only "Hazardous to the environment" pictograms are displayed (according to the European regulation for Chemicals). Asterisks correspond to levels of PNEC $_{water}$ (µg.L^{-1}): < 1 * ** , 1-50 * *, 51-100 * . Full list of pictograms and relevant toxicological values are presented in SI.

life-history traits. Both particles and leachates induced reprotoxicity and morphology alterations, but dose-inducing effects were contrasted. Tyre particles were visible in the digestive track of daphnids. Many chemicals quantified or detected in the leachates are known to be toxic/very toxic to aquatic life under global regulation classification and/or have $PNEC_{water}$ (predicted no effect concentration) below 100 µg.L^{-1}, which were derived using *D. magna* or other aquatic species. However, many lack toxicity information or could not be identified. There is an urgent need to characterise further the numerous additives in tyres (chemically and toxicologically). Mitigation measures could be *via* correct vehicle adjustment, driver behaviour and smarter tyre design (*i.e.*, safer-by-design).

3.2. Particle characterization

Particle length and width were compatible with a Gamma distribution (Fig. 2 and fits in Fig. S4-S7). The particle mixture dry-sieved through 300 µm mesh had an estimated length/width median of 57/40 µm (aspect ratio 1: 1.425), while the particle mixture retained between meshes of 300 and 600 µm had a median length/width of 89/56 µm (aspect ratio 1: 1.589). The particle morphology exhibited typical features of cryoground rubber particles with well-defined edges (Fig. 2B) (see *e.g.* Kreider et al. [29]). They were of irregular shapes, among which many were granular. The dispersion in water likely enhanced the detachment of adhered particles from each other that would not pass through the 300 µm mesh during the dry-sieving stage. After dispersion in water containing tween 80, 0.00025%, many of the smallest particles (size range 10–30 µm) detached from the larger particles (Fig. S2). Possibly, the rotation helped to break down the particles further. Particle masses were estimated to be of 0.37 µg.particle^{-1} on average (Fig. S9). As elements of comparison, two case-studies of pristine and worn tyre-wear particles were estimated at 0.51 µg.particle^{-1} and 0.29 µg.particle^{-1}, respectively [18].

3.3. Survival

The survival at 21 d was of 85% in "fresh" iso-media and surfactant controls [experiment 1 P, $n_i = 40$ each]; 70% in "fresh" iso-media controls and 77% in incubated iso-media controls [experiment 1 L, $n_i = 30$ each]; 80% in surfactant controls [experiment 2 P, $n_i = 69$]; 88% in incubated iso-media controls [experiment 2 L, $n_i = 66$] (Figs. 3 and 4). Note, in the survival models of experiments 1 P and 1 L, only the "surfactant controls" and "incubated isomedia controls" were included, respectively, as these corresponded to the diluent of every other concentration, contrary to the "surfactant-free fresh isomedia controls". Experiment calibration verification performed with zinc dichloride as referent toxicant resulted in an estimated $LC50_{21d}$ [95% CI] of 0.45 mg.L^{-1} [0.30; 0.65] (Fig. 3B and Fig. S12). This agrees with previously published studies [21,30].

The pristine tyre particle $LC50_{2d}$ is not presented because its estimation lies out of tested concentrations and a full sigmoidal curve was not yet achieved. At 21 d, a full sigmoidal curve was obtained in the

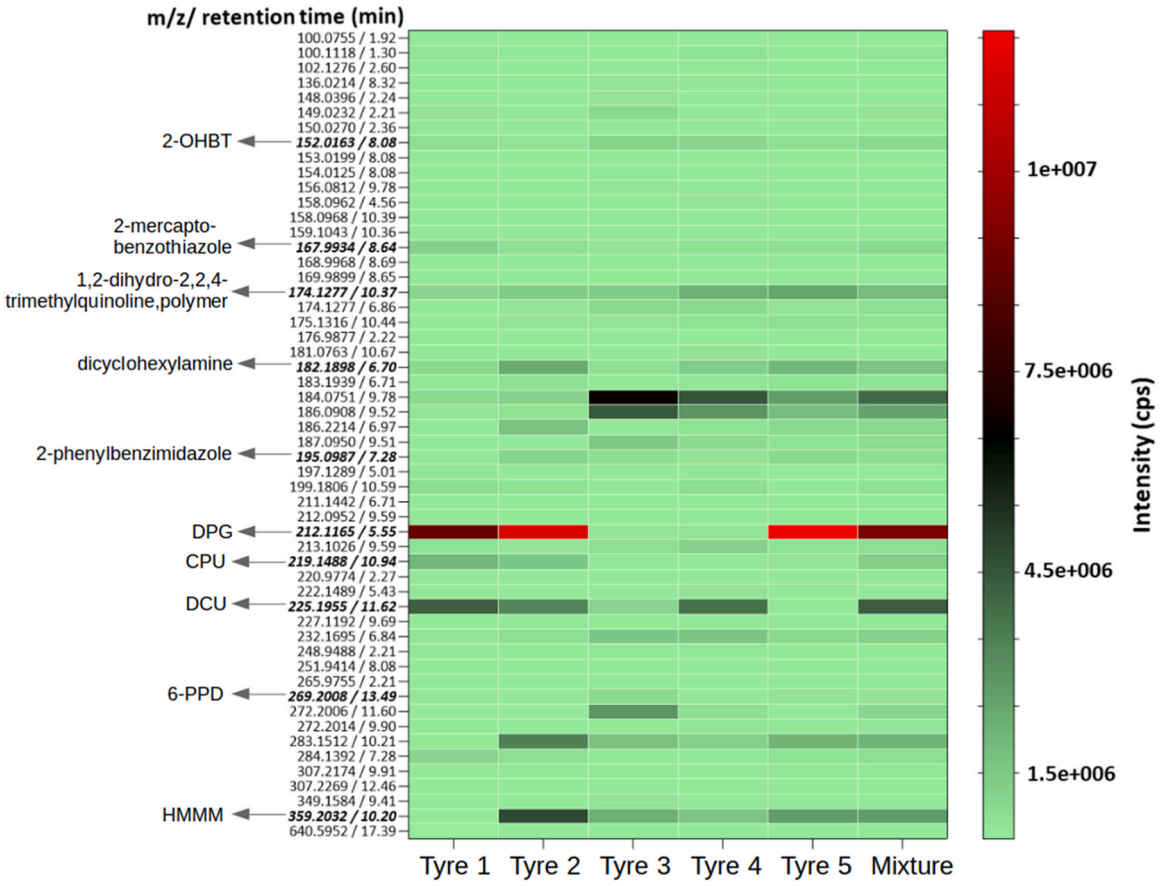


Fig. 8. Organic chemicals found in common in all tyre leachates and intensity across the samples (obtained by LC-QToF). Features identified are highlighted in black (bold/italic).

experiment 1 P and the corresponding LC50 $_{21d}$ [95% CI] was calculated to be 70 mg.L^{-1} [47; 112] (experiment 1 P, Fig. 3; see model toxicological values in Table S5). In the experiment 2 P, the 21 d survival at 50 mg.L^{-1} was 40% (Fig. 4). Accounting for mortality in controls, the log-logistic model of experiment 2 P calculated a LC50 $_{21d}$ of 49.5 mg.L^{-1} [33.2; 73.8] (Table S8 and Fig. S13). Accounting for both experiments, the most robust toxicological value we can provide on average is *D. magna* [tyre particle] LC50 $_{21d}$ = 60 mg.L^{-1}. Considering the equivalence estimation from measured particles (0.37 μg.particle^{-1}), this would correspond to an approximate number of 162 particle.mL^{-1}. As an element of comparison, pristine tyre-wear particles of larger size (210 μm on average) impacted the growth and reproduction of another freshwater zooplankton species *H. azteca* at 500 and 1000 particles.mL^{-1} for 21 d, respectively [27], but did not induce significant mortality at these concentrations. Hence the toxicity of the particles tested here was higher. This could be due to particle composition, and/or to the smaller size of tested particles and/or to a greater sensitivity of *D. magna*.

Considering only *D. magna*, tyre particles were of greater toxicity than common fossil-fuel microplastics. For example, an absence of chronic toxicity to *D. magna* was seen following a chronic exposure to polyamide microplastics up to 300 mg.L^{-1} [28] (size ranging below 180 μm). Maybe of closer size range (diameter 32–38 μm) were the additional controls of the present study, for which the PE microspheres at 50 mg.L^{-1} did not impact *D. magna* survival over 21 d (Fig. 4). This is also in line with another study using polyethylene microspheres for which 1 μm particles were acutely toxic to *D. magna* at this concentration range, whereas 100 μm were not [41], which emphasizes the importance of particle size in determining toxicity as recently demonstrated with tyre nanoparticles [9]. Our PE treatment was performed with spherical particles. It is known that particles shape is an important factor for their toxicity and that irregular shapes can clog the gut system of *D. magna* [44]. Therefore, the physical nature of tyre particles, being rubber of irregular shapes, may explain in part why they exerted greater toxicity than PE particles. It could be also because the tyre particles were pristine and so they may have leached many toxic chemicals.

Regarding tyre leachates (experiment 1 L), the LC50 $_{21d}$ [95% CI] was calculated at 542 mg: L [416; 613] (particle equivalence, Fig. 3B and Table S7). This is nearly 10-fold higher than for pristine particles (LC50 $_{21d}$ of 60 mg.L^{-1}), although the slightly bigger size of tyre particles in experiment 1 L may have reduced the overall leachate toxicity due to a lower surface contact (see Fig. 2A). With a smaller particle size, leachates would likely be more toxic and therefore the greater toxicity of pristine particles observed here – compared to leachates – could be less than by a factor of 10. At 21 d, the survival was 10% at 0.6 g.L^{-1} of leachate. At 9 d exposure, higher concentrations induced 100% mortality. From 0.3 g.L^{-1} of leachates downward, survival was near controls level for 21 d (Fig. 3B). Halsband et al. [20] observed approximately 50% mortality in the marine species *Calanus sp.* after 11 days of exposure to 5 g.L^{-1} of tyre particles leachates reaching 100% mortality after 17 days. The experiment 2 L of the present study (Fig. 4), however, showed that leachates did not impact daphnia survival at either of the environmentally relevant concentration of tyre leachates from 50 mg.L^{-1} downward. This also corroborates the fact that pristine particles were more toxic than their leachates only.

3.4. Reproduction rate

Stimulation of the reproduction rate was observed for daphnids exposed to tyre particles. This was consistent in both experiments 1 P

Table 2
Target organic chemical analyses of tyre leachates (LC-MS-MS).

Normalised concentrations per gram of tyre (in individual tyres and in the mixture, average of n = 3 samples, µg.g.L^{-1})								
	Tyre 1	Tyre 2	Tyre 3	Tyre 4	Tyre 5	Tyre mixture	Conc. range	Hazard
BTH	349	239	869	832	745	590	A	**
DPG	99	97	1.1	1.1	92	103	B	**
HMMM	0.34	70	29	17	42	42	C	*
DCU	26	20	2.9	23	0.33	26	C	?
2-OHBT	16	11	31	26	15	20	C	**
CPU	13	10	0.57	0.36	1.2	7.2	C	?
M-DCA	0.003	1.6	0.01	0.46	0.54	0.59	D	**
6PPDQ	< 0.008	< 0.008	0.34	0.01	< 0.008	0.41	D	***?
2-MTBT	0.08	0.27	0.08	0.14	0.1	0.3	D	? **
2-ABT	0.11	0.1	0.27	0.11	0.1	0.14	D	?
BTR	0.013	0.014	0.073	0.054	0.059	0.037	E	*
NCBA	0.023	0.028	0.074	0.029	0.001	0.033	E	?
24MoBT	< 0.001	0.008	0.011	0.012	0.007	0.009	E	?
C-DMU	< 0.008	< 0.008	0.013	0.015	0.014	< 0.008	F	?
5-MBTR	< 0.005	0.04	< 0.005	< 0.005	< 0.005	< 0.005	F	? *
D-DPU	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.001	F	**
Total	503.57	449.06	934.44	900.29	896.35	789.72		

Limits of detection (µg.L^{-1}) of individual organic chemicals								
BTH=0.1; DPG=0.66; HMMM=0.03; DCU=0.08; 2-OHBT=0.26; CPU=0.03; M-DCA=0.03; 6PPDQ=0.08; 2-MTBT=0.03; 2-ABT=0.03; BTR=0.04; NCBA=0.01; 24MoBT=0.01; C-DMU=0.08; 5-MBTR=0.05; D-DPU=0.01								

BTH= 0.1; DPG= 0.66; HMMM= 0.03; DCU= 0.08; 2-OHBT= 0.26; CPU= 0.03;
M-DCA= 0.03; 6PPDQ= 0.08; 2-MTBT= 0.03; 2-ABT= 0.03;
BTR= 0.04; NCBA= 0.01; 24MoBT= 0.01; C-DMU= 0.08; 5-MBTR= 0.05; D-DPU= 0.01
Note: Organic chemicals in leachates were quantified from an initial preparation at 10 g.L^{-1} of tyre particles and then divided by these 10 g to express concentration units as µg.g.L^{-1}. Bold & italic chemicals were also identified in the non-target method. Only "Hazardous to the environment" pictograms are displayed (according to the European regulation for Chemicals). Asterisks correspond to levels of PNEC $_{water}$ (µg.L^{-1}): < 1 * **, 1-50 * *, 51-100 * . Full list of pictograms and relevant toxicological values are presented in SI. Interrogation marks mean that insufficient toxicity information was found.

and 2 P (Fig. 5). The patterns were similar between these two experiments. They corresponded to a typical hormesis response. A dose-dependent increase occurred up to 32–47 mg.L^{-1} (concentrations giving the maximum values of the parabola of models 2 P and 1 P) and then the reproduction decreased up to 123 mg.L^{-1}. In experiment 1 P, the BMD 10% $_{21\ d}$ was estimated at 8 in the upward phase and at 101 mg.L^{-1} in the downward phase. In experiment 2 P, the BMD 10% $_{21\ d}$ (upward phase) was estimated at 1.3 mg.L^{-1}. Its BMD10% $_{21d}$ (downward phase) is not presented because it lies out of tested concentrations.

Tyre leachates mainly provoked a decrease in reproduction in the range of tested concentrations, being consistent in both experiments 1 L and 2 L. The lowest observed concentration of leachates for which this decrease occurred was 50 mg.L^{-1} (Fig. 5, exp. 2 L). In experiment 1 L and 2 L, the BMD 10% $_{21\ d}$ (downward phase) were calculated at 30 and 25 mg.L^{-1}, respectively. The BMD 50% $_{21\ d}$ (downward phase) can be calculated on those two experiments and are respectively at 199 and 39 mg.L^{-1}. The greater reprotoxicity observed in the experiment 2 L may be due to the smaller size distribution of the tyre particles, which were the same as the experiments 1 P and 2 P but not 1 L. Note that, to remain comparable to experiments 1 P and 2 P, we used a quadratic model for experiment 2 L which gave a good non-asymptotic fit to the data.

Overall, tyre particles exposure induced a contrasting response on reproduction to its leachate counterpart, a finding for which explanatory

hypotheses are not readily found. Nevertheless, experiment 1 L is not incompatible with a potential hormesis at concentrations lower than 157 mg.L^{-1}, but because this lower concentration range was not tested, the model is missing such experimental information. Interestingly, the additional controls with polyethylene microspheres (32–38 μm) at 50 mg.L^{-1} have also increased the reproduction rate (Fig. S19). An increase in reproduction is an ambivalent response as it can be the result of an exposure to healthy compounds (*e.g.*, some nutrients) or toxicants. In the case of tyre-tread particles, it is more likely a stress-response hormesis phenomenon [2,3,43,45]. In any case, pollution of this nature can be ecologically detrimental because emergent superabundant species can lead to chain reactions (*e.g.*, physiological trade-offs, community disequilibrium, etc) resulting in unhealthy ecosystems.

3.5. Morphometry

Tyre particles induced hypertrophia of daphnid tails (global model $p < 0.01$), but this occurred only at a concentration of 50 mg.L^{-1} (Fig. 6, *p.adj* < 0.01 for all pairwise combinations with this concentration). Regarding daphnia exposed to tyre particles, their body length and width showed an increasing trend along with concentrations (Fig. 6), however global model analyses were inconclusive ($p > 0.05$).

The leachates induced a more pronounced increase in body length (global model $p < 0.01$; *p.adj* of 0.048 and 0.001 for the pairwise comparisons of controls-12.5 and controls-50 mg.L^{-1}, respectively). The body width was also greatly increased as a result of leachate exposure (global model $p < 0.01$; *p.adj* of 0.002 and 6.1 $\times 10^{-06}$ for the pairwise comparisons of controls-12.5 mg.L^{-1} and controls-50 mg.L^{-1}, respectively). This occurred in a dose-response manner (*p.adj* of 0.03 for the pairwise comparison 3.1–50 mg.L^{-1}). No tail hypertrophia was observed for daphnids exposed to leachates (global model $p > 0.05$), but there was an increasing trend.

3.6. Tyre-tread particles uptake

A visible uptake of tyre particles occurred in daphnids' digestive track (Fig. 7). Particle uptake occurred in a dose-dependent manner up to 12.5 mg.L^{-1}, but was not significantly higher at 50 mg.L^{-1}. Particles of various sizes were observed in the range of few μm to around 100 μm. No particles were observed in tissues other than the digestive track. To our knowledge, the present study is the first to show dose-response semi-quantification of tyre particles uptaken in daphnia gut. Ingestion following acute exposure has previously been reported by [9].

3.7. Predicted no effect concentration (PNEC)

Based on the various results of the present study, we estimate the PNEC $_{freshwater}$ to be 137.5 μg.L^{-1} on average for mixture of tyre particles and their leachates (Table S10). Considering an estimated PEC $_{surface\ water}$ of tyre-wear particles of 120 μg.L^{-1} [50], tyre particles in the present study remain below the safety limits for the environment (risk quotient PEC/PNEC = 0.9 < 1). This value is close to the safety limit. However, as mentioned in the introduction, many environmental locations were quantified at much higher concentrations. Maximum PEC in surface water were reported to range from 0.03 to 56 mg.L^{-1} [57], whereas concentrations in run-off water average 30 mg.L^{-1} [50] and UK motorway ponds influents are in the range of 0.2–29.8 and 0.0–3.1 mg.L^{-1} at the outfalls [38]. It means that in many of these environmental locations, the risk quotient would be far above 1 and so far above the safety limits, considering the PNEC of 137.5 μg.L^{-1} from our study. Note, our PNEC $_{freshwater}$ calculation is much higher than another calculated PNEC $_{water}$ for microplastics in general of 0.33 μg.L^{-1} [7,50], for which the risk quotient would be above the safety limits even considering the lower PEC for surface water of 120 μg.L^{-1} (120/0.33, *i.e.*, PEC/PNEC > 1). This discrepancy may be due to the lack of multi-species comparison from our study, meaning the PNEC we provide could be an underestimation of real tyre toxicity.

3.8. Non-fully factorial design

Our "particle" treatments were done with pristine particles. Very likely, chemicals have leached during exposures. Therefore, these "particle" treatments must be interpreted as being the combination of particles mixture and some of their associated chemicals. The tyre leachates could only have induced chemical toxicity, whereas the pristine tyre particles treatment may have induced both, a physical and a chemical toxicity. This may explain why tyre particles were more lethal than their leachates. To have a fully factorial design, the study would need a "particle only" (additive free) treatment. We encourage future studies to tackle such an experimental design so to shed light on the relative contribution of particles *vs* leachates. Noteworthy, our "leachate" treatments were likely to have greater concentration of leached chemical additives than the "pristine particle" treatments. Indeed, they were subjected to 7 days of incubation, alternating temperatures (20 °C and 40 °C) and overhead rotations so to maximise the leaching process. This was not the case for the "pristine particle" treatments which, in all likelihood, contained less and fewer chemical additives.

3.9. Chemical characterization of tyre leachates

The predominant metal was zinc, which is in line with previous publications [18,20,8], followed by titanium and strontium (see Table 1). The predominance of zinc has been suggested to be a major driver of tyre leachates toxicity [61]. The picture is more complex for organic chemicals. In the non-target analysis (Fig. S34), the number of identified chemical features ranged from 2039 (Pirelli tyre) to 3424 (Nokian tyre). After applying the data processing filters (frequency, intensity and unicity), a total of 54 chemicals were all shared between the 5 tyres (heatmap shown in Fig. 8). In this shortlist of 54 chemicals, 10 were identified with a high degree of confidence, amongst which 6 were shared with the screening list used for the target analysis.

The most abundant molecule was 1,3-diphenylguanidine (DPG, red on heatmap, Fig. 8, *m/z* of 212.1165), which was also found in the target analysis (103 μg.g.L^{-1}, ranking 2nd, Table 2). DPG is a predominant tyre additive found in urban water system [63]. This vulcanization accelerator is toxic as shown for example by its estimated PNEC $_{freshwater}$ of 30 μg.L^{-1} (ECHA registration dossier, Oct. 2022, see Table S31). This PNEC for DPG would correspond to approximately 0.29 g.L^{-1} of rubber concentration equivalent for leachates in the experiment 1 L. Interestingly, this concentration corresponds to the safe zone of the survival curve (Fig. 3B). The tested concentrations in experiment 2 L were likely far below the PNEC for DPG. In the target list, the most abundant chemical was by far benzothiazole (BTH), at 590 μg.g.L^{-1} for the mixture (Table 2). This is also a common vulcanization accelerator and provides mechanical and abrasion resistance [64]. Its reported reproduction NOEC $_{21d}$ to *D. magna* is 1.5 mg.L^{-1} (ECHA registration website, Oct. 2022), from which a PNEC $_{freshwater}$ of 15 μg.L^{-1} was derived (Table S31).

The second most abundant organic molecule in the non-target analysis was DCU (*m/z* 225.1955, t = 11.62 min), ranking 4th in the target analysis (26 μg.g.L^{-1}). DCU is a potent inhibitor of epoxide hydrolase [33] and has been quantified at high concentrations in human serum [17]. Another chemical found at high concentrations in leachates was HMMM (*m/z* 359.2032/10.20), which ranked 3rd in the target analysis (42 μg.g.L^{-1}). This chemical is commonly used as a cross-linking agent and is widely found in waterways [40]. Recently, 15 transformation compounds from this parent molecule were quantified in the environment [25]. Its PNEC $_{surface\ water}$ is estimated at 54 μg.L^{-1}. The 5th and 6th most abundant target chemicals found were 2-OHBT and CPU (in the range 7–20 μg.g.L^{-1}). 2-OHBT was found to have LC50 $_{48\ h}$ and LC50 $_{7d}$ in *C. dubia* of 15.1 and 8.3 mg.L^{-1}, respectively [35], but no

ecotoxicity data was available for CPU. 2-OHBT is a by-product of the vulcanization accelerator 2-mercaptobenzothiazole [24] which was also identified in the non-target analysis (see below). The molecules DPG, HMMM, DCU, CPU and 2-OHBT were among the most abundant organic leachants in the present study, which is a pattern previously reported in stormwater [39].

A few other top-ranked chemicals were identified in the semi-quantitative non-target analysis, starting with 1,2-dihydro-2,2,4-trimethylquinoline polymer (*m/z* 174.1277/10.37) which is used in all types of rubber (tyre, shoes, toys) and has an estimated $PNEC_{freshwater}$ of 56 μg.L^{-1} [ECHA website, oct 2022]. Two very toxic molecules were identified, namely dicyclohexylamine (*m/z* 182.1898/6.70) [$PNEC_{freshwater}$ 1.6 μg.L^{-1}] and 2-mercaptobenzothiazole (*m/z* 167.9934/8.64) [$PNEC_{freshwater}$ of 4.1 μg.L^{-1}] (ECHA website, oct 2022). This latter molecule has anti-corrosive properties [60] and is used as a vulcanization accelerator. It has been quantified at high concentrations in human urine of occupationally exposed workers (up to 6210 μg.L^{-1} of urine) [14]. All other chemicals of the screening list were found at much lower concentration (decreasing order being M-DCA, 6-PPD-Q, 2-MTBT, BTR, NCBA and 24MoBT). Three suspected chemicals were found only at trace levels (C-DMU, D-DPU and 5-MBTR). Note, the toxicity of 6PPD-Q is species-specific as it was found to be acutely toxic to coho salmon at very low concentrations ($LC50_{24\ h}$ in the range 95–800 ng.L^{-1}) [52,53] but not to *D. magna*, whereas its parent 'anti-degradant' compound 6-PPD was found to be toxic for the latter [23].

3.10. Estimation of tyre-tread and chemical burden released in the environment

On average, over its product lifetime, a car passenger tyre loses 40 ± 10% of its rubber content through wear, corresponding to approximately 0.03 g.km^{-1} per tyre [5]. This means that a realistic average driver mileage of 7400 miles.$year^{-1}$ (11 909 km) [62] will release approximately 357 g.tyre.$year^{-1}$, so 1.43 kg of tyre-tread rubber per year considering a car with four tyres. This corresponds to 3.9 g.day^{-1}. In turns, this would correspond to a release of 1.13 g.$year^{-1}$ of potentially toxic organic chemicals and 285 mg.$year^{-1}$ of potentially toxic metals. Considering that an estimated 18% of tyre particles are reaching freshwater habitats [19,55,56], an average car passenger releases approximately 257 g.$year^{-1}$ of tyre-tread particles in freshwater ecosystems.

4. Conclusion

The current societal use of vehicles generates considerable amounts of tyre-tread particles in the environment. The present study provides critical evidence that tyre particles can release chemicals that are known to be toxic and many unknown or insufficiently characterised chemicals. Tyre particles can be extensively ingested by organisms at the base of the food chain, as observed in the present study. The highest environmental concentrations of these particles were demonstrated to induce massive mortality to *D. magna*. Tyre particle concentrations closer to environmental reality and their leachates were demonstrated to strongly impact the reproduction and morphology development of *D. magna*. In conclusion, tyre particles and associated leachates are hazardous pollutants of particular concern that in some locations may exceed the chronic safety limits for the environment.

CRediT authorship contribution statement

PB: Conceptualization, Experiments, Data analysis, Coordination, Writing – original draft preparation. **CR:** Organic chemistry, Data analysis, Funding acquisition, Writing. PD: Organic chemistry, Data analysis, Funding acquisition, Writing. **MLDM:** Data analysis, Writing. **RB:** Inorganic chemistry, Data analysis, Writing. **LD:** Inorganic chemistry, Data analysis, Writing. **FP:** Conceptualization, Coordination, Scanning Electron Microscopy, Writing; RT: Conceptualization, Coordination, Funding acquisition, Writing. **KT:** Organic chemistry, Funding acquisition, Writing. **TG:** Conceptualization, Coordination, Funding acquisition, Writing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.133580.

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