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Evaluation of tire tread particle toxicity to fish using rainbow trout cell lines

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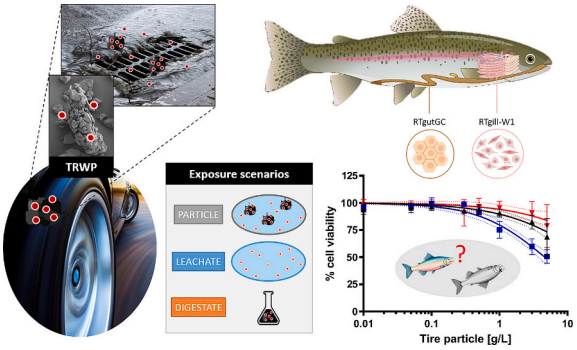
HIGHLIGHTS

- Assessment of tire and road wear particles' toxicity to fish using fish cell lines
- Specific toxicity of tire particles, leachates and *in vitro* digestate investigated
- Acutely toxic concentration of tire particles exceeds environmental concentrations
- Tire particles continuously leach chemicals, Zn and 6PPD main drivers of toxicity
- 6PPD-quinone (6PPD-Q) detected but not toxic to RTgill-W1 and RTgutGC cell lines

GRAPHICAL ABSTRACT



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ABSTRACT

Tire and road wear particles (TRWP) resulting from tire abrasion while driving raise concerns due to their potential contribution to aquatic toxicity. Our study aimed to assess cryogenically milled tire tread (CMTT) particle toxicity, used as a proxy for TRWP, and associated chemicals to fish using two Rainbow Trout (*Oncorhynchus mykiss*) cell lines representing the gill (RTgill-W1) and the intestinal (RTgutGC) epithelium. CMTT toxicity was evaluated through several exposure pathways, including direct contact, leaching, and digestion, while also assessing the impact of particle aging. Following OECD TG249, cell viability was assessed after 24 h acute exposure using a multiple-endpoint assay indicative of cell metabolic activity, membrane integrity and lysosome integrity. *In vitro* EC50 values for the fish cell lines exceeded river TRWP concentrations (2.02 g/L and 4.65 g/L for RTgill-W1 and RTgutGC cell lines, respectively), and were similar to *in vivo* LC50 values estimated at 6 g/L. Although toxicity was mainly driven by the leaching of tire-associated chemicals, the presence of the particles contributed to the overall toxicity by inducing a continuous leaching, highlighting the importance of considering combined exposure scenarios. Aging and digestion conditions were also found to mediate CMTT toxicity.

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Thermooxidation resulted in a decreased chemical leaching and toxicity, while *in vitro* digestion under mimicked gastrointestinal conditions increased leaching and toxicity. Specific chemicals, especially Zn, 2-mercaptobenzothiazole, 1,3-diphenylguanidine, and N-(1,3-dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine (6PPD) were identified as contributors to the overall toxicity. Although 6PPD-quinone was detected in CMTT digestate, cytotoxicity assays with RTgill-W1 and RTgutGC cell lines showed no toxicity up to 6 mg/L, supporting the notion of a specific mode of action of this chemical. This study provides insights into the toxicological mechanisms induced by tire particles and their associated chemicals and can help in the evaluation of potential risks to aquatic life associated with TRWP.

1. Introduction

Tire and road wear particles (TRWP) are generated during the abrasion of tires while driving. These particles are heterogeneous and complex as they are composed of the tire rubber polymer with tire additives, but also road minerals, bitumen and various chemicals originating from other materials found on road surfaces (Klöckner et al., 2021b; Kreider et al., 2010). Several metals were previously detected in the composition of tire particles (Klöckner et al., 2021b; Kreider et al., 2010; Masset et al., 2021) because they are commonly added into the rubber polymer to improve tire performance and durability. For example, zinc (Zn) is commonly used as a sulfur vulcanization catalyst during the curing process of rubber and can represent up to 2.5 % mass of the final tire composition (Bocca et al., 2009). Other metals, such as lead (Pb), manganese (Mn), cobalt (Co), chromium (Cr), barium (Ba), and nickel (Ni) were also measured in traces in the tire rubber, and at higher concentrations in TRWP, revealing the contribution of the road constituents to the overall metal burden of TRWP (Klöckner et al., 2021b; Kreider et al., 2010; Masset et al., 2021). Several organic chemicals are added to the composition of tire rubber to facilitate polymerization during manufacturing or to increase performance and longevity. Among the numerous organic compounds added, 2-mercaptobenzothiazole (2-MBT) and 1,3-diphenylguanidine (DPG) are intensively used as vulcanization agents and can represent up to 0.5 % of the tire rubber (Masset et al., 2022; Unice et al., 2015). Phenylenediamine compounds, such as N-isopropyl-*N*′-phenyl-*p*-phenylenediamine (IPPD) and N-(1,3-dimethylbutyl)-*N*′-phenyl-1,4-phenylenediamine (6PPD), are additionally used as antioxidants and antiozonants (up to 4 % of the tire tread) in the final product to prevent cracking and degradation of the rubber during wear (Layer and Lattimer, 1990; Masset et al., 2022).

Once TRWP are generated, they are expected to settle on the roadside (Panko et al., 2013a) and be transferred into the nearby soil, from which a fraction will eventually reach aquatic systems (Werbowski et al., 2021), potentially causing ecotoxicological effects. These particles have been detected in various compartments of the environment, with concentrations ranging from 0.1 to 100 g/kg on the road side, 0.5–1.2 g/kg in river sediment and 0.5–5 mg/L in river water (Wagner et al., 2018), which raises the question of their potential ecotoxicological impact. Moreover, TRWP have been found in the stomachs of several wild fish species (Parker et al., 2020), indicating that fish can ingest them, and recent research demonstrated that tire-associated chemicals may become more bioaccessible under gastrointestinal conditions (Masset et al., 2022, 2021). Additionally, TRWP are susceptible to environmental weathering and aging, which can lead to changes in their chemical composition and physical properties (Wagner et al., 2022, 2018). This can result in leaching of associated chemicals into aquatic systems and potential toxicological impacts on aquatic species.

A recent example is the 6PPD oxidation product, 2-((4-methylpentan-2-yl)amino)-5-(phenylamino)cyclohexa-2,5-diene-1,4-dione (6PPD-Q) (Tian et al., 2022, 2021), which was identified in urban runoff as the primary causal toxicant for the long-running issue of Coho Salmon (*Oncorhynchus kisutch*) mortality. 6PPD-Q is highly toxic to several other salmonid species (Brook Trout (*Salvelinus fontinalis*), Rainbow Trout (*Oncorhynchus mykiss*) (Brinkmann et al., 2022) and White-Spotted Char (*Salvelinus leucomaenis pluvius*) (Hiki and Yamamoto, 2022)), while other fish species (Arctic Char (*Salvelinus alpinus*), White Sturgeon (*Acipenser transmontanus*), Zebrafish (*Danio rerio*) and Japanese Medaka (*Oryzias latipes*)) and invertebrates (*Daphnia magna* and *Hyalella Azteca*) were found to be significantly less sensitive (Brinkmann et al., 2022; Hiki et al., 2021; Varshney et al., 2022), suggesting a species-specific mode of action. These recent findings highlight the importance of studying the combined effect of TRWP and their associated chemicals.

Previous studies investigating the potential toxic impact of TRWP and tire-associated chemicals on aquatic life are scarce, and have mainly been conducted by exposing organisms to tire crumbs (Cunningham et al., 2022; LaPlaca et al., 2022; LaPlaca and van den Hurk, 2020; Redondo-Hasselerharm et al., 2018) or aqueous leachates (Capolupo et al., 2021, 2020; Kolomijeca et al., 2020; Marwood et al., 2011; Panko et al., 2013b; Stephensen et al., 2003). No lethal effects were recorded following acute exposure of Fathead Minnow (*Pimephales promelas*) and *Daphnia magna* to 10,000 mg/L TRWP leachates (Marwood et al., 2011), and others observed only slightly diminished survival in larval Fathead Minnow over 32 exposure days (Panko et al., 2013b). Both studies concluded that TRWP should be considered a low risk to aquatic ecosystems. Similarly, no adverse effect on survival, growth or feeding rate of benthic macroinvertebrates were observed following exposure to TRWP up to 10 % sediment dry weight (Redondo-Hasselerharm et al., 2018). Moreover, only partial mortality was observed after exposure of Fathead minnow to 6000 mg/L crumb rubber (LaPlaca and van den Hurk, 2020), despite an accumulation of particles in the intestinal tract and presence of Zn and PAH compounds leaching from the particles. Yet, when looking into sublethal effects, deformities and increase of heart rate were observed when exposing Fathead minnow embryos to a 10,000 mg/L TRWP leachate (Kolomijeca et al., 2020). Moreover, developmental abnormalities of Zebrafish embryos were reported after exposure to 80 mg/L TRWP, with both chemical and particle specific toxicity (Cunningham et al., 2022). Finally, induction of CYP1A1 was measured in Rainbow Trout exposed to tires, associated with the release of PAHs from the rubber (Stephensen et al., 2003). Some studies also investigated the toxicity of leachates from aged tire particles (Camponelli et al., 2009; Day et al., 1993; Halle et al., 2021). These studies found that aging increased Zn leaching from the particles, extending the metamorphosis time for *Rana Sylvatica*, which could ultimately influence population dynamics.

Although previous studies have reported some sublethal effects and low acute toxicity risk of tire particles and associated chemicals, there are still certain data gaps and limitations in the existing research. These limitations arise from the lack of standardized experimental conditions, which include varying protocols for generating tire particles and the use of different parameters for temperature, pH, and salinity to produce leachates. These parameters can significantly impact the solubilization and bioaccessibility of tire-associated chemicals (Kolomijeca et al., 2020; Masset et al., 2022, 2021), potentially leading to different toxicity outcomes. Furthermore, mainly *in vivo* acute toxicity studies have been performed to evaluate the effects of tire particles and associated chemicals. However, *in vivo* studies have limitations, as they do not allow for an optimal control over the exposure conditions, making it challenging to establish a consistent and standardized experimental setup. Additionally, *in vivo* studies are resource-intensive and time consuming, limiting the ability to achieve a high throughput and conduct a

comprehensive assessment of toxicity across various exposure scenarios. Lastly, although *in vivo* studies can offer valuable insights into the overall effects of a toxicant, they often face limitations in fully elucidating the underlying toxicity mechanisms.

Therefore, this study aimed to strategically address this knowledge gap and assess the toxicity of tire particles and associated chemicals to fish using two Rainbow Trout cell lines representing the gill and the intestinal epithelium of the fish, namely RTgill-W1 and RTgutGC (Bols et al., 1994; Kawano et al., 2011). These cell lines were proven to give *in vitro* data comparable to acute toxicity *in vivo* data (Schug et al., 2020; Tanneberger et al., 2013) and serve as models to investigate acute toxicity of water samples and chemicals (Dayeh et al., 2003, 2002; Minghetti et al., 2019; Scott et al., 2022). The RTgill-W1 cell line assay have also been accepted as a standard method by the International Organization for Standardization (ISO), and as a test guideline by the Organisation for Economic Co-operation and Development (OECD) (ISO, 2019; OECD, 2021). Furthermore, these cell lines allow for precise and well-defined toxicity testing with minimal volumes, excellent comparability between different exposure scenarios, including particles *versus* leachates, and mimicking various exposure pathways. Additionally, these cell lines are well suited for predicting mixture toxicity effects. We used standardized cryogenically milled tire tread (CMTT) as a surrogate material for environmental TRWP (Cardno ChemRisk, 2020; Kovochich et al., 2021), and investigated the toxicity through several exposure pathways. These included (i) direct contact with cells to quantify a combined particle/leachate effect; (ii) exposure to leachate only to assess the toxicity of the leached chemicals; and (iii) exposure to thermooxidized particles to determine the effect of aging (Klöckner et al., 2021a). The RTgutGC cells were further (iv) exposed to CMTT digestate to investigate if fish gastro-intestinal conditions could result in a different chemical leaching profile and alter toxicity.

2. Material and methods

2.1. Materials

All chemicals used were from Sigma Aldrich and of analytical grade or higher. The compound 6PPD-Q, resulting from the oxidation of 6PPD, was not available commercially when our study was carried out. Therefore, it was synthesized in-house in our synthesis chemistry laboratory following a protocol from the literature (Agua et al., 2021). The synthesized 6PPD-Q was then characterized by Carbon-13 Nuclear Magnetic Resonance (NMR ^{13}C) and Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) and results were compared to the characterization of the compound features previously obtained (Tian et al., 2022, 2021). When a commercial 6PPD-Q commercial standard became available (HPC Standards®), the quality of our in-house 6PPD-Q was assessed by comparing the high-resolution mass spectra of the commercial 6PPD-Q with our in-house 6PPD-Q using an Orbitrap Exploris 120 mass spectrometer (Thermo-Fisher Scientific®). The results confirmed that the structure of the product synthesized in-house was similar and of similar quality to the 6PPD-Q purchased from HPC Standards® (Masset et al., 2022).

Foetal bovine serum (FBS) was purchased from Eurobio Scientific, trypsin solution was from Pan Biotech, the Leibovitz L-15 medium from PAN Biochem and Versene from Gibco™. The 75 cm^2 cell culture flasks were from Techno Plastic Product AG. 24-well culture plates were from Greiner Bio-One. The cytotoxicity assays were performed in L-15/ex, which has identical salts and sugar composition to the commercial cell culture medium Leibovitz's L-15 but does not contain amino acids and vitamins (OECD, 2021; Schirmer et al., 1997).

2.2. CMTT particles

The generation of CMTT was previously described (Masset et al., 2021). Briefly, the upper layer of the tire tread from Pirelli® (Sottozero 3), Michelin® (Primacy 3) and Bridgestone® (Saetta Touring 2) tires (ratio 1:1:2, respectively) was removed, cut into small pieces of 1 cm^3 using industrial scissors and a water jet machine, and finally cryogenically milled using a model A Hammer Mill (Pulva®). This process resulted in particles in the size range of 15 to 300 μm with a mean size of 188 μm.

Artificially aged CMTT were generated by thermooxidation by placing the particles in a drying cabinet at 80 °C for 20 days, simulating extreme conditions on the road surface (Klöckner et al., 2021a). The particles were collected and stored in amber glass vials in darkness at room temperature. More details regarding the physicochemical characteristics of CMTT and aged CMTT (size distribution, electron microscopy images) as well as chemical compositions can be found in our previous studies (Masset et al., 2022, 2021).

2.3. CMTT leachates

CMTT leachates were produced by incubating CMTT particles under gentle agitation at 20 °C for 24 h in different media to assess whether the media's chemical composition, in the presence or absence of sediments, could influence chemical leaching from the tire particles, potentially resulting in different toxicity outcomes: (i) mineral water (Evian®, composition described in Supplementary Table S1) at 100 g CMTT/L, (ii) a mixture of mineral water and artificial sediment (consisting of a mixture of fine sand (Neogard®) with 0,02 % of fish food (Tetramin®) (3:1, vol;vol)) at 100 g CMTT/L and (iii) in L-15/ex at concentrations of 0.5, 1, 3, 5, 10, 20 and 50 g CMTT/L. At the end of the incubation, the leachates were collected, filtered through 0.45 μm glass fiber filters (GFF, Whatman®) and stored at −20 °C until use. The chemical composition of the different types of leachate are summarized in Supplementary Table S2.

2.4. CMTT digestate

Digestion of CMTT particles was performed by applying an *in vitro* digestion protocol adapted for Rainbow Trout (Masset et al., 2022, 2021; Oldham et al., 2023; Siri et al., 2021). It consists of 3 h of digestion in simulated gastric fluid ($SF_{GASTRIC}$) to mimic the transit time in the fish stomach, followed by 24 h in simulated intestinal fluid ($SF_{INTESTINAL}$), which was estimated as an average transit time in the fish small intestine. The composition of the fish simulated gastric and intestinal fluids used in this study is described in the supplementary material (Supplementary Table S3). Briefly, both $SF_{GASTRIC}$ and $SF_{INTESTINAL}$ consisted of a luminal buffer adapted from Leibovitz's L-15 cell culture medium to mimic the composition of the lumen of fish intestine and were designed to be used in combination with the RTgutGC cell line, isolated from Rainbow Trout intestine. Purified pepsin (Sigma-Aldrich®) was added to the luminal buffer at a concentration of 12.5 U/mg of protein and pH was adjusted to 2 with 32 % HCl to obtain $SF_{GASTRIC}$. A concentration of 4 mg/mL of porcine bile extract (Sigma-Aldrich®) and 2 mg/mL of pancreatin (Sigma-Aldrich®) were added to the luminal buffer to obtain $SF_{INTESTINAL}$ with a pH of 7.4. The *in vitro* digestion was performed at 20 °C under gentle agitation at a concentration of 100 g CMTT/L of digestive fluid by introducing 5 g of CMTT in amber glass vessels containing 25 mL of $SF_{GASTRIC}$. Twenty-five mL of $SF_{INTESTINAL}$ were added to the digestion vessels after 3 h of digestion. After digestion of the CMTT, the samples were collected and centrifuged at 950*g*-force for 5 min to remove large CMTT particles and bile aggregates and the supernatant was filtered through 0.45 μm GFF filters (Whatman®). Samples were then transferred to an Amicon Ultra-4 Centrifugal Filter (Amicon ultra-15, Millipore®; cut-off = 10 kD) and centrifuged at 4000*g* for 30 min at 20 °C to remove large biological molecules and produce the digestate samples. Experimental blanks with digestives fluids without CMTT were also prepared and analysed. All samples were stored at −20 °C until use.

2.5. Cell culture: RTgill-W1 and RTgutGC

RTgill-W1 and RTgutGC cells were cultured in 75 cm^2 cell culture flasks (Techno Plastic Product AG) in Leibovitz L-15 medium (PAN Biotech) supplemented with 5 % Fetal Bovine Serum (FBS, Eurobio Scientific) at 19 ± 1 °C in an incubator at normal atmosphere. Once the cells reached a confluency of around 90 %, they were washed twice with Versene (Gibco™) and detached from the flask using 0.25 % trypsin (PAN Biotech). After trypsinization, cells were resuspended in L-15/FBS, centrifuged for three min at 875*g* at 19 ± 1 °C and counted using an electronic cell counting system CASY TCC (Biovendis Products GmbH). The cell suspensions were then diluted with L-15/FBS to allow for a seeding density appropriate for each cell line: RTgill-W1 cells were seeded at 350,000 cells per mL (equivalent to 184,000 cells per cm^2), and RTgutGC cells were seeded at 150,000 cells per mL (78,500 cells per cm^2) in 24-well culture plates, then incubated at 19 ± 1 °C for 48 h to allow formation of a confluent cell monolayer before exposure.

2.6. Exposure solution preparation and cell exposure

CMTT particle and aged-CMTT particle exposure suspensions were prepared individually by adding the corresponding amount of CMTT in 20 mL of L-15/ex to reach the desired concentration and vortexed for 1 h before use. CMTT/water and CMTT/water/sediment leachate exposures were prepared by diluting a CMTT leachate stock (100 g of CMTT per L of water, see 2.3.) in L-15/ex. Dilution controls without particles were run in parallel to monitor any potential effect due to Evian water and/or control sediment. CMTT digestate dilutions were prepared by diluting a CMTT digestate stock solution (prepared at 100 g of CMTT per L of digestive fluids, see 2.4.) in L-15/ex. Similarly, to the leachate's experiments, dilution controls of digestive fluids without CMTT were run in parallel to monitor potential effect of the digestive fluids. CMTT/L-15/ex leachate concentrations (0.5, 1, 3, 5, 10, 20 and 50 g CMTT/L) were prepared individually by incubating the correct amount of CMTT particles in 40 mL of L-15/ex for 24 h, before removal of the particles by filtration at 0.45 μm.

Stock solutions of 2-MBT, DPG, 6PPD and 6PPD-Q were prepared in Dimethyl sulfoxide (DMSO). Exposure solutions were prepared by diluting the stock solutions in L-15/ex while keeping DMSO percentage at 0.5 % (*v*/v).

Before exposure, cells were washed once with 1 mL of L-15/ex to remove any traces of L-15/FBS from the well. Then, cells were exposed to 2 mL of L-15/ex containing the desired concentration of CMTT particles, CMTT leachate or CMTT digestate, prepared as described above. For 2-MBT, DPG, 6PPD and 6PPD-Q assays, cells were exposed to 2.5 mL of the respective dosing mixture, and 500 μL were immediately sampled (t0) and stored at −20 °C before chemical analysis (see 2.8.). After 24 h incubation at 19 ± 1 °C in the dark, 500 μL of the media were sampled for chemical analysis (t24), and the rest was removed to allow cell viability assessment following the protocol described below. All concentrations are expressed as measured concentrations (geometric mean of t0 and t24 measured concentrations, OECD, 2021).

2.7. Cell viability assay

Following exposure, cell viability was assessed in accordance with OECD TG249 (OECD, 2021) by using a multiple-endpoint cytotoxicity assay indicative of metabolic activity (alamarBlue™ (AB); Invitrogen), cell membrane integrity (CFDA-AM, 5-carboxyfluorescein diacetate acetoxymethyl ester; Invitrogen) and lysosome integrity (Neutral Red (NR); Sigma-Aldrich). After incubation, the exposure solution was removed and the cells were washed once with 1 mL phosphate-buffered saline (PBS), and incubated for 30 min in 400 μL 5 % (v/v) AB and 4 μM CFDA-AM in PBS. A Tecan plate reader (Infinite M200, Tecan) was used to record the fluorescence at respective excitation/emission wavelengths of 530/595 nm for AB and 493/541 nm for CFDA-AM. After reading of the fluorescence, the AB/CFDA-AM working solution was removed and the cells were incubated with 400 μL of 1.5 % (v/v) NR for 1 h in the dark at 19 ± 1 °C. The cells were then washed with 400 μL fixative solution (0.5 % v/v formaldehyde and 1 % *w/v* $CaCl_2$) for a few seconds, then incubated in 400 μL extractive solution (1 % v/v acetic acid and 50 % v/v ethanol) for 10 min in the dark at room temperature under gentle horizontal agitation. The fluorescence was then measured at excitation/emission wavelengths of 530/645 nm. Results obtained were reported as percent viability based on fluorescent units (FU) of the L-15/ex control.

2.8. Chemical analyses of CMTT particles, leachates and digestate

2.8.1. Metals

The metal content of CMTT and aged CMTT particles was determined after an acid digestion of the particles (Masset et al., 2021). Triplicates of 250 mg of each material were placed into Teflon vessels, and 9 mL of 69 % HNO_3 with 1 mL of milliQ® water were added. These vessels were placed into a microwave reactor (multiwave Pro Anton Paar®) and heated at 200 °C under the following conditions: 15 min heating up to 200 °C, 45 min steady conditions at 200 °C and 20 bars, and finally 10 min of cooling to 70 °C. The resulting samples were filtered through 0.45 μm filters (Chromafil®), diluted 50-fold and analysed for metals. Analyses of Zn were performed on ICP-OES (Shimadzu® ICPE-9000) for microwave digested extracts (CMTT) since Zn was present in high amounts in CMTT. Analyses of Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Mo, Ba, and Pb, present in low quantities in all samples, were performed on an ICP-MS/MS (Agilent® 8900). For both instruments, calibration curves were established with 10 points of appropriate concentrations from a multi-elements standard solution (Inorganic Ventures®). Quality control solutions were processed between series of 10 samples. More information regarding instrument settings and QA/QC are available in our previous publication (Masset et al., 2021).

2.8.2. Organic chemicals

The organic chemical composition of CMTT and aged CMTT was determined as described in Masset et al., 2022. Briefly, the particles were spiked with deuterated internal standards (benzothiazole-d4, aniline-d5, diphenylurea-d10 and 6PPD-Q-d5 and a mix of 16 deuterated PAHs) and Soxhlet-extracted with 150 mL of methanol for 16 h, followed by 150 mL of dichloromethane for another 16 h. The resulting fractions were combined and concentrated to 2 mL using a rotavapor (Büchi®), and filtered through a 0.45 μm GFF. A 1 mL aliquot was prepared without further clean-up for direct analysis with Ultra Performance Liquid Chromatography coupled with a tandem mass spectrometer (UPLC-MSMS). Another aliquot underwent additional purification by passing through a chromatographic column filled with 3 g of silica-gel previously activated at 180 °C for 8 h and eluted with 50 mL of hexane. After concentration to 2 mL with a rotavapor, the extracts were concentrated near dryness under nitrogen and solvent exchanged to 500 μL of isooctane for Gas Chromatography coupled with a tandem mass spectrometer (GC-MSMS) analyses. For each digestate sample and CMTT leachate sample, a sub-sample (1 mL) was spiked with an appropriate amount of deuterated internal standard (benzothiazole-d4, aniline-d5, diphenylurea-d10 and 6PPD-quinone-d5). All samples were analysed directly with UPLC-MSMS without further clean up. The following tire-associated compounds in the samples were analysed: Benzothiazole (BTH), 2-aminobenzothiazole (2-A-BTH), 2-hydroxybenzothiazole (2-H-BTH), 2-MBT, cyclohexylamine (CHA), 2-Methylthiobenzothiazole (MTBT), 2,2′-dithiobisbenzothiazole (MTBS), aniline (ANI), DPG, 6PPD and 6PPD-Q. Six calibration standards were analysed for each batch of samples (1 ng/mL to 500 ng/mL, linearity $R^2 > 0.99$). Details regarding the chemicals used, UPLC-MSMS methods and QA/QC for chemical analyses of CMTT particles, leachates and digestates are provided in our previous study (Masset et al., 2022).

2.9. Data analysis

The data presented are mean values ± standard deviation (SD) of at least three different experiments performed on different passages of cells, with each experiment containing three technical replicates. All results are expressed as a function of measured concentrations (geometric mean of t0 and t24 measured concentrations, OECD, 2021). Statistical analysis was performed using GraphPad Prism Version 8.4.3 (GraphPad Software, San Diego, CA USA). Fluorescent units obtained in the cell viability assays were converted to percentage viability of control. The non-linear regression sigmoidal dose-response curve fitting module using the Hill slope equation was used to fit the dose-response curve data.

3. Results and discussion

3.1. Comparative assessment of CMTT and aged-CMTT particle toxicity to fish

CMTT particles caused acute cytotoxicity in the fish cell lines (Fig. 1). Metabolic activity was the most sensitive endpoint, yielding an EC50 of 2.023 g/L CMTT (1.599 to 2.560, 95 % CI) and 4.651 g/L CMTT (3.598 to 6.011, 95 % CI) for RTgill-W1 and RTgutGC cell lines, respectively. These effect concentrations are in the same range as concentrations previously reported in *in vivo* fish acute toxicity studies, with 40 % mortality observed after exposure of Fathead minnow to 6 g/L crumb rubber (LaPlaca and van den Hurk, 2020). Yet, they are about three orders of magnitude above the ~4 mg/L of TRWP detected in river water. On the other hand, road runoff has been reported to potentially exceed 100 mg/L, *i.e.* being within one order of magnitude of our effect concentrations (Wagner et al., 2018). Further, our results revealed that the non-toxic concentration, *i.e.* the highest concentration not yet producing a toxic response (Stadnicka-Michalak et al., 2018), was 43 mg/L for the RTgill-W1 and 130 mg/L for the RTgutGC. These concentrations bracket the 80 mg/L TRWP which had induced developmental abnormalities, *i.e.*, sublethal effects, in Zebrafish embyos (Cunningham et al., 2022). Hence, repeated exposure to such high concentrations might lead to potential chronic toxicity, which remains to be investigated.

When both cell lines were exposed to aged-CMTT, the induced toxicity decreased compared to unaged CMTT (Fig. 1). The EC50 values were determined at 11.44 g/L (9.046 to 14.46, 95 % CI) for RTgill-W1 and predicted at 114 g/L (extrapolated value, 54.02 to 240.4, 95 % CI) for RTgutGC. Analyses of CMTT and aged CMTT particles (Supplementary Table S2) revealed changes in the chemical composition of the particles due to aging, which were also reflected in the composition of their respective leachates. Specifically, there was a decrease in Zn and several organic compounds, including BTH, 2-MBT, DPG and 6PPD, along with an increase in 2-H-BTH and 6PPD-Q (Supplementary

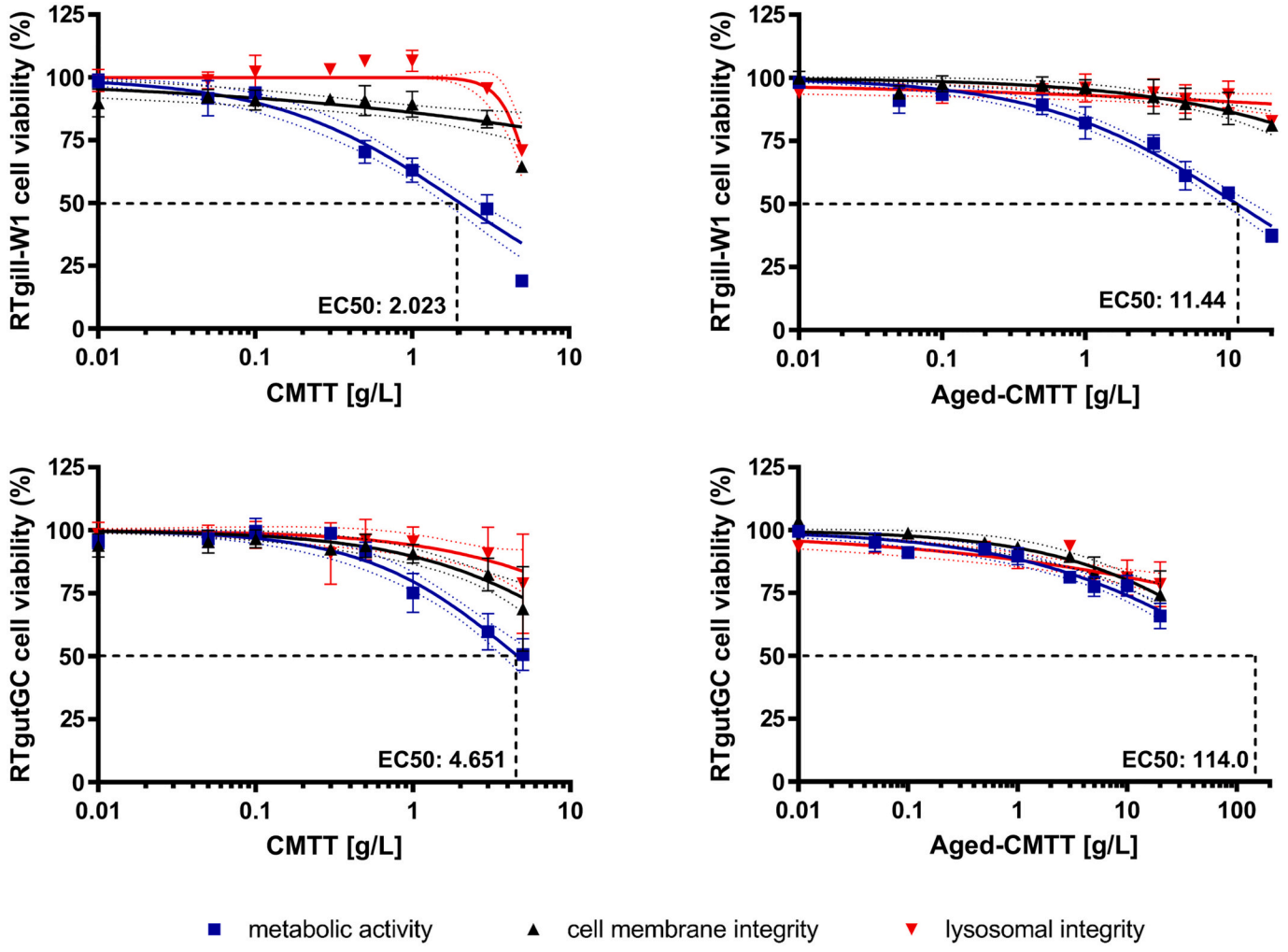

Fig. 1. Acute cytotoxicity of CMTT (left) and aged-CMTT particles (right) to RTgill-W1 (top) and RTgutGC cell lines (bottom). Dose-response curves reflect cell metabolic activity (alamarBlue™), cell membrane integrity (CFDA-AM) and lysosome integrity (Neutral Red) after 24 h of exposure. Values shown are averages and standard deviations (dashed lines) of independent experiments (n = 3–5), expressed as a percentage of control (L-15/ex). Dashed lines highlight the EC50 values determined or estimated for the most sensitive endpoint of the cytotoxicity assay.

Table S2). Since the aged and unaged particles have similar physical properties (size distribution and electron microscopy images can be found in Masset et al., 2021 and Masset et al., 2022), this decrease in leaching chemicals may explain the observed lower toxicity of aged-CMTT compared to unaged particles, and hint that Zn, along with BTH, 2-MBT, DPG, and 6PPD, may contribute to CMTT toxicity. This suggests that the toxicity could primarily be driven by the leaching chemicals rather than a physical effect. Furthermore, as the exposure medium consists of a mixture of chemicals, the differential toxicity observed between the two cell lines could be attributed to their varying sensitivity to specific leaching chemicals. For example, previous studies determined the EC50 values for Zn to be 15.37 ± 1.7 μM for RTgill-W1 and 98.38 ± 9.68 μM for RTgutGC cell line (Dayeh et al., 2005; Ibrahim et al., 2020) which could explain the overall higher sensitivity observed with the RTgill-W1 cells.

3.2. Comparative toxicity assessment of CMTT leachates and digestate: influence of media on chemical desorption and toxicity

To experimentally assess the contribution of CMTT-associated chemicals to the overall toxicity of CMTT, the toxicity of CMTT leachates in L-15/ex was tested in parallel. Interestingly, the toxicity induced by CMTT/L-15/ex leachates was lower for both tested cell lines (Fig. 2) compared to the toxicity previously observed with CMTT particles (Fig. 1). The estimated EC50 values were above the highest tested concentration, and estimated at 110.8 g/L (extrapolated value, 39.54 to 316.6, 95 % CI) and 156.0 g/L (extrapolated value, 35.04 to 694.3, 95 % CI) for RTgill-W1 and RTgutGC cell lines, respectively. These *in vitro* data are in accordance with previously reported *in vivo* data, which showed no lethal effects following acute exposure of Fathead Minnow (*Pimephales promelas*) and *Daphnia magna* to 10 g/L tire wear particle leachates (Marwood et al., 2011). Moreover, these results demonstrate that, while the leaching chemicals were the primary drivers of toxicity when exposing to CMTT, the presence of the particles also contributed to the observed toxic effects. Given that some tire chemicals, such as 6PPD and IPPD, are prone to blooming (Choi, 1997; Lewis, 1986), *i.e.* gradually migrating toward the tire's surface, and thereby supplying a continuous source throughout the entire lifespan of the tire, our hypothesis is that the particles could continuously leach chemicals in the exposure medium, therefore increasing cell exposure and resulting in greater toxicity. Furthermore, given that the exposure was conducted under static conditions, a concentration gradient may have led to the accumulation of chemicals at the particle-liquid interface. As the particles tended to sink toward the cell monolayer, this could have created a localized microenvironment near the cells with high concentrations of chemicals, thereby leading to a greater toxicity. Based on these

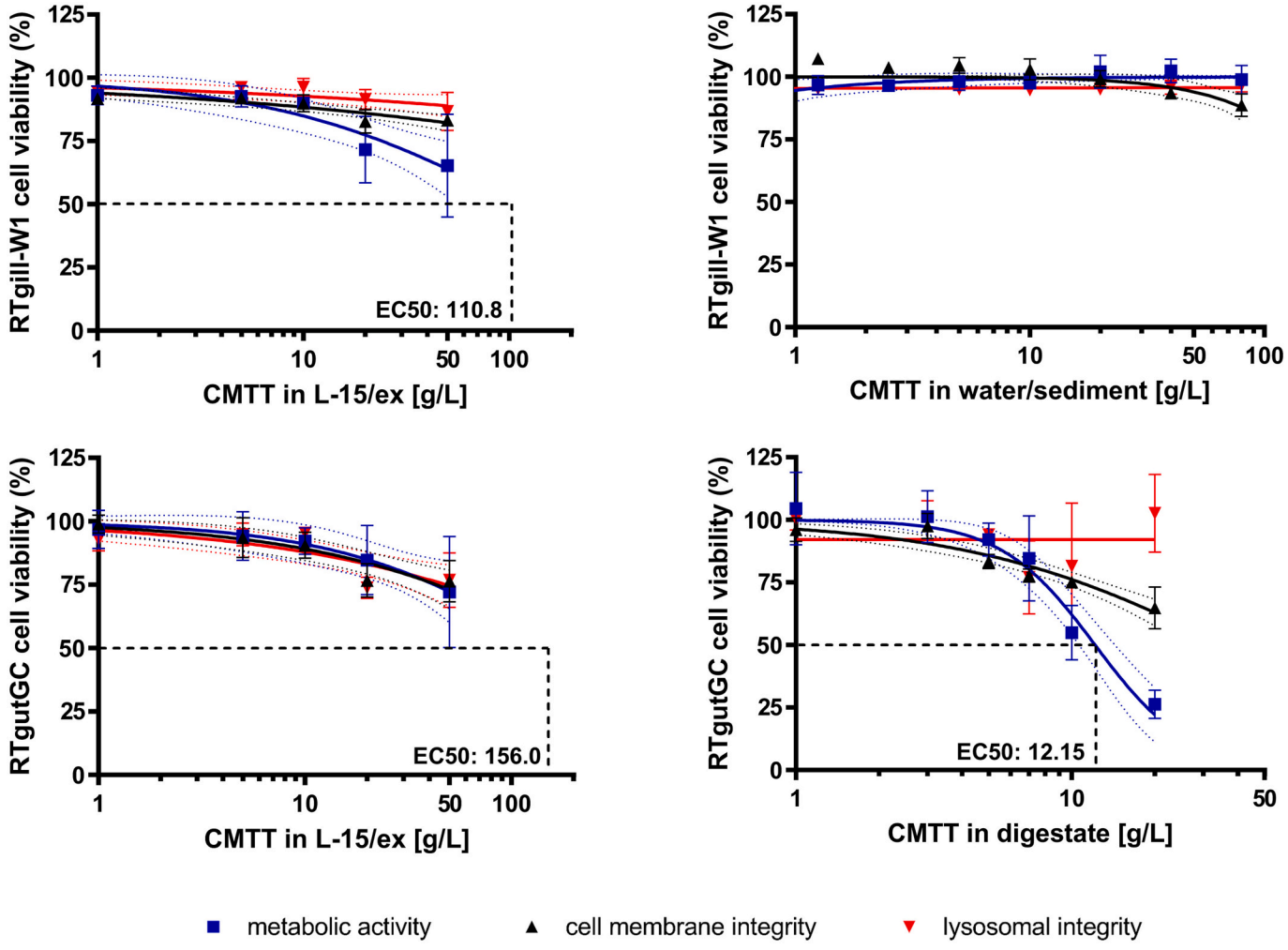

Fig. 2. Acute cytotoxicity of CMTT/L-15/ex leachates to the RTgill-W1 and RTgutGC cell lines (left), CMTT/water/sediment leachate to the RTgill-W1 cell line (top right), and CMTT digestate acute toxicity to the RTgutGC cell line (bottom right). Dose-response curves reflect cell metabolic activity (alamarBlue™), cell membrane integrity (CFDA-AM) and lysosome integrity (Neutral Red) after 24 h of exposure. Values shown are averages and standard deviations (dashed lines) of at least three independent experiments (n = 3–5), expressed as a percentage of control (L-15/ex). Dashed lines highlight the EC50 values determined or estimated for the most sensitive endpoint of the cytotoxicity assay.

considerations, an integrative assessment of tire particle ecotoxicity should take the combined exposure, *i.e.* particles and leachate, into account.

When the RTgill-W1 cell line was exposed to the CMTT/water/sediment leachate, the induced toxicity was even lower than for the CMTT/L-15/ex leachate (Fig. 2). In fact, no toxicity was observed at the highest concentration tested, which was a leachate obtained from 80 g/L CMTT. Similar results were obtained when exposing RTgill-W1 to a CMTT/water leachate, where no toxicity was observed at the highest concentration tested (data not shown). These data are in accordance with a recent study in which no effects were observed on *Daphnia pulex* survival exposed to tire wear leachate (1 g/L) before 10 days of leaching incubation time (Li et al., 2023a).

Furthermore, the RTgutGC cells were exposed to CMTT digestate to investigate if fish gastro-intestinal conditions could result in a different chemical profile and induce changes in toxicity (Fig. 2). The toxicity observed in this case was greater compared to the L-15/ex and water/sediment leachates, with an EC50 of 12.15 g/L (10.51 to 14.05, 95 % CI) of CMTT in digestive fluids.

Overall, the toxicity of the CMTT leachates and digestate to RTgutGC cells correlated with their chemical composition (Supplementary Table S2). The CMTT/digestate exhibited the highest toxicity, and analyses revealed higher concentrations of both metal and organic compounds compared to the CMTT/water and CMTT/water/sediment leachates (Supplementary Table S2). Specifically, there were higher concentrations of Zn (12,018 μg/L *vs* 25.1 μg/L), CHA (3236.7 μg/L *vs* 711.7 μg/L) and DPG (5386.0 μg/L *vs* 2376.9 μg/L) in the CMTT digestate compared to the CMTT/water/sediment leachate. The increase in concentrations of both metal and organics in the digestate was likely due to the acidic pH of the gastric phase and the presence of bile salts in the intestinal phase of the digestion (Masset et al., 2022, 2021).

Similarly, the analysis of CMTT/water/sediment leachate unveiled lower concentrations of Zn (600.0 μg/L *vs* 1480.2 μg/L) and CHA (171.0 μg/L *vs* 1199.5 μg/L) compared to the CMTT/L-15/ex leachates, likely explaining the difference of toxicity between these two samples.

These results underscore the importance of the surrounding media in regards to the desorption of associated chemicals and resulting toxicity, and highlight the significance of considering the digestion of tire particles in the overall assessment of their toxicity.

3.3. Identification of metals and organic compounds in CMTT digestate and their contribution to overall toxicity

Chemical analyses of the CMTT digestate and leachates showed the presence of several metals, such as Zn, Fe, Ba and Pb, and organic compounds, such as DPG, 6PPD and 6PPD-Q (Supplementary Table S2). Among the chemicals detected in the digestate, Zn stood out as one that might dominate the overall toxicity. Zn concentration in the EC50 digestate (produced at 12 g of CMTT/L of digestive fluids) was calculated at 1.442 mg/L while an EC50 of 6.039 mg/L for Zn was previously reported for the RTgutGC cell line (Ibrahim et al., 2020). However, based on *in vivo* literature toxicity data, three other compounds detected in the digestate might also have contributed to the overall toxicity observed in our *in vitro* experiment. These chemicals are 2-MBT (183.5 μg/L, known EC50 at 34.7 ± 1.9 mg/L using RTgill-W1 cell line (Zeng et al., 2016)), DPG (646.3 μg/L, known LC50 at 4.2 mg/L, Fathead minnow (*Pimephales promelas*), (French Agency for Food, 2020)), and 6PPD (13.7 μg/L, known LC50 at 28 μg/L, Japanese rice fish (*Oryzias latipes)*, (OECD, 2004)), which were all measured at high level in the digestate that had induced the 50 % decrease in cell viability. Finally, traces of 6PPD-Q (0.58 μg/L) were also detected in the digestate prior to Amicon-filtration. This compound has been previously identified as acutely toxic to various aquatic species, with however highly species-specific sensitivities (Brinkmann et al., 2022; Hiki et al., 2021; Tian et al., 2022; Varshney et al., 2022). To determine the contribution of these chemicals in the overall observed toxicity of the digestate, we tested them separately using our cell line models.

3.4. Individual chemical assessment of 6PPD, 6PPD-Q, 2-MBT and DPG: contribution to overall CMTT toxicity

The toxicity assessments of 6PPD using RTgill-W1 and RTgutGC cell lines revealed that this compound was a significant contributor to the overall toxicity observed in our assay (Fig. 3). The *in vitro* results were consistent with *in vivo* data, with an EC50 of 121.6 μg/L (86.9 to 170.1, 95 % CI) and 177.1 μg/L (166.5 to 188.4, 95 % CI) for RTgill-W1 and RTgutGC cell lines, respectively, compared to an LC50 of 28 μg/L reported for Japanese rice fish (*Oryzias latipes*, OECD, 2004). Furthermore, we calculated a concentration of 13.7 μg/L of 6PPD in the digestate that resulted in a 50 % decrease in viability. This concentration is less than 10 times lower than the determined EC50, indicating that 6PPD contributed to the overall toxicity.

When exposing RTgill-W1 and RTgutGC cell lines to 6PPD-Q (Fig. 3), we observed a 25 to 30 % decrease in metabolic activity at 20 μg/L. This was in accordance with the recent observations that RTgill-W1 cell viability decreased at 80 μg/L (<20 % decrease in metabolic activity determined by MTT assay, (Mahoney et al., 2022)). However, we did not observe a concentration-dependent increase in toxicity beyond that concentration, even at concentrations up to 6200 μg/L for RTgill-W1 and 1450 μg/L for RTgutGC (Fig. 3). This result means that 6PPD-Q was not contributing to the toxicity observed in our *in vitro* assays, despite the reported toxicity of this compound *in vivo*, including toward Rainbow Trout, at far lower concentrations (Brinkmann et al., 2022). This lack of toxicity to the RTgill-W1 and the RTgutGC cell lines may imply that 6PPD-Q acts on a different target organ with a specific mode of action not addressed by these cell lines. For example, recent *in vivo* studies argue that 6PPD-Q's mode of action could be neurotoxicity. This mode of action was suggested based on symptoms exhibited following juvenile Coho Salmon exposure to roadway runoff (Blair et al., 2021), and observed in Roundworm (*Caenorhabditis elegans*) exposed to environmentally relevant concentrations of 6PPD-Q (0.1 to 10 μg/L), leading to abnormal locomotion behaviors and neurodegeneration. Despite the insensitivity of the RTgill-W1 and RTgutGC cell lines to 6PPD-Q, these cell lines remain suitable for the assessment of CMTT toxicity to fish, as the concentrations of 6PPD-Q in the CMTT exposure solutions were measured <LOQ, hence well below the 6PPD-Q LC50 value reported for Rainbow Trout of 1 μg/L. Moreover, the CMTT toxicity results obtained *in vitro* aligned with previous *in vivo* data, supporting the suitability of the RTgill-W1 and RTgutGC models.

2-MBT and DPG toxicity assessments using RTgill-W1 revealed that both compounds were not major contributors to the overall toxicity observed in our assay (Fig. 4). The assessment of 2-MBT revealed an EC50 of 18.92 mg/L (17.52 to 20.42, 95 % CI) for metabolic activity, which was in accordance with previous *in vitro* data (34.7 ± 1.9 mg/L, (Zeng et al., 2016)) and close to *in vivo* LC50 values reported for *Ceriodaphnia dubia* (4.19 mg/L, Nawrocki et al., 2005). In contrast, the concentration was calculated at 0.1835 mg/L in the EC50 digestate, which is approximately 100 times lower than the EC50 determined at 18.92 mg/L.

Likewise, DPG yielded an EC50 of 88.84 mg/L (82.17 to 96.05, 95 % CI), which was over 130 times higher than the concentration in the EC50 digestate (646.3 μg/L), and 20 times higher than its LC50 reported for Fathead minnow (*Pimephales promelas*) (4.2 mg/L, French Agency for Food, 2020).

3.5. Contribution of chemicals to CMTT digestate toxicity: Application of the concentration addition principle

Using the concentration addition principle of mixture toxicity, we estimated the relative contribution of each chemical toward the total toxicity in our cell viability assay. For that, we used measurements from the digestate stock (100 g CMTT/L, see Supplementary Table S2) and

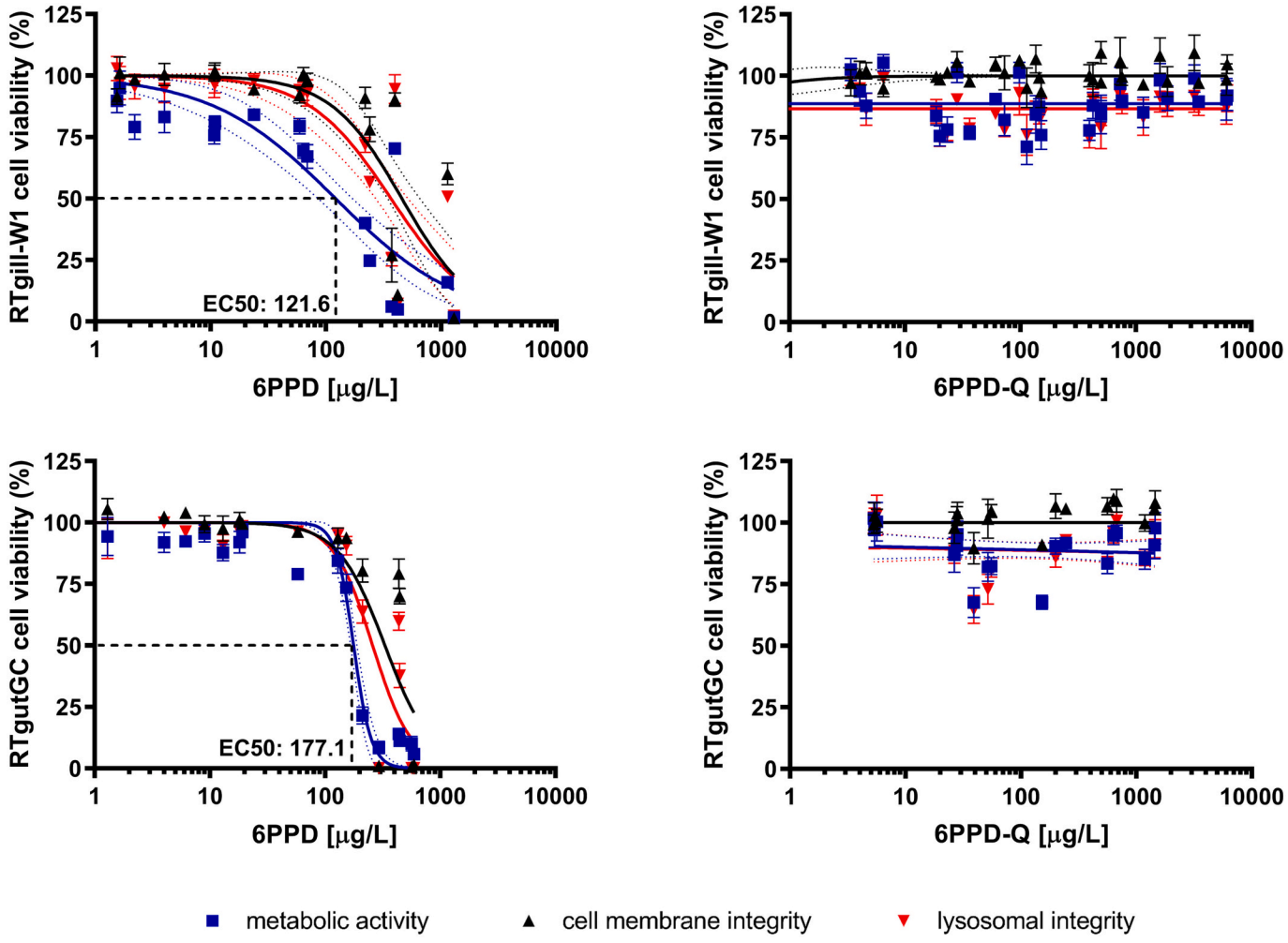

Fig. 3. Acute cytotoxicity of 6PPD (left) and 6PPD-Q (right) to RTgill-W1 (top) and RTgutGC cell lines (bottom). Dose-response curves reflect cell metabolic activity (alamarBlue™), cell membrane integrity (CFDA-AM) and lysosome integrity (Neutral Red) after 24 h of exposure. Data points represent the average and standard deviation (dashed lines) from at least three independent experiments ($n = 3$–5), expressed as a percentage of control (L-15/ex). Black dashed lines highlight the EC50 values for the most sensitive endpoint of the cytotoxicity assay.

calculated the concentrations of associated chemicals that were present in the EC50 digestate (12 g CMTT/L digestive fluids). Then, we combined these concentrations with the previously determined EC50 values for each individual chemical (see Supplementary Table S4). This analysis revealed that Zn was the main driver of toxicity in CMTT digestate, contributing 22.5 % to the overall toxicity, followed by 6PPD (7.7 % of overall toxicity), whereas DPG and 2-MBT only accounted for 0.7 % and 0.9 % of the overall toxicity. These results are consistent with previous studies in which Zn and PAHs were identified and listed as the toxic candidates in TRWP leachates (Camponelli et al., 2009; Kolomijeca et al., 2020; LaPlaca and van den Hurk, 2020; Marwood et al., 2011; Stephensen et al., 2003). In total, Zn, 6PPD, DPG and 2-MBT accounted for 32 % of the total toxicity. The remaining 68 % might be explained by additive effects of unmeasured chemicals, including degradation products formed *via* photodegradation or oxidation of the tire antioxidants (Hu et al., 2022; Li et al., 2023b; Zhou et al., 2023), present in the mixture in high enough concentrations to contribute to the toxicity. The leaching chemicals might also have non-additive or synergistic effects, resulting in a greater toxicity. To determine the cause of the missing 68 % of toxicity, an effect-directed analysis approach, following the procedure that allowed the discovery of 6PPD-Q (Tian et al., 2021), is indicated.

3.6. Limitations and environmental implications

The main limitation of this study is the use of the standardized tire particle "CMTT" as a proxy for environmental TRWP. It has been demonstrated that the chemical content of TRWP is not identical to that of pure tire tread due to encrustation of minerals and organic constituents originating from the road pavement. Moreover, the different surface areas of CMTT and TRWP could impact the solubilization kinetics of the associated compounds as well as their overall bioaccessibility and resulting toxicity. Furthermore, even though the effects of aging on tire particles was considered in this study *via* exposure of CMTT to thermooxidative conditions, TRWP will undergo various types of weathering that were not addressed (photodegradation, mechanical shear stress, biodegradation). Further studies should take these other aging processes into account and investigate the toxicity of such aged tire particles and TRWP.

4. Conclusions

Our study assessed the toxicity of a standardized TRWP proxy (CMTT) to fish *in vitro*, using two fish cell lines representing the gill and the gut epithelium, namely RTgill-W1 and RTgutGC. We found that the EC50 of CMTT was in the same range as what was previously reported *in vivo* and far above TRWP environmental concentrations. Furthermore,

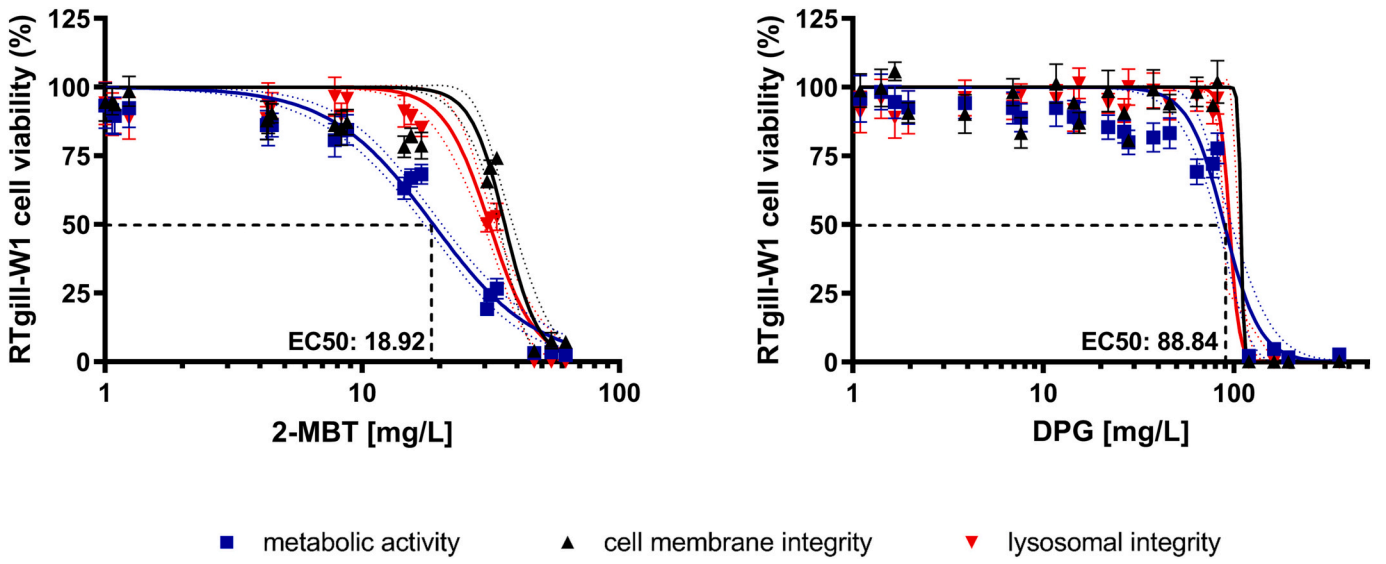

Fig. 4. Acute cytotoxicity of 2-MBT and DPG to the RTgill-W1 cell line. Dose-response curves reflect cell metabolic activity (alamarBlue™), cell membrane integrity (CFDA-AM) and lysosome integrity (Neutral Red) after 24 h of exposure. Data points represent the average and standard deviation (dashed lines) from at least three independent experiments ($n = 4$), expressed as a percentage of control (L-15/ex). Black dashed lines highlight the EC50 values for the most sensitive endpoint of the cytotoxicity assay.

we showed that the toxicity could primarily be driven by the leaching chemicals rather than a physical effect, but that the presence of the particles contributed to the overall toxicity, probably by inducing a continuous leaching of chemicals. This finding highlights the need to consider combined exposure to particles and leachates in the future. Aging and digestion conditions were also found to affect the toxicity of CMTT: thermooxidation resulted in less chemical leaching associated with a decrease of toxicity, while gastrointestinal conditions during *in vitro* digestion increased the leaching of metals and organic compounds, resulting in greater toxicity. Our results emphasize the importance of considering the potential ingestion of TRWP and microplastics in general, as well as the role of gastrointestinal conditions in mediating the bioaccessibility of chemicals, to conduct a proper risk assessment. We also identified specific chemicals, such as Zn, 2-MBT, 6PPD and DPG, as contributors to the overall toxicity. Finally, while 6PPD-Q was detected in the CMTT digestate, it was not toxic to the RTgill-W1 and RTgutGC lines when tested individually, which suggests an alternative target organ and a specific mode of action such as neurotoxicity, which will require further investigation. Overall, these findings highlight the need for further research to assess the toxicity of TRWP in the environment, as well as to evaluate the risk associated with unknown and uncharacterized compounds that may present a risk to the environment.

CRediT authorship contribution statement

W. Dudefoi: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **B.J.D. Ferrari:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. **F. Breider:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **T. Masset:** Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. **G. Leger:** Investigation, Methodology. **E. Vermeirssen:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **A.J. Bergmann:** Conceptualization, Writing – review & editing. **K. Schirmer:** Conceptualization, Funding acquisition, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dudefoi William reports financial support was provided by World Business Council for Sustainable Development. Masset Thibault reports financial support was provided by World Business Council for Sustainable Development. Bergmann Alan reports financial support was provided by World Business Council for Sustainable Development.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.168933.

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