Nanoparticle-specific and Chemical-specific Effects of Tire Wear Particle Leachate on Amphibian Early Life Stages

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Highlights

- TWP leachate was separated into fractions containing particles, chemicals, or both.
- Results revealed nanoparticle- and chemical-specific effects of TWP leachate.
- Fractions containing leached chemicals had higher teratogenicity after 60 h.
- Locomotion was reduced after 9 days in fractions containing leached chemicals.
- Nanoparticle fraction significantly increased brain size after 9 days.

Nanoparticle-specific and Chemical-specific Effects of Tire Wear Particle Leachate on Amphibian Early Life Stages

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Abstract

Tire wear particles (TWP) have been identified as a potentially toxic form of plastic in the environment. The exact mechanisms of toxicity of TWP are poorly understood, especially the possibility of specific toxicity pathways due to nanoparticles and leached chemicals. The amphibian *Silurana tropicalis* was exposed to stock dispersions of different fractions of TWP leachate (nanoparticles and leached chemicals; leached chemicals; nanoparticles) during its early development for 60 h and 9 days, and endpoints such as mortality, malformations and behavior were recorded. In the 60-h exposure, individuals were exposed to treatments ranging from 0-100% of the stock dispersions. The nanoparticle fraction caused a significant decrease in larval survival at all concentrations. The proportion of malformed tadpoles and tail abnormalities were impacted by all fractions, but fractions containing leached chemicals exhibited more head, gut, and edema malformations. For the 9-day exposure, individuals were exposed to treatments ranging from 0-10% of the stock dispersions. At almost all concentrations, survival was significantly reduced. Tadpoles exposed to the nanoparticle fraction had significantly larger brains. Tadpoles in fractions containing leached chemicals swam significantly less in the 10% treatment. These results reveal particle-specific and chemical-specific effects of TWP leachate which may have negative repercussions at the population level.

Environmental Implications

The mechanisms of toxicity of tire wear particle leachate are currently poorly understood, especially in freshwater organisms. Our study sheds light on the differing toxicities of the nanoparticulate and dissolved chemical constituents of tire wear particle leachate on a highly relevant model amphibian, *Silurana tropicalis*. Leached tire wear chemicals, tire wear nanoparticles, and their mixture affected survival and physiologically relevant endpoints of the larval amphibian such as malformations, swimming behavior and brain morphometry, which can lead to adverse impacts at the population and ecosystem levels. The physiological impacts varied for the different tire wear leachate fractions, suggesting that the environmental impacts may vary with the weathering conditions and the dispersal patterns of dissolved and nanoparticulate species of tire wear leachate.

Keywords: Tire toxicity; Urban runoff; Plastic pollution; Aquatic vertebrates; Swimming behavior; Sustainability

1. Introduction

Concomitant with the rapid expansion of the automotive industry and the exponential increase in production of the modern radial tire since the 1950s [1], tire wear particles (TWP) have become a ubiquitous environmental contaminant that has been identified in the air, soil, and water [2, 3]. The generation of this contaminant is inevitable as TWP are formed from the abrasion of tire tread during the driving of automobiles [4, 5], with peak emissions typically occurring in urban areas adjacent to high traffic roadways [6]. One study has estimated that over 5.9 million tons of TWP are released into the environment annually [7] and these particles can vary in size from the micrometer range (1 μm to 5 mm) to the nanometer range (0.001 to 1 μm) [4].

The fate and toxicity of TWP in freshwater ecosystems are especially concerning, firstly due to the potential for accumulation of particles in this environmental compartment [8], and secondly, due to the leaching of chemical additives from the particles once in an aqueous environment [9, 10]. Tire tread contains a proprietary formulation of rubbers (natural or synthetic), softeners, vulcanization agents, antioxidants, plasticizers, fillers, *etc.* all in varying quantities depending on the brand, type, and model of tire [4, 11, 12]. In addition to the toxicity caused by the leached chemicals [13-15], cytotoxicity may also result from the interaction of the particles with cellular and biomolecular entities leading to a disruption of physiological processes [16].

Most ecotoxicological studies have focused on the overall effects of TWP leachate [10, 12, 17, 18], and only a few recent studies have begun to isolate the particulate component and leached chemicals component to understand their respective contributions to the overall toxicity. Among these recent studies, Cunningham *et al.* found that their nano-TWP dispersion (< 1 μm)

and leachate (< 0.02 µm) produced different teratogenic effects on zebrafish (*Danio rerio*) larvae suggesting different modes of toxic action [9]. A difference in the mechanism of toxicity between TWP dispersion and leachate was also suggested by Khan *et al.* after these authors observed distinct differences in the acute (48 h) mortality of the freshwater amphipod *Hyallela azteca* upon exposure to the two treatments [19]. Liu *et al.* reported that their leachate (< 0.45 µm) reduced the cell viability of the marine sediment-dwelling bacteria (*Bacillus subtilis*) compared to their TWP dispersion (6 – 130 µm) [20]. Finally, Halle *et al.* discovered that the relative toxicity of their leachate and TWP dispersion to *H. azteca* varied based on the age of the tire and the particle concentration [21]. Unfortunately, the lack of standardization in TWP leachate generation and nomenclature (i.e., TWP dispersion versus leachate), makes inter-study analyses difficult. Despite these recent efforts, it is still unclear whether the toxicity of TWP in freshwater ecosystems is mainly attributable to the tire wear chemical leachate, nanoparticulates or a combination of both.

This study aims to address these knowledge gaps by performing toxicity tests using the model amphibian, *Silurana (Xenopus) tropicalis*. *S. tropicalis* is a highly fecund, diploid organism capable of being hormonally induced to breed at any time of the year [22]. *S. tropicalis* produces relatively large eggs that develop *ex vivo* facilitating direct observation of its early developmental stages [23]. Exposure to TWP leachates is likely to occur during the embryonic to metamorphic stages of development for most amphibians, and throughout adulthood for fully aquatic species like *S. tropicalis*. The presence of amphibian populations in the ecosystem is often an indication of good environmental health, since anurans are highly susceptible to contaminants in their surroundings as a result of their permeable skin [24]. However, only a few studies have used amphibians to evaluate the toxicity of TWP leachates and all these studies

found that TWP leachate is toxic to amphibians during their early development [15, 17, 18, 25]. Among these studies, Gualtieri *et al.* began to distinguish the toxicity of TWP dispersions (50 and 100 g TWP/L stock concentrations) and organic extracts (0.05-0.12 g organic extract/L) to *Xenopus laevis*, but no conclusions were drawn regarding which component was mainly responsible for the toxic effects observed [25]. Furthermore, no amphibian study has focused solely on the nano-TWP portion of TWP leachate.

In this study, *S. tropicalis* was exposed to different fractions of a TWP mixture (i.e., leached chemicals, nanoparticles (< 0.2 µm), and a combination of both) in 60-h and 9-day toxicity tests, and endpoints such as survival, malformations, swimming behavior and brain morphometry were analyzed. The objectives of this study were: 1) to elucidate the relative toxicity of these fractions in freshwater ecosystems, 2) to investigate any synergistic or antagonistic effect on toxicity from a combination of the particle and leached chemical constituents, and 3) to investigate the connection between any behavioral effects caused by TWP mixture exposure and potential changes in brain morphometry.

2 Materials and Methods

2.1 Generation and Characterization of Tire Wear Particle Leachate Fractions

The generation of TWP leachate fractions was performed following procedures used in a previous study [26]. Briefly, TWP were generated by abrading the tread of five different end-of-life tires (obtained from an auto repair shop in Montreal, Quebec, Canada) using a hand drill equipped with a diamond drill bit (Big Horn Diamond Burr Set, model number: 19391) (Figure 1). Each tire was made by a different manufacturer. Equal masses of particles from each tire were mixed to represent the variability of tire particles present in the environment. This particle

mixture was subsequently used to generate a TWP dispersion. A dispersion of 40 g TWP/L of moderately hard reconstituted water (MHRW) [27] was stirred at room temperature for 7 days in the dark. The particle dispersion was then sequentially filtered using a 1 mm sieve, followed by a 1.5 µm glass microfiber filter (Whatman) and finally a 0.20 µm nitrocellulose mixed ester filter (Advantec) to obtain fraction 1 (F1) containing leached chemicals and nanoparticles (< 0.2 µm). A portion of F1 was filtered through a 20 kDa GE osmonics flat sheet membrane (Sterlitech) in an Amicon® stirred cell (Millipore Sigma). The filtrate from the 20 kDa membrane, i.e., fraction 2 (F2), was collected, and the retentate was resuspended to obtain fraction 3 (F3, nanoparticles < 0.2 µm). Based on the results of Nanoparticle Tracking Analysis (NTA) (LM14 instrument with 532 nm green laser, NanoSight Ltd.), F3 was diluted so that its particle number concentration was equivalent to that of F1. Most of the mass of TWP was removed by sequential filtration so that the actual concentration of TWP in the different fractions was markedly less than the initial 40 g/L TWP dispersion. In addition to an unfiltered water control (Control), an F1 procedural control (PC F1) and an F3 procedural control (PC F3) were prepared in which MHRW was sequentially filtered in the same manner as F1 and F3, respectively. Finally, to ensure that the media was suitable for amphibian exposures, salts were added to all treatments according to the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) protocol [28]. Fractions F1, F2 and F3 as prepared are referred to hereafter as stock dispersions.

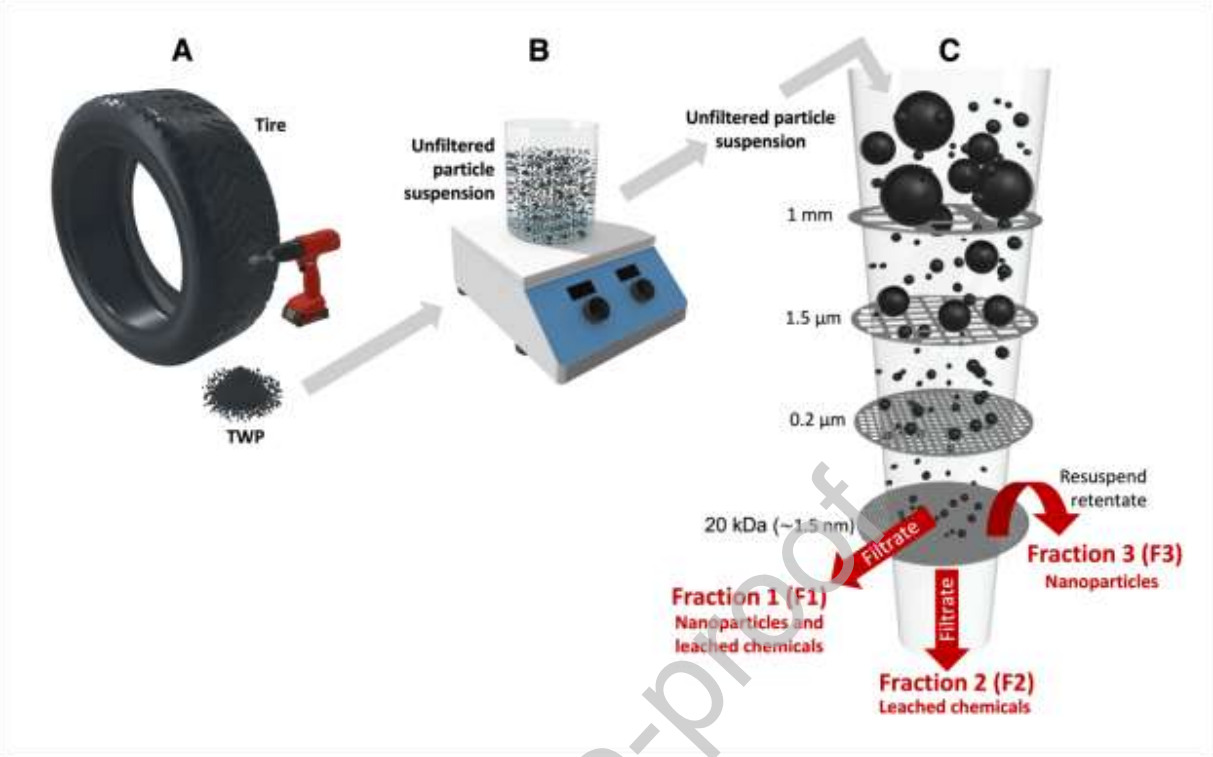

Figure 1. Procedure for generating the different tire wear particle leachate fractions. (A) Five end-of-life tires were abraded using a drill with a diamond drill bit. (B) 40 g TWP/L of moderately hard reconstituted water were stirred for one week at room temperature. (C) The TWP dispersion was sequentially filtered using filters/membranes of reducing pore sizes to obtain the three different fractions of the tire wear particle leachate. The media was converted to FETAX media prior to toxicity exposures.

Using dynamic light scattering (DLS, Zetasizer Ultra, Malvern Panalytical), the hydrodynamic diameter of particles in the stock dispersions was obtained. For DLS, styrene butadiene was selected as a proxy for rubber polymers present in the TWP leachate, and measurements were taken at room temperature.

For each treatment, five metal elements, ^{27}Al, ^{63}Cu, ^{66}Zn, ^{111}Cd, ^{208}Pb, were monitored using inductively coupled plasma – mass spectrometry (ICP-MS, Thermo iCAPQ c, quadrupole ICP-MS, Teledyne Cetac ASX-560 autosampler). Calibration standards (SCP Science Quality standard 4) between 20 ng/L and 1 mg/L were prepared in 2% nitric acid matrix. Linear relationship between counts and concentration for each metal element was achieved (R^2 > 0.9999). For measurement accuracy, quality control samples of predetermined concentrations were monitored at the middle and end of the ICP-MS sequence. To prepare samples for ICP-MS analysis, samples were first diluted with water (Optima™ LC/MS Grade, Fisher Chemical™), filtered through 0.22 μm filters (InnoSep™ SF13, 13 mm, CA, Syringe Filter) and acidified to 2-3% using concentrated nitric acid (70%, Thermo Scientific™).

Liquid chromatography-quadrupole time of flight mass spectrometry (LC-QTOF-MS) (Agilent 6545 LC/Q-TOF) was also performed to determine the concentration of organic chemicals. Fourteen organic chemicals were chosen as targeted analytes in the present study, and detailed information about these analytes is shown in Table S1. Bisphenol AF-$^{13}C_{12}$ (BPAF-$^{13}C_{12}$; purity ≥ 98%) and bisphenol S-$^{13}C_{12}$ (BPS-$^{13}C_{12}$; purity ≥ 98%) analytical standards were purchased from Toronto Research Chemicals (Toronto, Canada). Bis(2-ethylhexyl) phthalate-d_{38} (DEHP-d_{38}; purity ≥ 98%), diethyl phthalate-d_{14} (DEP-d_{14}; purity ≥ 99%), diallyl phthalate-d_4 (DAP-d_4; purity ≥ 99%), butyl benzyl phthalate-d_4 (BBzP-d_4; purity ≥ 99%), di-n-octyl phahalte-d_4 (DnOP-d_4; purity ≥ 99%), diisobutyl phthalate-d_4, (DiBP-d_4; purity ≥ 99%), diisodecyl phthalate-d_4 (DiDP-d_4; purity ≥ 99%), mono-n-butyl phahalte-d_4 (MBP-d_4; purity ≥ 99%), dicyclohexyl phthalate-d_4 (DcHP-d_4; purity ≥ 99%), and didecyl phthalate-d_4 (DDP-d_4; purity ≥ 99%) were purchased from CDN isotopes (Pointe-Claire, Canada). Bisphenol A-$^{13}C_{12}$ (BPA-$^{13}C_{12}$; purity ≥ 98%) was purchased from Cambridge Isotope Laboratories (Tewksbury, USA).

The isotope-labelled internal standard mixture solution (BPAF-$^{13}C_{12}$, BPS-$^{13}C_{12}$, BPA-$^{13}C_{12}$, BPF-$^{13}C_{12}$, DEP-d_{14}, DiBP-d_4, BBzP-d_4, DcHP-d_4, DEHP-d_{38}, DnOP-d_4, DAP-d_4, DiDP-d_4, MBP-d_4, DDP-d_4) was prepared at 1 μg mL^{-1} in methanol the day before use. For sample preparation, each water sample was filtered through a 0.22 μm PTFE filter (Fisher Scientific, Whitby, Canada), prepared in 25% methanol (water sample : methanol, 3:1 v/v) and stored in a glass vial. One milliliter of diluted water sample was transferred to a high-performance liquid chromatography (HPLC) glass vial, and 50 μL of internal standard mixture at 1 μg mL^{-1} was spiked into the water sample, followed by vortexing for 1 min. Detailed parameters for LC-QTOF-MS are provided in Table S2 and Table S3.

The morphology of raw particles generated from drilling (Figure 1A) was observed using a darkfield microscope (Olympus® BX43 microscope). Furthermore, enhanced darkfield hyperspectral imaging (CytoViva® hyperspectral microscope) was used to spatially map selected metals such as zinc, aluminum, and copper onto these raw TWP (Figure 1A). Spectral signatures of each metal were overlaid onto the corresponding TWP in ImageJ [29] so that all metals were displayed in one image. The presence of nanoparticles in the different fractions was investigated using transmission electron microscopy (TEM) (Talos F200X G2 TEM, Thermo Fisher Scientific). For TEM, grids were prepared by pipetting 2 μL of the TWP fraction dispersions on a copper 400-mesh coated with a thin carbon film (CF-400-CU) followed by water evaporation. The contrast of images was adjusted using ImageJ (version 1.53k) [29]. TEM images were not used to determine the particle size distribution since this method may have introduced drying artifacts and altered particle aggregation/dispersion [30].

2.2 Amphibian Husbandry

All experiments using the amphibian, *S. tropicalis*, were conducted at the Institut national de la recherche scientifique (INRS) (Quebec, Canada) (protocol #2201-02) in accordance with the guidelines of the Canadian Council on Animal Care (CCAC). To induce spawning, a healthy male and female adult frog were each injected with a priming dose of 12.5 international units (IU) of human chorionic gonadotropin hormone (hCG) (Sigma-Aldrich) in the dorsal lymph sac. About 20 h later, each frog was injected with a boosting dose of 200 IU of hCG. The male and female frogs were then paired and left in the dark to promote amplexus. The deposited eggs were de-jellied with 2% w/v L-cysteine solution (MP Biomedicals), and healthy, viable embryos were selected after two sorting phases [31]. All exposures were done in a climate controlled room at 27 ± 1 °C, 50% humidity with a 12 h day/12 h night cycle [28].

2.3 Acute Exposure Tests

The FETAX protocol [28] with slight modifications was used as a guide for conducting the amphibian exposures. The treatments used were control (0%), PC F1 (0%), PC F3 (0%), F1 (10, 25, 50, 75, and 100%), F2 (10, 25, 50, 75, and 100%) and F3 (10, 25, 50, 75, and 100%). Treatment levels were expressed as volume percentages, for example, 100% treatment represents the undiluted stock dispersion, whereas 75% treatment represents a 25% dilution of the stock dispersion with FETAX salt media. All treatments were done in quadruplicate (n = 4). The pH of all stock dispersions was adjusted to 7.6-7.9 using hydrochloric acid and sodium hydroxide, followed by aeration whereby air was pumped through the media through a glass Pasteur pipette. For the exposure, 15 embryos at Nieuwkoop-Faber (NF) stages 12-13 were haphazardly selected and placed in each experimental unit containing 30 mL of treatment media [32]. Following the FETAX protocol, all embryos were taken from a single mating pair to minimize genetic variability [28]. The placement of the experimental units was randomized daily according to the

results from a random number generator (randomizer.org). Dead individuals and debris were removed daily, and the media aerated manually with a glass Pasteur pipette. The acute exposure ended after 60 h when most of the tadpoles in the control group reached NF stage 46. The surviving tadpoles were euthanized by immersion in 0.2% w/v tricane methanesulfate (MS-222) solution (Sigma-Aldrich) and then fixed in 10% neutral buffered formalin (NBF) (Epredia). Survival and malformation data were obtained from the preserved tadpoles. Malformations were determined using the *Atlas of Abnormalities for Xenopus* [31], and a tadpole was counted as malformed if it had at least one malformation (head, gut, tail or edema). Water samples from time 0 h and 60 h of the acute exposure were analyzed using ICP-MS and LC-QTOF-MS.

2.4 The 9-Day Exposure Test

A longer 9-day exposure was also conducted to assess the toxicity of low concentrations of the TWP leachate on *S. tropicalis* during the embryonic to early pre-metamorphic stages of development. This exposure lasted for 9 days post-fertilization. The treatments used were control (0%), PC F1 (0%), PC F3 (0%), F1 (1 and 10%), F2 (1 and 10%) and F3 (1 and 10%) with n = 4 replicates. Fifteen embryos at NF stages 12-13 were randomly selected and placed in each experimental unit initially containing 30 mL of treatment media. All embryos were from a single mating pair. The total volume of treatment media in the experimental units was gradually increased from 30 mL (day 0) to 100 mL (day 3) and finally 300 mL (day 6) with fresh treatment media. On day 3, daily feeding of the tadpoles commenced. Tadpoles were fed 2 mg of powdered tadpole food (Ward's Science, VWR) twice a day. Dead tadpoles, food detritus and feces were removed daily. The placement of experimental units was randomized on days 0, 1, 2, 3 and 6. From day 3 onwards, the experimental units were aerated and covered to minimize evaporation.

At the end of the 9-day exposure, tadpole swimming behaviour was assessed with a startle response assay and a feeding assay. Swimming assays were developed according to the *Standard Guide for Behavioral Testing in Aquatic Toxicology* [33]. The startle response is a useful metric for assessing the avoidance behavior of animals to a simulated predator; the normal response for tadpoles is to dart away from a predator [34, 35]. The design of the startle response assay was based on that of previous behavioral studies [36-38]. Briefly, a tadpole was haphazardly selected from an experimental unit and transferred to a Petri dish (4 cm internal diameter) filled with 9 mL of its treatment media (0.7 cm liquid depth). Ten Petri dishes each with one tadpole were then placed on a light board and the tadpoles were left to acclimatize for 10 min, after which a rod was dropped from a height of 2.5 cm onto the center of the light board, exposing all animals to the same standardized vibrational force. The swimming behavior of the animals was recorded for 1 min using a video camera positioned above the animals (Figure S1). The tadpoles were then left to acclimatize for another 2 min, and the feeding assay was performed in which the animals' response to a food stimulus was tested. The tadpoles were not fed 24 h prior to the feeding assay. For this assay, 30 µL of Sera Micron tadpole food (5g/L) (Sera GmbH) was added to each Petri dish and the behaviour of the tadpoles was recorded for 1 min (Figure S2). The video analysis software, Kinovea (version 0.9.5), was used to track the swimming behavior of the tadpoles in each assay. From the video footage of both assays, the total swimming distance in 1 min after the stimulus was introduced was obtained. More details of the experimental set up are described in the Supplementary Information. Water samples from the 9-day exposure were analyzed using ICP-MS and LC-QTOF-MS as previously described.

After completion of the swimming assays, the surviving tadpoles were euthanized by immersion in 0.2% w/v MS-222, and their spines were severed as a secondary physical method

to confirm death. The animals were then fixed in 10% NBF. Preserved tadpoles were primarily imaged ventrally and laterally using a stereomicroscope (Olympus SZX16, Olympic Scientific Solutions), and these images were used to assess growth (head-to-tail body length, body width and tail length) and malformations. ImageJ was used to obtain growth measurements.

Next, X-Ray nano-computed tomography (CT) scans of haphazardly selected, similarly sized individuals from the control, 10% F1, F2 and F3 groups (n = 3) were taken using the Zeiss Xradia 520 Versa (Carl Zeiss Canada Limited). These tadpoles had no visible malformations. To prepare the samples for the CT scans, the fixed tadpoles were washed in deionized water, followed by 25, 50 and 70% ethanol, then stained with 1% phosphotungstic acid in 70% ethanol for about 2 weeks at 4 ℃. Immediately prior to imaging, the animals were washed in 70% ethanol and embedded in a 0.5% agarose gel. All scans were obtained at 3-4 μm resolution using a 4× objective lens with 2 × 2 camera binning over a 360 degree-rotation. Each scan consisted of 1600 projections and taken at 60 kVp and 82 μA (Table S4). The scans obtained were used to construct 3D images in the Dragonfly image analysis software (Object Research Systems Inc.), and linear measurements of the brain were taken (Figure S3 and Figure S4) according to the procedure described previously [39].

2.5 Statistical Analyses

Acute Exposure Data

R statistical software version 4.2.1 [40] and RStudio software [41] with agricolae [42] and MASS packages [43] were used to perform all statistical analyses. Normality was checked using the Shapiro-Wilk test, and a square-root transformation was applied to the response variables that did not meet the normality assumption. Comparisons between groups were done using two-way analysis of variance (ANOVA) with post-hoc Tukey HSD test with treatment and level as factors (Table S5). Differences were considered statistically significant at $p \leq 0.05$. For edema malformation data, a non-parametric Kruskal-Wallis test was applied when normality could not be met with data transformations. Concentration-response modelling and regression analyses were not often supported by the data; hence we used ANOVA's to provide consistent comparisons for the exposures and endpoints.

The 9-day Exposure Data

At exposure completion, survival and malformation data were analyzed using two-way ANOVAs as described previously. For all other endpoints such as morphometrics (i.e., growth and brain size) and swimming behavior, generalized linear mixed models (GLMM) were fitted to the data using the lme4 package [44] and lmerTest package [45] in R and RStudio. This kind of modeling was selected as it incorporates both random effects of the experimental units (tadpoles in the same jar are not independent from each other) and additional biologically relevant fixed effects besides treatment and level like tadpole density and body length which significantly affected growth morphometrics and swimming behavior, respectively [46, 47]. Data exploration and GLMM validation procedures were done following a previous study ensuring that the assumptions of homoscedasticity and homogeneity were met by each model [48]. The procedure

for selecting the best GLMM for each endpoint is described in the Supplementary Information and in Tables S6 and S7. Differences were considered statistically significant at $p < 0.05$.

For all boxplots, the thick horizontal bars represent the median values, and the upper and lower hinges of the boxplot represent the 75^{th} and 25^{th} percentiles, respectively. The upper whisker (vertical line) of the boxplot is 1.5 times the inter-quartile range (IQR), and the lower whisker is 1.5*IQR. Dots represent the average value of the response variable per replicate. The diamond (◇) represents the average of the four replicates per treatment level.

3. Results

3.1 Tire Wear Particle Leachate Characterization

The particle concentration of F1 stock dispersion was $3.5 \times 10^8 \pm 1.6 \times 10^7$ particles/mL from NTA analyses, and the concentrated F3 retentate was diluted to match this particle number concentration so that these two fractions had comparable particle numbers. Using NTA, no particles were detected in the controls nor in F2. From the NTA size distributions, the average diameter of nanoparticles in F1 was 275 ± 14 nm, and that of F3 was 139 ± 1 nm (Table S8). From DLS size distributions based on intensity, the average hydrodynamic diameter of the nanoparticles in F1 was 252 ± 12 nm, and that of F3 was 135 ± 11 nm for the most prominent peak (Figure 2A). The presence of peak 2 in the DLS size distribution of F3 (Figure 2B) was indicative of either micrometer-sized aggregates or other large particles present in the media. The polydispersity index (PDI) of F1 was 0.16 ± 0.03, and F3 was more polydisperse with a PDI of 0.42 ± 0.01.

The morphology of the TWP generated from drilling was examined (Figure S6), and the particles were found to be amorphous with a rough surface texture resembling TWP previously

generated from mechanical abrasion processes [49, 50]. The imaging also confirmed that the method of abrasion used in this study produced particles that have similar morphologies to real-world environmental tire and road wear particles [51]. TEM images of F1 and F3 confirmed the presence of nanoparticles in the leachate (Figure 2C-D and Figure S7). Using the NTA data for particle concentration and average particle hydrodynamic diameter, the mass concentrations of F1 and F3 stock dispersions were calculated to be 4.61 mg/L and 0.60 mg/L, respectively, assuming that the particles were spherical and had the density of rubber (1.2 g/cm^3) [52].

Enhanced darkfield hyperspectral microscopy (CytoViva) was used as a rapid, qualitative method to chemically map metals on the surface of TWP. Selected metals such as zinc, aluminum, and copper were identified on the surface of the TWP (Figure 2E). Previous studies that used scanning electron microscopy - energy dispersive X-ray spectroscopy (SEM/EDX) also confirmed the presence of zinc, aluminum, and copper on tire tread and TWP [11, 53].

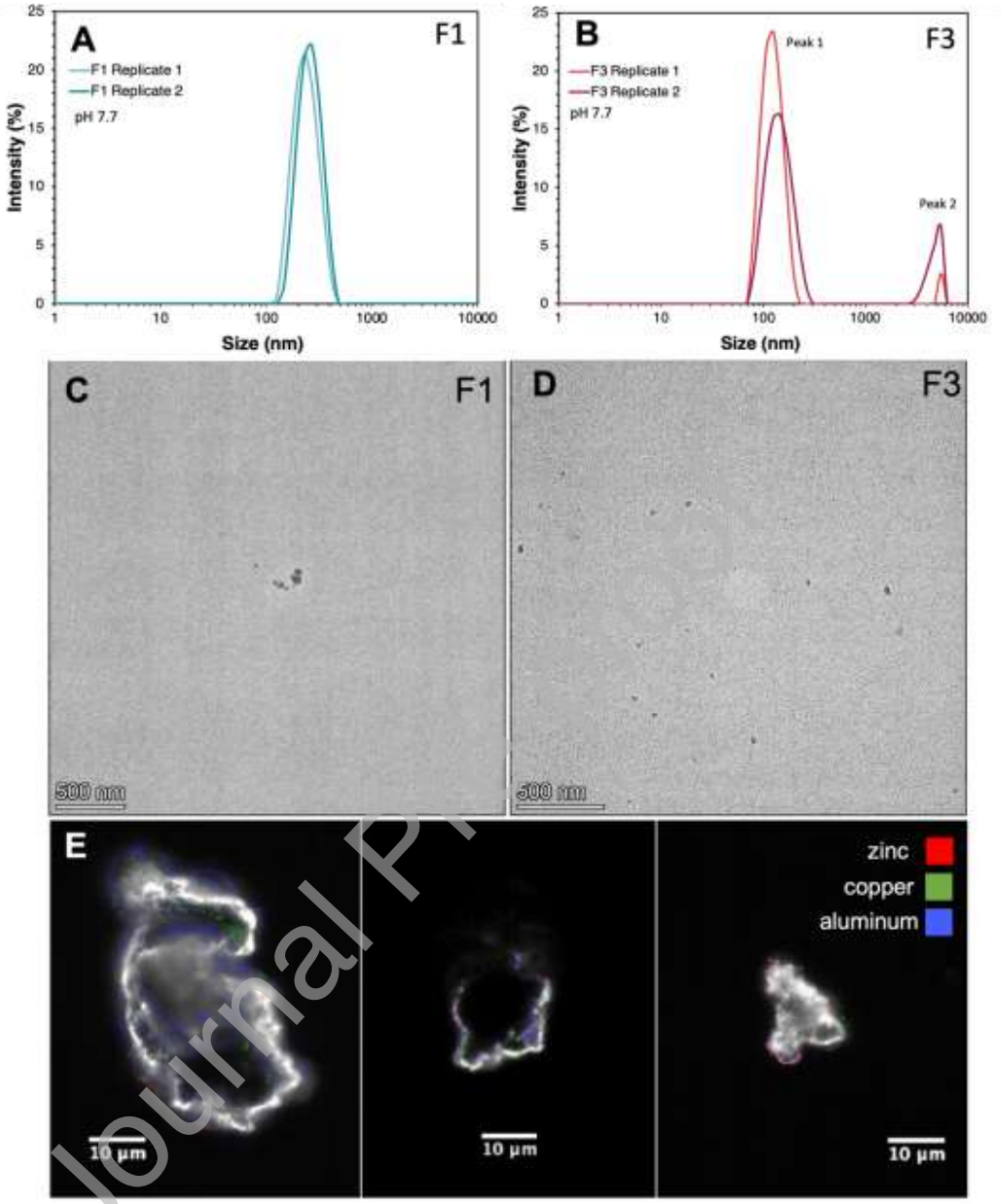

Figure 2. Particle characterization of the exposure fractions F1 and F3. (A) Hydrodynamic size distribution of particles in F1. (B) Hydrodynamic size distribution of particles in F3. (C) TEM image of nanoparticles in F1. (D) TEM images of nanoparticles in F3. (E) CytoViva® Enhanced Darkfield Hyperspectral Images showing spatially mapped metals on tire wear

particles. Metals such as zinc, copper, aluminum were identified on the surface of tire wear particles.

ICP-MS analysis was used to quantify the metal concentrations in the 100% exposure treatments and controls, and the results are shown in Table 1. Overall, zinc was the most prominent heavy metal detected at concentrations of 3.04 mg/L and 2.21 mg/L in F1 and F2, respectively, and in low amounts in F3 (0.151 mg/L). The same trends in zinc concentration were observed at the 10% treatment level used in the 9-day exposure (Figure S8). Aluminum, copper, cadmium, and lead were detected in low amounts in F1 and F2 compared to the controls.

LC-QTOF-MS was utilized to quantify targeted organic compounds in the 100% exposure treatments and controls, and the results are shown in Table 1. Of the targeted compounds, benzothiazole, a vulcanization accelerator [54], was the most abundant organic compound detected at concentrations of 1.15 mg/L and 1.01 mg/L in F1 and F2, respectively. Benzothiazole is often cited in literature as one of the key components of leachates derived from TWP [14, 54]. Hexa(methoxymethyl) melamine (HMMM), another vulcanization agent [55, 56], was the second most concentrated chemical detected at concentrations of 1.035 mg/L and 0.865 mg/L in F1 and F2, respectively, and in low quantities in F3. Mercaptobenzothiazole, a vulcanization accelerator [57], was detected in low quantities for F1 and F2, but was not detected in F3. Other targeted chemicals such as N-phenyl-N'-(1,3-dimethylbutyl)-p-phenylenediamine quinone (6-PPD quinone), dibutyl phthalate, diphenyl phthalate, diheptyl phthalate, dihexyl phthalate, di(2-ethylhexyl) phthalate (DEHP), decyl octyl phthalate, bisphenol A, Irganox 1081, Irganox 1330 and Irganox 1010 were not detected in any treatments. The results of these chemical characterizations demonstrate that most of the leached chemicals were successfully

removed from F3, and that F1 and F2 retained most of the leached chemicals. The concentration of organic chemicals at 0 h and 60 h of acute and 9-day exposures is recorded in Table S9.

Table 1 Concentration of metals and organic compounds (mean ± standard error, n = 4) in controls and 100% F1, F2 and F3.

		Control	PC F1	PC F3	F1	F2	F3
Metal	Aluminum (µg/L)	38 ± 2	26 ± 0.4	28 ± 2	72 ± 2	72 ± 3	27 ± 2
	Copper (µg/L)	33 ± 0.5	34 ± 0.9	35 ± 0.3	111 ± 3	101 ± 2	30 ± 1
	Zinc (µg/L)	24 ± 1	22 ± 0.2	36 ± 2	3035 ± 673	2208 ± 49	151 ± 14
	Cadmium (µg/L)	31 ± 13	24 ± 4	28 ± 2	86 ± 4	68 ± 4	13 ± 0.5
	Lead (µg/L)	8 ± 0.7	7 ± 0.1	8 ± 0.5	24 ± 0.1	24 ± 0.1	5 ± 0.02
Organic Compound	Benzothiazole (µg/L)	n.d.	n.d.	n.d.	1153 ± 20	1007 ± 45	n.d.
	Mercaptobenzothiazole (µg/L)	n.d.	n.d.	n.d.	7 ± 2	12 ± 0.3	n.d.
	Hexa(methoxymethyl) melamine (µg/L)	n.d.	n.d.	n.d.	1035 ± 37	865 ± 215	13 ± 3
	6-PPD quinone (µg/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Dibutyl phthalate (µg/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Dipentyl phthalate (µg/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Diheptyl phthalate (µg/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Dihexyl phthalate (µg/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Di(2-ethylhexyl) phthalate (µg/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Decyl octyl phthalate (µg/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Bisphenol A (µg/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Irganox 1081 (µg/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Irganox 1330 (µg/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Irganox 1010 (µg/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

F1: Fractions 1; F2: Fraction 2; F3: Fraction 3; n. d.: not detected; PC F1: Procedural control for fraction 1; PC F3: Procedural Control for fraction 3; 6-PPD quinone: N-phenyl-N'-(1,3-dimethylbutyl)-p-phenylenediamine quinone.

3.2 Acute Exposure

No dose-response effect on the survival of *S. tropicalis* was observed for any treatment. Most F1 and F2 treatments did not have a statistically significant effect on survival compared to the control (ANOVA, $p > 0.05$; Figure 3A; Table S5), and the mean survival rate was as high as 72% and 73% for F1 and F2, respectively. However, at all concentrations of F3, the survival rate was significantly lower than the control (ANOVA, $p < 0.05$; Figure 3A; Table S5), with the lowest mean survival rate at 47%.

For the proportion of malformed tadpoles, a roughly proportional increase with increasing concentrations of each treatment was observed, with the greatest number of malformed tadpoles found at the 100% treatment level (Figure 3B). When investigating the frequency of the different types of malformations, the proportion of tadpoles with gut abnormalities was significantly higher in the leached chemical-containing fractions compared to the control (i.e., 75 - 100% F1 and 50 - 100% F2) (ANOVA, $p < 0.05$; Figure 3C; Table S5). Tadpoles in F3, did not show significant gut malformations when compared to the control (ANOVA, $p > 0.05$; Figure 3C; Table S5). Head malformations were significant at 100% F1 and 25% - 100% F2 (ANOVA, $p < 0.05$; Figure 3E; Table S5), but not for F3 (ANOVA, $p > 0.05$; Figure 3E; Table S5). Similar trends were observed for the proportion of edematous tadpoles, in which F1 and F2 tadpoles exhibited higher average rates of edema compared to F3, though none of the treatments were significantly different from the control according to the results of the Kruskal Wallis test (Kruskal Wallis, $p > 0.05$; Figure 3D). At almost all treatment levels for F1, F2 and F3, tail malformations were significantly higher than the control (ANOVA, $p < 0.05$; Figure 3F; Table S5).

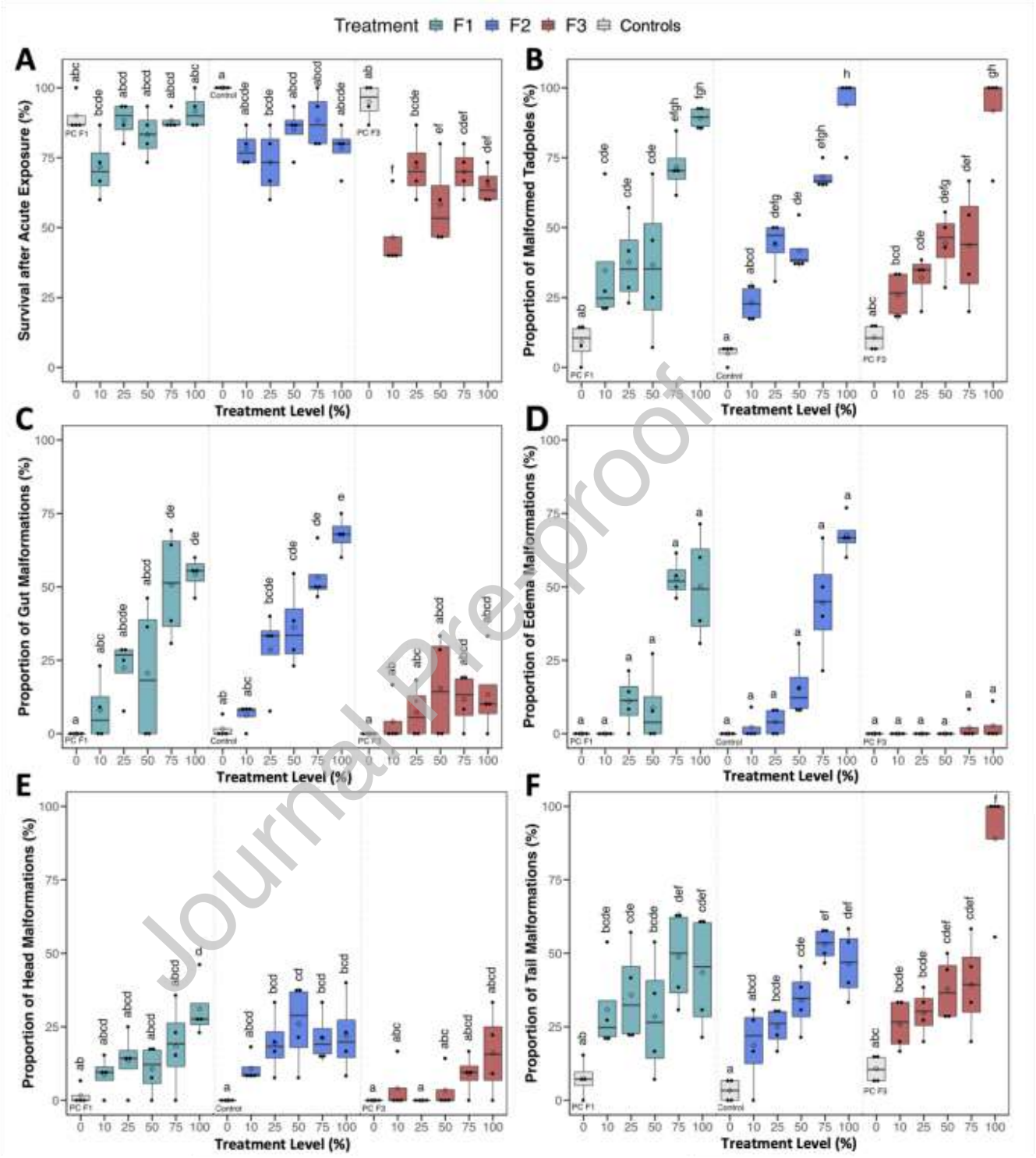

Figure 3. Treatment comparisons of *S. tropicalis* percent survival and malformations after 60-h acute exposure to tire wear particle leachate fractions. Boxplot of proportion of (A)

surviving tadpoles; (B) surviving tadpoles that were malformed; (C) surviving tadpoles with gut malformations; (D) surviving tadpoles with edema; (E) surviving tadpoles with head malformations; and (F) surviving tadpoles with tail malformations. Treatments were control (0%), PC F1 (0%), PC F3 (0%), F1 (10, 25, 50, 75, and 100%), F2 (10, 25, 50, 75, and 100%) and F3 (10, 25, 50, 75, and 100%). For (A), (B), (C), (E) and (F) letters correspond to significant differences ($p < 0.05$) according to two-way ANOVA with treatment (F1/F2/F3) and level (0-100%) as factors. For better visualization of trends, boxplots are grouped according to treatment. Boxplots with the same letter are not considered significantly different ($p > 0.05$) by Tukey-HSD test. For (D) no significant differences were found using the Kruskal-Wallis test.

3.3 The 9-Day Exposure

Compared to the control, almost all treatments significantly decreased the rate of survival of *S. tropicalis* after a 9-day exposure period (ANOVA, $p < 0.05$; Figure 4A; Table S5). Survival rates were comparable between 1% and 10% treatments levels for all fractions (ANOVA, $p > 0.05$; Figure 4A; Table S5), and at each respective treatment level there was no significant difference among the fractions (ANOVA, $p > 0.05$; Figure 4A; Table S5), though the mean survival rate dropped to approximately 50% in F2 and F3, and to 62% in F1.

For the proportion of malformed tadpoles, there was no significant effect of any treatment compared to the control, likely due to the death of highly malformed animals (ANOVA, $p > 0.05$; Figure 4B; Table S5). Categories of malformations (i.e., head, gut, tail and edema) are shown in Figure S9.

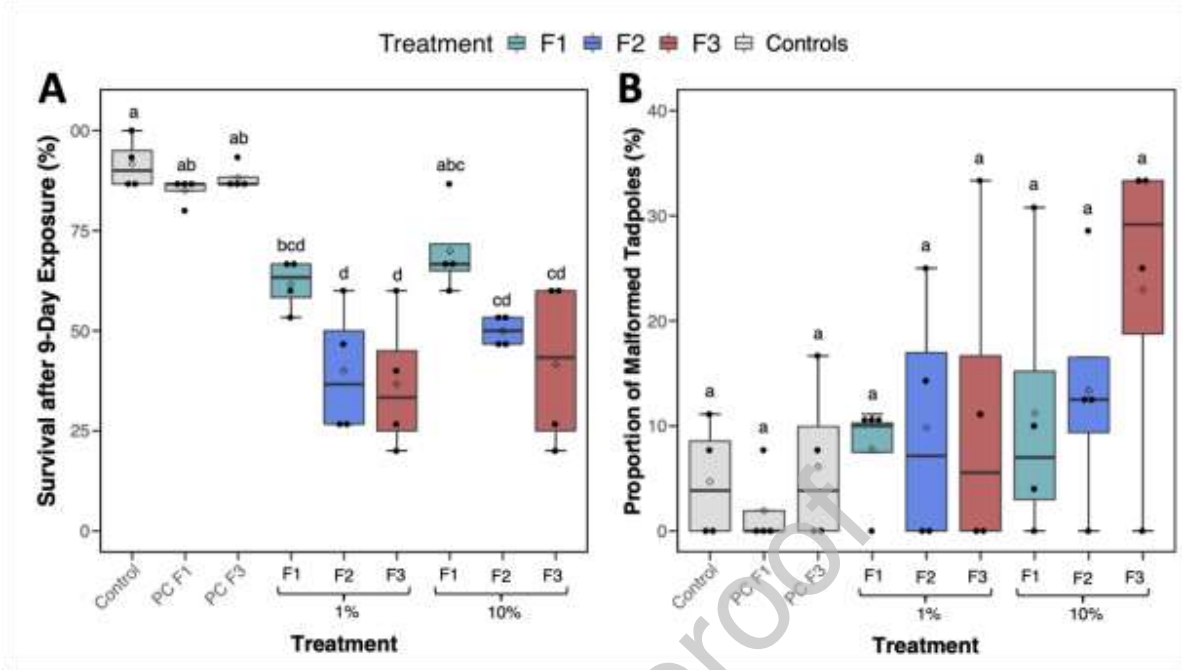

Figure 4. Treatment comparisons of *S. tropicalis* percent survival and malformations after 9-day exposure to tire wear particle leachate fractions. Boxplots of (A) percentage of surviving tadpoles; and (B) proportion of surviving tadpoles that were malformed. Treatments were control (0%), PC F1 (0%), PC F3 (0%), F1 (1 and 10%), F2 (1 and 10%) and F3 (1 and 10%). Letters correspond to significant differences ($p < 0.05$) according to two-way ANOVA with treatment (F1/F2/F3) and level (0 - 10%) as factors. Boxplots with the same letter are not considered significantly different ($p > 0.05$) by Tukey-HSD test.

Regarding morphometry, 9-day exposure to all fractions of TWP leachate did not significantly affect the body length of the tadpoles when compared to the controls (GLMM, $p > 0.05$; Figure 5A; Table S7). However, the density of tadpoles in the experimental unit did significantly affect the growth rate in an inverse manner – the lower the tadpole density in the experimental unit, the higher the growth rate (GLMM, $p < 0.05$; Table S7). The relationship between tadpole density and growth rate has previously been reported [58]. Similar results were

obtained for other growth morphometrics such as tail length and body width (Figure S10A-B). Measurements were obtained by one individual, and the mean coefficient of variance for 20 repeated morphological measurements for five different tadpoles was ≤ 0.01 for the tail length, body length and body width measurements [59].

For brain morphometry (Figure S3 and Figure S4), exposure to 10% F3 significantly increased the size of the telencephalon width (Figure 5B), diencephalon width (Figure S10C), optic tectum width (Figure S10D), and medulla width (Figure S10E) (GLMM, $p < 0.05$; Table S7). These results indicate that the brains were significantly larger for tadpoles that were exposed to F3. Similarly, measurements were obtained by one individual, and the mean coefficient of variance for 20 repeated morphological measurements for five different tadpoles was ≤ 0.01 for all brain width measurements.

Regarding swimming behavior during the startle response assay, at the 10% treatment level tadpoles exposed to F1 and F2 swam significantly less than those exposed to the controls, but there was no significant difference in swimming between tadpoles in F3 and those in the controls (GLMM, $p < 0.05$; Figure 5C; Table S7). However, the trend was different at the 1% treatment level in which F3 was the only treatment that was significantly different from the controls (GLMM, $p < 0.05$; Figure 4C; Table S7). For the total distance travelled while feeding, only 10% F2 was significantly different from the control, as these tadpoles exhibited lower feeding activity (Figure 5D).

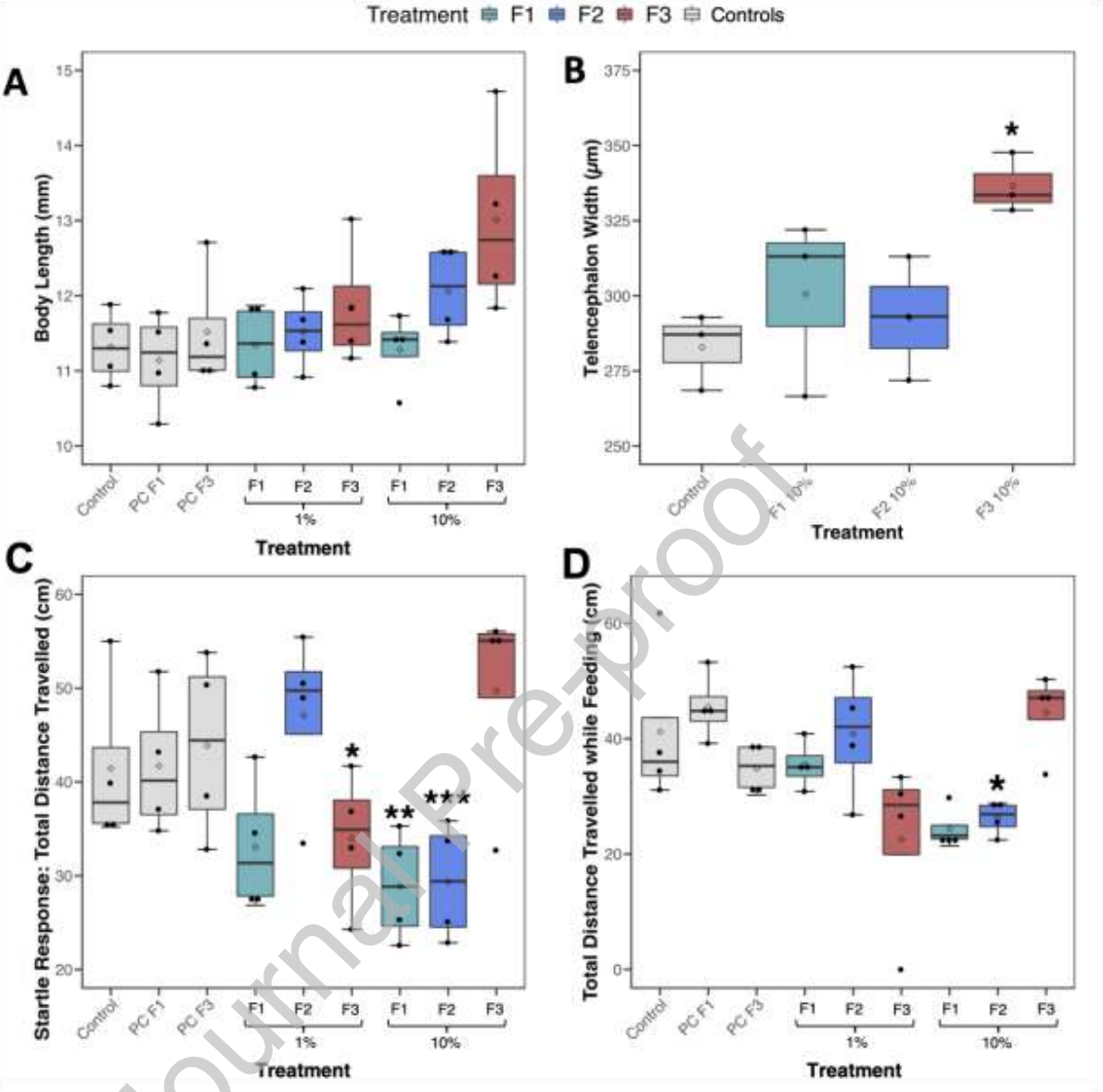

Figure 5. Morphometrics and locomotory behavior of *S. tropicalis* tadpoles after 9-day exposure to tire wear particle leachate fractions. Boxplots of (A) total body length; (B) telencephalon width; (C) total distance travelled during the startle response assay; and (D) total distance travelled while feeding. Statistical analyses were conducted using generalized linear

mixed models (GLMM). Significance codes correspond to p values as follows: *** $0 \leq p \leq 0.001$; ** $0.001 < p \leq 0.01$; * $0.01 < p \leq 0.05$ when compared to the control.

4. Discussion

4.1 Tire Wear Particle Stability

Both DLS and NTA results confirmed that mean particle sizes were larger in F1 than in F3. The greater extent of particle aggregation in F1 was likely caused by a more extensive metal-polymer complexation related to the higher concentration of multivalent metal cations and organic compounds present in that fraction [60, 61]. Chemical characterization results confirmed elevated concentrations of the heavy metal zinc and organic compounds like benzothiazole and HMMM in F1 compared to F3. Several other studies have reported similar findings whereby zinc was the most concentrated heavy metal in TWP leachate [21, 62, 63]. Zinc is used as vulcanization agent in the tire manufacturing process [11]. Low amounts of aluminum, copper, cadmium, and lead were also detected in F1 and F2, and these metals, among others, have also been previously identified in TWP [10, 64]. Due to the lack of covalent bonding between many of these chemical constituents and the tire rubber polymer, the continuous leaching of chemicals from TWPs is likely to occur throughout exposure [10]. Another possible reason for the smaller particle sizes in F3 is that some of the larger nanoparticles may have remained embedded in the 20 kDa membrane during resuspension of the retentate (Figure 1C).

4.2 Impact on Survival

Overall, exposure to F3 caused a significant decrease in the survival of *S. tropicalis* tadpoles after both acute and 9-day exposures. Though F1 and F2 were generally not acutely

toxic to this species after a 60-h exposure period, these two fractions did significantly reduce survival after a 9-day exposure period at low concentrations.

When comparing particle-containing fractions, F3 appeared to exert greater toxicity compared to F1. One hypothesis is that the larger particles in F1 caused them to be less bioavailable to the organism; thereby reducing their uptake and cytotoxicity [16]. A similar pattern of size-dependent toxicity has been previously observed in which exposure to a 100 g/L TWP dispersion resulted in lower acute toxicity to embryos of the amphibian *X. laevis* when compared to a 50 g/L TWP dispersion - these authors attributed the reduced toxicity to enhanced aggregation and larger particle size at the higher concentration [18, 25]. Mantecca *et al.* concluded that particle size and surface area play crucial roles in the toxicity of TWP as these physical properties can elicit different mechanisms of toxicity [65]. Jeong *et al.* suggested that the increased surface area to volume ratio of the smaller particles likely results in greater leaching of additives from the TWPs [66]. Numerous other studies have concluded that smaller particles generally cause more cytotoxic responses in living organisms compared to larger particles [9, 67, 68]. The exact mechanism of toxicity of nano-TWP remains to be elucidated and the uptake of nano-TWP within the organism remains to be demonstrated.

When comparing chemical constituent fractions, there was no statistically significant difference in survival between F1 (particles and leached chemicals) and F2 (leached chemicals) for both the acute and 9-day exposures. Cunningham *et al.* reported different findings whereby their TWP dispersion was more toxic than their filtered leachate to *D. rerio* (zebrafish) embryos and *Daphnia magna* (crustacean) [9]. However, their nanoparticle (< 1 µm) concentrations ranged from $0 – 3.6 \times 10^9$ particles/mL compared to the nanoparticle (< 0.2 µm) concentrations in the present study which ranged from $0 – 3.5 \times 10^8$ particles/mL.

Though not acutely toxic, F2 significantly diminished survival after 9-day exposure with the average survival being almost as low as that of F3. These results indicate that a chemical-induced mode of toxicity comes into play with longer exposure time [69]. The increased mortality may be associated with genotoxic mechanisms such as damage to DNA/RNA/proteins or oxidative stress [70], however, further studies are needed to reveal the precise mechanism of toxic action in *S. tropicalis* since this objective was beyond the scope of this study. Based on the TWP leachate generated in this study, nanoparticles were more toxic to *S. tropicalis* compared to the leached chemicals after a 60-h exposure period.

The results showed that there was a general lack of significant acute toxicity for the chemical constituent fractions (i.e., F1 and F2), and one possible reason for this is that the concentration of leached chemicals may be near the non-observable effect concentration (NOEC) for this exposure time, though this metric could not be obtained for this study due to the lack of a dose-response effect for survival. Even at the 100% treatment level, average survival was greater than 75%. For *X. laevis*, an amphibian species closely related to *S. tropicalis* [22], Mantecca *et al.* reported a NOEC of 50 mg/L of concentrated organic extracts from tire debris [15], but did not report the concentrations of metals and organic compounds within their organic extracts. In the present study, concentrations of most leached metals and organic chemicals were either not detected or in the µg/L range for both F1 and F2. Only zinc, benzothiazole and HMMM reached concentrations in the low mg/L range (< 3.04 mg/L) for F1 and F2.

4.3 Impact on Malformations

After the 9-day exposure, there was no significant difference in the proportion of living, malformed tadpoles between the control and treatment groups (Figure 4B). Conversely, for the acute exposure, trends in the overall proportion of malformed tadpoles were similar for F1, F2 and F3 – malformations were higher with increasing concentration (Figure 3B). A similar trend of increasing *X. laevis* tadpole malformations with increasing concentrations of TWP leachates after 120 h exposure has been previously reported [18].

Distinct differences between fractions were observed when examining the frequency of single malformations. Fractions with leached chemicals (i.e., F1 and F2) had significantly higher gut and head malformations, and on average higher instances of edema compared to F3. Gut and head abnormalities have been previously identified as common malformations in *X. laevis* tadpoles upon exposure to organic extracts from tire debris [15]. These authors concluded that organic extracts were potent teratogens causing abnormal morphogenesis in larval amphibians, and in the present study both F1 and F2 contained organic chemicals. For another freshwater species, *D. rerio*, pericardial edema has been observed in larvae after exposure to nano TWP dispersions (< 1 µm, $EC_{50} = 8.05 \times 10^8$ particles/mL) and leachate (EC_{50} = 84.1%) [9]. Chibwe *et al.* found higher instances of severely malformed fathead minnow (*Pimephales promelas*) embryos upon exposure to TWP leachate [14]. A higher frequency of malformations has previously been linked to DNA damage [71] or changes in enzymatic activities [72].

Due to the complex composition of the leachate, pinpointing the main chemicals responsible for these teratogenic effects remains a challenge, especially for amphibians. Furthermore, in combination, chemicals could exert antagonistic or synergistic effects based on

their relative concentrations as seen with the combinations of zinc and benzothiazole [63], and zinc and lead [73].

4.4 Impact on Body Morphometrics after 9-Day Exposure

For the 9-day exposure, TWP leachates had no significant effect on any growth parameter. However, the inverse relationship between tadpole density and growth was significant along the body and tail lengths (craniocaudal axis), but not along the body width (mediolateral axis). Gillespie also reported increased tadpole growth along the craniocaudal axis with lower tadpole densities [58]. A recent study, reported that nano-TWP dispersions did not affect the growth of mysid shrimp (*Americamysis bahia*), but did reduce growth of Inland Silverside fish (*Menidia beryllina*) at higher particle concentrations [74]. Siddiqui *et al.* also found that leached chemicals did not have a significant impact on growth for both these species.

Further investigations into brain morphometrics at the 10% treatment level revealed that tadpoles exposed to F3 had significantly larger brains compared to the control - the telencephalon, diencephalon, optic tectum, and medulla were all larger in width. According to the Cognitive Buffer Hypothesis, one possible explanation for the increased brain size is to confer greater locomotory abilities to compensate for environmental stressors [75]. We acknowledge that the increased growth in treatments with reduced survival may be related to density effects that were not fully captured in the statistical models, and we recommend that future studies be performed with treatment levels that support higher survival or with one animal per experimental unit.

4.5 Impact on Swimming Behavior after 9-Day Exposure

The startle response of tadpoles is an ecologically relevant endpoint. When exposed to a predator, tadpoles exhibit a sharp, jerking motion accompanied by accelerated swimming to avoid predation [76, 77]. At the 10% treatment level, tadpoles in F1 and F2 showed significantly lower swimming activity when compared to the controls, indicating a potentially diminished ability for predator avoidance. Tadpoles in F3 were on average more active at this concentration compared to those in F1 and F2, possibly due to stress [70] or the F3 tadpoles' increased brain size - a larger telencephalon in anurans may lead to improved navigation and cognitive function [78]. One possible cellular mechanism of toxic action that could be responsible for the increased swimming activity is overstimulation of the cholinergic system which controls movement [70]. At the 1% treatment level, however, the tadpoles in F3 swam significantly less than the controls. A similar trend was observed when comparing the feeding activity, though only 10% F2 was significantly lower than the controls, and this reduction in locomotion may have negative implications on the animal's ability to forage for food.

Overall, chemical constituent fractions (*i.e.*, F1 and F2) had a negative impact on swimming behavior at the 10% treatment, but the trend was not so obvious at the lower 1% treatment. From an extensive behavioral study, Siddiqui *et al.* reported significant behavioral changes for two different estuarine species, *A. bahia* and *M. beryllina*, upon exposure to both the leached chemicals and nano-sized TWP (< 1 µm) at particle concentrations of 60, 6,000 and 60,000 particles/mL [74].

5. Conclusions

Prolonged 9-day exposure to low concentrations of all fractions of tire wear particle leachate (F1, F2, and F3) at almost all treatment levels significantly decreased the survival of the amphibian *S. tropicalis* during its embryonic to larval stages. However, the nanoparticulate fraction (F3) was the most acutely lethal fraction, likely due to the presence of smaller, more bioavailable nanoparticles exerting greater cytotoxic effects. Enhanced aggregation of particles in F1 may be responsible for its lower acute toxicity compared to the other nanoparticle-containing fraction, F3. For the acute exposure, the frequency of malformed tadpoles increased in a roughly proportional manner to increasing concentrations of all fractions. Distinct differences between fractions were observed for single malformations, whereby the fractions containing leached chemicals (*i.e.*, F1 and F2) exhibited higher instances of head, gut, and edema malformations suggesting that the leached chemicals exerted a more teratogenic than lethal effect at the concentrations used in the present study. Prolonged 9-day exposure to F1 and F2 also had significant negative effects on both the tadpole startle response and feeding activity at the 10% treatment though the results were different at the 1% treatment. This altered behavior is a sign of reduced individual fitness that could have ramifications at the population level. Brain morphometrics (telencephalon width, diencephalon width, optic tectum width and medulla width), were impacted after 9 days, and tadpoles in F3 had significantly larger brains indicative of exposure to a higher degree of environmental stress according to the cognitive brain hypothesis.

These results reveal that there are distinct adverse effects associated with the nanoparticle and leached chemicals in TWP leachate, and suggests that the relative proportion of these constituents in the environment will influence the overall toxicity to freshwater organisms. The

leached chemicals and nanoparticles have different modes of toxic action, but further research is needed to elucidate the precise cellular mechanisms of toxicity of these different constituents, as well as to identify the specific metals/chemicals driving toxicity. Finally, the results were inconclusive regarding any synergistic or antagonistic effect on toxicity from a combination of the particle and leached chemical constituents, and more research should be done to investigate this.

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CRediT Authorship Contribution Statement

Rachel S. Cheong: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Validation; Visualization; Writing - original draft; Writing - review and editing. **Eva Roubeau Dumont:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Validation; Visualization; Writing - review and editing. **Paisley E. Thomson:** Investigation; Methodology; Project administration; Writing - review and editing. **Diana C. Castañeda-Cortés:** Investigation; Methodology; Project administration; Writing - review and editing. **Laura M. Hernandez:** Conceptualization; Investigation; Methodology; Writing - review and editing.

Xiaoyu Gao: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Writing - original draft; Writing - review and editing. **Jingyun Zheng:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Writing - original draft; Writing - review and editing. **Anca Baesu:** Conceptualization; Data curation; Formal analysis; Methodology; Validation; Writing - review and editing. **Jun-Ray Macairan:** Conceptualization; Investigation; Methodology; Writing - review and editing. **Anthony J. Smith:** Conceptualization; Investigation; Methodology; Writing - review and editing. **Hoai-Nam N. Bui:** Conceptualization; Investigation; Methodology; Writing - original draft; Writing - review and editing. **Hans C. E. Larsson:** Conceptualization; Methodology; Project administration; Resources; Writing - review and editing. **Subhasis Ghoshal:** Conceptualization; Methodology; Project administration; Resources; Validation; Writing - review and editing. **Stéphane Bayen:** Conceptualization; Methodology; Project administration; Resources; Validation; Writing - review and editing. **Valérie S. Langlois:** Conceptualization; Methodology; Project administration; Resources; Writing - review and editing. **Stacey A. Robinson:** Conceptualization; Data curation; Formal analysis; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing - review and editing. **Nathalie Tufenkji:** Conceptualization; Funding acquisition; Methodology; Project administration; Resources; Supervision; Writing - review and editing.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

Appendix A. Supporting Information

Materials and methods, including the details of liquid chromatography-quadrupole time of flight mass spectrometry (Table S1, Table S2, Table S3), experimental design of behavioral assays (Figure S1, Figure S2), tadpole nano-CT scanning parameters (Table S4), and tadpole brain morphometrics (Figure S3, Figure S4).

Results, including statistical analyses (Table S5, Table S6, Table S7), particle size characterization (Table S8, Figure S5), particle morphology (Figure S6, Figure S7), metal and organic chemicals characterization (Figure S8, Table S9), and additional endpoints from toxicity experiments (Figure S9, Figure S10).

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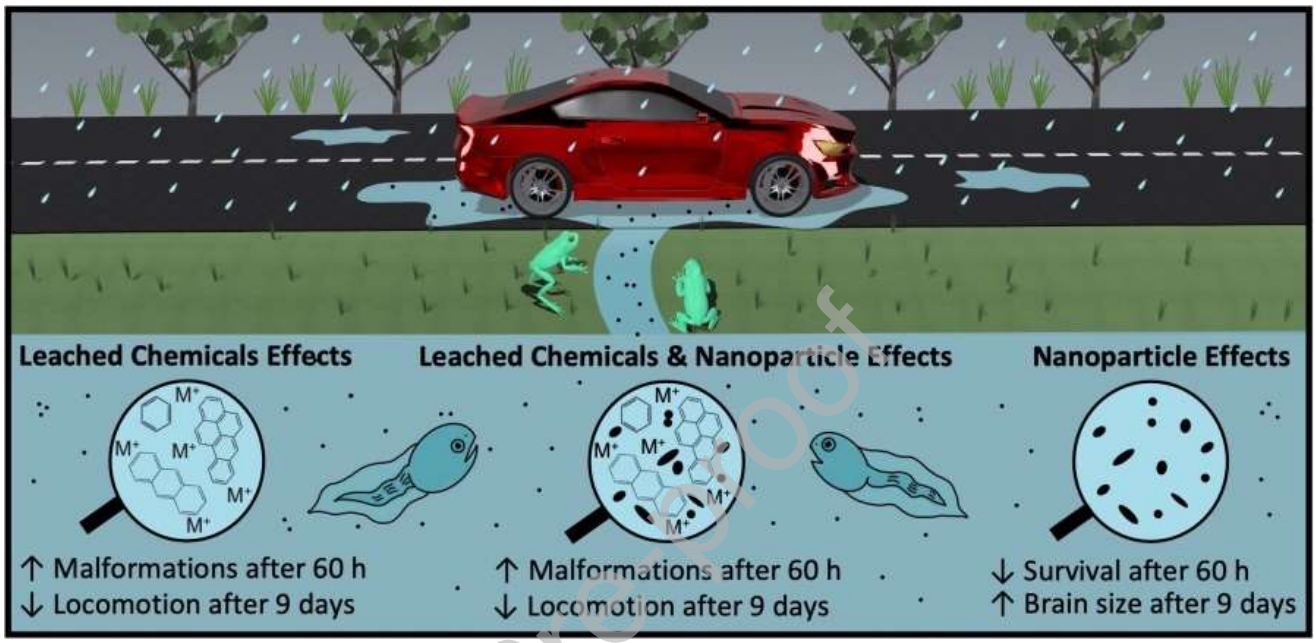
Graphical Abstract:



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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: