

## Environmental Chemistry

## Estrogenic, Genotoxic, and Antibacterial Effects of Chemicals from Cryogenically Milled Tire Tread

Alan J. Bergmann,<sup>a,\*</sup> T. Masset,<sup>b</sup> F. Breider,<sup>b</sup> W. Dufefoi,<sup>c</sup> K. Schirmer,<sup>c,d,e</sup> B. J. D. Ferrari,<sup>a,f</sup> and E. L. M. Vermeirssen<sup>a</sup><sup>a</sup>Swiss Centre for Applied Ecotoxicology, Dübendorf, Switzerland<sup>b</sup>Central Environmental Laboratory, Ecole Polytechnique Fédérale de Lausanne–EPFL, Lausanne, Switzerland<sup>c</sup>Department Environmental Toxicology, Eawag, Dübendorf, Switzerland<sup>d</sup>Institute of Biogeochemistry and Pollutant Dynamics, ETHZ, Zürich, Switzerland<sup>e</sup>Laboratory of Environmental Toxicology, Ecole Polytechnique Fédérale de Lausanne–EPFL, Lausanne, Switzerland<sup>f</sup>Swiss Centre for Applied Ecotoxicology, Lausanne, Switzerland

**Abstract:** Tire and road wear particles (TRWP) contain complex mixtures of chemicals and release them to the environment, and potential toxic effects of these chemicals still need to be characterized. We used a standardized surrogate for TRWP, cryogenically milled tire tread (CMTT), to isolate and evaluate effects of tire-associated chemicals. We examined organic chemical mixtures extracted and leached from CMTT for the toxicity endpoints genotoxicity, estrogenicity, and inhibition of bacterial luminescence. The bioassays were performed after chromatographic separation on high-performance thin-layer chromatography (HPTLC) plates. Extracts of CMTT were active in all three HPTLC bioassays with two estrogenic zones, two genotoxic zones, and two zones inhibiting bacterial luminescence. Extracts of CMTT artificially aged with thermooxidation were equally bioactive in each HPTLC bioassay. Two types of aqueous leachates of unaged CMTT, simulating either digestion by fish or contact with sediment and water, contained estrogenic chemicals and inhibitors of bacterial luminescence with similar profiles to those of CMTT extracts. Of 11 tested tire-associated chemicals, two were estrogenic, three were genotoxic, and several inhibited bacterial luminescence. 1,3-Diphenylguanidine, transformation products of *N*-(1,3-dimethylbutyl)-*N'*-phenyl-*p*-phenylenediamine, and benzothiazoles were especially implicated through comparison to HPTLC retention factors in the CMTT samples. Other bioactive bands in CMTT samples did not correspond to any target chemicals. Tire particles clearly contain and can leach complex mixtures of toxic chemicals to the environment. Although some known chemicals contribute to estrogenic, genotoxic, and antibacterial hazards, unidentified toxic chemicals are still present and deserve further investigation. Overall, our study expands the understanding of potential adverse effects from tire particles and helps improve the link between those effects and the responsible chemicals. *Environ Toxicol Chem* 2024;43:1962–1972. © 2024 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

**Keywords:** 6PPD; Antimicrobials; Bioassay-directed fractionation; Estrogenic compounds; Genotoxicity; Microplastics; Tire wear particles

## INTRODUCTION

Tire and road wear particles (TRWP) are formed by abrasion of tires with road surface. They currently make up a large proportion of primary microplastics released to the environment (Sieber et al., 2020). Release of TRWP might continue to

increase in part because of rising use of electric vehicles, which produce more TRWP than conventional vehicles (Beddows & Harrison, 2021). These particles are nanometer- to micron-sized with heterogeneous compositions including rubber and carbon black from the tires and bitumen and minerals from road surfaces. Such small particles can have biological effects due to their physical properties, but TRWP are of particular concern because of the many unbound chemical additives of tire rubber, which may leach from the particles into the environment (Wik & Dave, 2009).

Although metals in, and leaching from, tires can be well characterized through target chemical analysis, organic chemical content is less well defined due to potential impurities of

This article includes online-only Supporting Information.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

\* Address correspondence to alanjames.bergmann@oekotoxzentrum.ch

Published online 21 June 2024 in Wiley Online Library

(wileyonlinelibrary.com).

DOI: 10.1002/etc.5934

ingredients and unanticipated transformation products. Many tire additives are employed for their reactivity, for example, vulcanizers or antioxidants. During production and use, the additives transform into various products that are still being extensively characterized (Seiwert et al., 2022). Thus, organic chemical constituents of tires and their transformation products may pose unintended hazards for aquatic biota. Effects of tire particles detected in *in vivo* laboratory studies have included mortality (Chow et al., 2019; Li et al., 2023), teratogenicity (Gualtieri, Andrioletti, Vismara, et al., 2005), endocrine disruption (Eriksson et al., 2022; Li et al., 2006; Wang et al., 2023), and genotoxicity (Gualtieri, Andrioletti, Mantecca, et al., 2005). Recently, species-specific mortality in stormwater-receiving streams was attributed to a tire-associated antiozonant transformation product, 2-((4-methylpentan-2-yl)amino)-5-(phenylamino)cyclohexa-2,5-diene-1,4-dione (6PPD-Q; Tian et al., 2021). The other laboratory and field studies of toxic environmental samples and tire leachates did not typically identify the responsible toxicant(s). However, commonly supposed culprits are known additives, such as benzothiazole and its derivatives (Halsband et al., 2020; Reemtsma et al., 1999; Sørensen et al., 2023).

Few previous studies have evaluated estrogenicity from tire particles. Li et al. (2006) found estrogenicity of tire-water leachates with an enzyme-linked reporter assay but not in a yeast estrogen screen (YES). They did not identify the responsible chemicals. Eriksson et al. (2022) observed estrogenic activity of tire particle extracts in organic solvent with ER $\alpha$ -CALUX. The responsible toxicants were also not identified, but the authors point to detected polycyclic aromatic compounds (PAHs) that have known estrogenicity and might contribute to bioactivity of tire particle extracts. Another study found no estrogenic activity but did report antiestrogenic effects of tire particle Soxhlet extracts (Zhang et al., 2002). In summary, although estrogenicity from tires has been observed, it has only occasionally been investigated, and the responsible chemicals remain only suggestive.

Genotoxicity has been a more studied effect of tire particles than estrogenicity. Damage to DNA was observed in *in vitro* studies with tire particles, their extracts, and leachates (Gualtieri, Andrioletti, Mantecca, et al., 2005; Karlsson et al., 2006; Poma et al., 2019) and *in vivo* studies with developing *Xenopus laevis* and adult *Fundulus heteroclitus* (Gualtieri, Andrioletti, Mantecca, et al., 2005; LaPlaca et al., 2022). Some known constituents of tire particles have been shown to be genotoxic, such as PAHs and their derivatives (LaPlaca et al., 2022) and benzothiazoles (Zeng et al., 2016). However, the chemicals causing genotoxicity in tire particle leachates are still unconfirmed, and potential toxicity is likely attributable to a mixture of known and the many unknown chemicals originating from tire tread.

Tire particles have also been reported to interfere with general metabolic activity of cells, potentially leading to cytotoxicity (Day et al., 1993; Gualtieri, Andrioletti, Mantecca, et al., 2005; Hartwell et al., 2000). A bacterial luminescence inhibition test (BLIT) with naturally luminescent bacteria is a sensitive method to detect disruption of cellular processes. In Germany,

a BLIT is recommended as the first step in ecotoxicity screening of construction material eluates (Deutsches Institut für Bautechnik, 2011), so it is an apt screening tool for materials in contact with the environment (Bell et al., 2020). Day et al. and Hartwell et al. showed that aqueous leachates of tires inhibited luminescence in BLIT, which was more severe for new tires than used (Day et al., 1993) and if leached into freshwater rather than salt water (Hartwell et al., 2000). Sørensen et al. (2023) found that methanol extracts of rubber products, including tires, were toxic to luminescent bacteria, more so than other plastic types. Despite detecting many chemicals in the toxic samples, only a minority could be tentatively identified. This exemplifies the need for additional methods to close the gap between biological effects and chemical drivers of toxicity.

Bioassays applied to high-performance thin-layer chromatography (HPTLC) can help link specific chemicals to toxic effects. By separating chemicals before applying a bioassay, HPTLC bioassays provide a profile, or chromatogram, of toxicity. They can thereby detect multiple bioactive substances, allowing comparison of individual chemicals between samples and to standard substances. These are sensitive methods, outperforming microtiter plate versions of bioassays (Bergmann et al., 2020; Meyer et al., 2021). The YES, genotoxicity detection with *umuC* SOS response (*umuC*), and BLIT are some of the best-established assays on HPTLC. Using the HPTLC-YES and -BLIT, Bell et al. (2021) attributed estrogenicity and bacterial luminescence inhibition to 4-*t*-butylphenol as the causative leachate from epoxy coatings. Others have used an HPTLC-*umuC* assay to help attribute genotoxicity of paper food packaging migrates to linoleic acid and its epoxides (Meyer et al., 2023).

To improve our understanding of the chemical profile of toxicity and which chemicals are driving the effects, we investigated toxic chemicals leaching from tire particles with HPTLC bioassays. We applied HPTLC-YES, -*umuC*, and -BLIT to determine estrogenicity, genotoxicity, and bacterial luminescence inhibition of extractable and leachable chemicals from cryogenically milled tire tread (CMTT), a surrogate for TRWP. Thermooxidized CMTT was also extracted to provide insight into the effects of aging processes. By comparing the chromatographic behavior of individual bioactive chemicals with the unknown toxicants in the CMTT samples, we help identify suspected chemical drivers of the effects. Our study expands the understanding of chemical hazards in tire particles and helps improve the link between those effects and the responsible chemicals.

## METHODS

### Materials

Eleven tire-associated chemicals (see Table 1; Supporting Information, Table S1) based on the work of Masset et al. (2022) were purchased and prepared in methanol or acetone at 0.5 to 1 g/L and tested in dilution series in HPTLC-YES, -*umuC*, and -BLIT. Growth media, buffers, and standards were prepared for HPTLC-YES and -*umuC* as described previously (Bergmann et al., 2020, 2023). Reagents for HPTLC-BLIT were prepared based on published protocols (Bell et al., 2020, 2021).

**TABLE 1:** Chemicals detected in cryogenically milled tire tread (CMTT) extracts and their bioactivity in high-performance thin-layer chromatography (HPTLC) bioassays

Compound	CASRN	Acronym	Indication of bioactivity		
			HPTLC-YES	HPTLC- <i>umuC</i> (-S9)	HPTLC-BLIT
<i>N</i> -(1,3-Dimethylbutyl)- <i>N'</i> -phenyl- <i>p</i> -phenylenediamine	793-24-8	6PPD	Yes <sup>a</sup>	No	Yes
2-((4-Methylpentan-2-yl)amino)-5-(phenylamino)cyclohexa-2,5-diene-1,4-dione	2754428-18-5	6PPD-Q	No	No	No
2-Mercaptobenzothiazole	149-30-4	SBT	No	No	Yes
2-(Methylthio)benzothiazole	615-22-5	MTBT	No	No	Yes
2,2'-Dithiobisbenzothiazole	120-78-5	MBTS	Yes <sup>a</sup>	No	Yes
Benzothiazole	95-16-9	BT	No	No	Yes
2-Aminobenzothiazole	136-95-8	ABT	No	No	Yes
2-Hydroxybenzothiazole	934-34-9	HBT	No	No	Yes
Aniline	62-53-3	ANI	No	No	Equivocal <sup>b</sup>
1,3-Diphenylguanidine	102-06-7	DPG	No	Yes	No
Cyclohexylamine	108-91-8	CHA	No	Yes	No

<sup>a</sup>Attributed to impurities or transformation products of the parent chemical (see Discussion section).

<sup>b</sup>Aniline produced a large, lightly suppressed zone in HPTLC-BLIT, possibly due to diffusion on the HPTLC plate.

BLIT = bacterial luminescence inhibition test; CASRN = Chemical Abstracts Service registration number; YES = yeast estrogen screen.

## CMTT generation

The generation of CMTT is described by Masset et al. (2022). New tires from three manufacturers (Pirelli Sottozero 3, Michelin Primacy 3, and Bridgestone Saetta Touring 2) were processed and combined into a mixture of particles. The upper layer of tire tread was removed and cut with scissors, then cryogenically milled into small particles. The particles had a mean size of 188 μm (see Masset et al., 2022 main text and supplemental data for particle characteristics). A subsample of CMTT was artificially aged with thermooxidation at 80 °C for 20 days as described previously (Klößner et al., 2021; Masset et al., 2022).

## CMTT extraction

Two hundred milligrams of CMTT were sequentially extracted with methanol and dichloromethane for 16 h each in a Soxhlet apparatus. The methanol and dichloromethane extracts were combined and reduced to 2 mL with a rotary evaporator (Büchi, Flawil, Switzerland). The final concentration was 100 mg CMTT equivalent/mL extract. Extracts, undiluted and diluted in methanol, were applied to HPTLC plates for HPTLC bioassays.

## Simulated digestion of CMTT

Simulated gastrointestinal digestion of CMTT was performed according to Masset et al. (2021, 2022). The CMTT (400 mg) was combined with 20 mL artificial gastric fluid and incubated at 20 °C with gentle agitation. After 3 h, 20 mL artificial intestinal fluid was added and incubated at 20 °C with gentle agitation for 24 h. The resulting digestate was filtered through 0.45-μm glass fiber filters to remove large particulates, then with Amicon<sup>®</sup> ultracentrifugation filters with a 10-kDa cutoff to remove biomolecules of the artificial digestate fluids. Aliquots were taken after each filtration step for spiking experiments to evaluate the efficiency of liquid–liquid extraction

(LLE; see LLE section below). The final concentration was 100 mg CMTT equivalent/mL digestate. Digestive fluids that were processed without the addition of CMTT served as a negative control for the digestion procedure. The digestates were prepared for HPTLC bioassays with LLE.

## Leachates of CMTT in sediment/water

Aqueous leachates of CMTT were prepared with and without sediment. For the former, 5 g CMTT was mixed with 20 g artificial sediment (Neogard<sup>®</sup> sand with 0.02% v/v Tetramin<sup>®</sup>) and 50 mL mineral water, as described in Masset et al. (2022). For leaching into only water, 5 g CMTT was combined with 50 mL mineral water. The CMTT was incubated with sediment/water or only water for 24 h at 20 °C. Aliquots of overlying water were collected in glass vials and filtered with 0.45-μm glass fiber filters. The final concentration of the leachates was 100 mg CMTT equivalent/mL leachate. As negative process controls, water with and without sediment that was never in contact with CMTT was processed the same as treated sediment and water. Leachates were prepared for HPTLC bioassays with LLE.

## Liquid chromatography–tandem mass spectrometry

Extracts, digestates, and leachates were analyzed with high-performance liquid chromatography (HPLC)–tandem mass spectrometry (Xevo TQ MS; Waters) for target chemicals according to Masset et al. (2022). The targeted chemicals are listed in Table 1.

## LLE

Digestates and leachates were exchanged to organic solvents with LLE to focus on organic compounds and reduce

matrix effects. In addition, we evaluated the effectiveness of LLE with aliquots of the samples spiked with genotoxicants and estrogenic chemicals (more in Supporting Information, Text S1). Digestates of CMTT in simulated gastrointestinal fluid, blank digestate fluid, and a nanopure water control were spiked with a mixture of known genotoxicants at 0.5 µg/mL: 4-nitroquinoline-*N*-oxide (4-NQO), nitrofurantoin, mitomycin-*C*, 2-nitrofluorene, 5-chloro-2-methyl-4-isothiazolin-3-one (CMIT), and nalidixic acid. Liquid–liquid extraction of CMTT leachates in sediment/water was additionally tested with spiked 17β-estradiol. The spiked samples were split in half (500 µL each). One aliquot was set aside to be tested as an aqueous sample.

Spiked and unspiked aliquots of digestates and leachates were processed with LLE as follows: 1 mL of 9:1 ethyl acetate:*n*-hexane was added to the aqueous aliquots and incubated at room temperature for 5 min, during which the samples were mixed by vortexing for a few seconds at 0, 2.5, and 5 min. The samples were centrifuged for 5 min at 5000 rcf before 900 µL of supernatant was carefully removed with a pipette and collected in separate amber glass vials, premarked at 500 µL. The process was repeated two times with 900 µL of fresh ethyl acetate:*n*-hexane added to the samples each time. The three iterative extracts were combined and concentrated from 2700 to 500 µL with a gentle stream of nitrogen and external heating at 40 °C. The concentration was maintained at 100 mg CMTT equivalent/mL, or 50 mg in 500 µL. Both the spiked aqueous samples and liquid–liquid extracts were tested in HPTLC-YES and *-umuC* bioassays by applying 50 µL to the HPTLC plates. Unspiked LLE samples of digestates and leachates were tested in all three HPTLC bioassays.

## HPTLC

HPTLC plates (Silica gel 60) were washed by developing with methanol to approximately 90 mm in a twin trough chamber (CAMAG, Muttenz, Switzerland), incubated at 110 °C in a drying oven (WTC Binder, Tuttlingen, Germany) for 0.5 h, and stored in aluminum foil at ambient conditions. Standard chemicals and samples were applied to HPTLC plates in 6-mm bands with an Automated TLC Sampler 4 (CAMAG) at 10 mm from plate bottom, at least 20 mm from the sides, and at least 12 mm between the centers of applied bands. For initial screening of samples in all bioassays, chromatographic development was performed with an Automated Multiple Development 2 (CAMAG): twice with methanol to 20-mm distance from plate bottom, acetone to 40 mm, 3:1 acetone:ethyl acetate to 50 mm, ethyl acetate to 60 mm, 2:1 ethyl acetate:*n*-hexane to 70 mm, 1:1 ethyl acetate:*n*-hexane to 80 mm. Atmospheric conditioning solution was 10 mL 25% NH<sub>4</sub>OH in 200 mL distilled deionized water.

## YES on HPTLC plates

As described previously, HPTLC-YES was performed (Bergmann et al., 2020). Briefly, yeast (*Saccharomyces cerevisiae*, as prepared by McDonnell et al. 1991) were incubated

in growth medium overnight and reconstituted in fresh medium, and 3 mL was applied to prepared HPTLC plates with a Derivatizer (CAMAG; red nozzle, level 6). After 3 h of incubation on HPTLC plates, the plates were dried and estrogenic signals were detected with 4-methylumbelliferyl-β-D-glucuronide (MUG) as described for HPTLC-*umuC*. A positive control (17β-estradiol) accompanied every plate at 0.8 pg. After an initial screening, the AMD2 chromatograph was adjusted to improve separation of estrogenic signals clustered near the solvent front: twice with methanol to 20 mm, 1:1 methanol:ethyl acetate to 40 mm, 1:1 ethyl acetate:*n*-hexane to 60 mm, 1:9 ethyl acetate:*n*-hexane to 80 mm. Images of HPTLC plates were collected after every step with a Visualizer 2 (CAMAG) with white light and 366 nm illumination. The HPTLC plate tracks were scanned for fluorescence with Scanner 3 (CAMAG) at 366 nm excitation.

## *umuC* on HPTLC plates

As described previously, HPTLC-*umuC* was performed (Bergmann et al., 2023). An aliquot of the bacterial morning culture was centrifuged, the supernatant was removed, and the bacteria were resuspended in fresh medium at a density of 380 ± 20 formazine attenuation units (FAU). A Derivatizer (CAMAG) with red nozzle at spraying level 6 was used to spray 3 mL of bacteria to a prepared HPTLC plate, and the plates were incubated at 37 °C for 2 h. After incubation, the HPTLC plate was removed from the incubator and dried. The Derivatizer was used with the blue nozzle at spraying level 6 to spray 2 mL of MUG (0.5 mg/mL in buffer) onto the HPTLC plate, which was then incubated at 37 °C and >90% relative humidity for 0.5 h. The HPTLC plate was removed and dried, then exposed to NH<sub>3</sub> vapor in a twin trough chamber with the silica layer facing a trough with 20 mL deionized water and 3 mL 25% NH<sub>4</sub>OH for 10 min. Detection of genotoxic signals was performed with a Visualizer 2 and Scanner 3 (CAMAG) as described for HPTLC-YES. Blank acetone or methanol was applied as a negative control to every plate. Two levels (0.31 and 2.5 ng/band) of 4-NQO served as positive controls with each HPTLC-*umuC* plate.

## Bacterial luminescence inhibition on HPTLC plates

The BLIT was established on HPTLC based on previous work (Azadnia & Morlock, 2019; Bell et al., 2021). Bacteria (*Aliivibrio fischeri*) were stored at –80 °C in cryostocks of 300 µL of a growth culture that had reached at least 10<sup>8</sup> luminescence units (Infinite 200; Tecan, Männedorf, Switzerland). Bacterial cells for a bioassay were prepared by thawing a cryostock and adding 200 µL to 20 mL of *A. fischeri* growth medium. The bacterial culture was incubated at 20 °C, 150 rpm, and 60% humidity for at least 24 h. The optical density and the luminescence of the bacteria were evaluated in a microplate reader (Infinite 200; Tecan). If the luminescence was above 10<sup>8</sup> units and FAU was above 1500, we proceeded with the bioassay. Developed HPTLC plates were allowed to dry at room conditions for at



least 2 h before bacteria were applied. 3,5-Dichlorophenol (65 ng) and caffeine (625 ng) served as positive controls. Then, 3 mL of overnight culture was pipetted directly from the overnight culture flask into a red Derivatizer nozzle and sprayed with level 6. After spraying and settling of the bacterial mist, the plate was removed from the Derivatizer and immediately documented with the Bioluminizer (CAMAG), a dark chamber with a cooled charge-coupled device camera. Ten 60-s images were collected with 1.1-min intervals.

## Data evaluation

The HPTLC-*umuC* and HPTLC-YES bioassay track profiles were generated and evaluated using VisionCats (Ver. 2.4). The HPTLC-BLIT track profiles were generated with ImageJ (Ver. 1.54d; Schneider et al., 2012) and further processed using Excel (Microsoft Office, 2016) (described further in caption to Supporting Information, Figure S9). Results of the HPTLC bioassay were assessed qualitatively to determine the presence and absence of estrogenic, genotoxic, and luminescence inhibiting chemicals. Background variation was determined in the HPTLC-bioassay chromatogram adjacent to fluorescence or inhibition zones of interest. Signals >3 times the background variation in duplicate HPTLC plates were further considered in comparison to the positive control. Fluorescent signals were considered bioactive if they reached peak heights of at least 10% of the positive controls, which were applied at amounts targeting 100% effect (0.8 pg estradiol or 2.5 ng 4-NQO) based on previous work (Bergmann et al., 2020, 2023). Potentially interfering native fluorescence was determined by imaging HPTLC plates before applying MUG.

## RESULTS

### Sample preparation, LLE

Standard genotoxicants were spiked in blank and CMTT digestates at 0.5 µg/mL. Both the spiked aqueous digestates and liquid–liquid extracts were active in HPTLC-*umuC* (Supporting Information, Figure S1). Genotoxicants in spiked water were all detected, as compared with the reference mixture in organic solvent. In contrast, the aqueous spiked digestate control produced a dark zone near retention factor (Rf)=0 that possibly interfered with detection of nalidixic acid (Rf=0.1) and disrupted chromatography of the other spiked chemicals. Notably, CMIT was reduced in aqueous sample signals compared with the reference mixture. However, digestate fluid that was processed with Amicon filters allowed detection of CMIT. Spiked samples processed with LLE did not have a dark zone at Rf=0, and every spiked chemical was detected. It is possible that acidic components of the aqueous digestates interfered with the yeast response, resulting in the dark zones, which were not extracted during LLE. However we did not further investigate the cause of these dark signals. Amicon filtered samples in LLE showed additional improvement to the CMIT signal. There was no apparent difference between CMTT digestates and the digestate control. In addition, LLE improved chromatography of unknown

estrogenic chemicals in digestion simulants compared with application as aqueous samples (Supporting Information, Figure S2). Spiked 17β-estradiol was detected in all leachate samples (Supporting Information, Figure S3).

## Estrogenicity

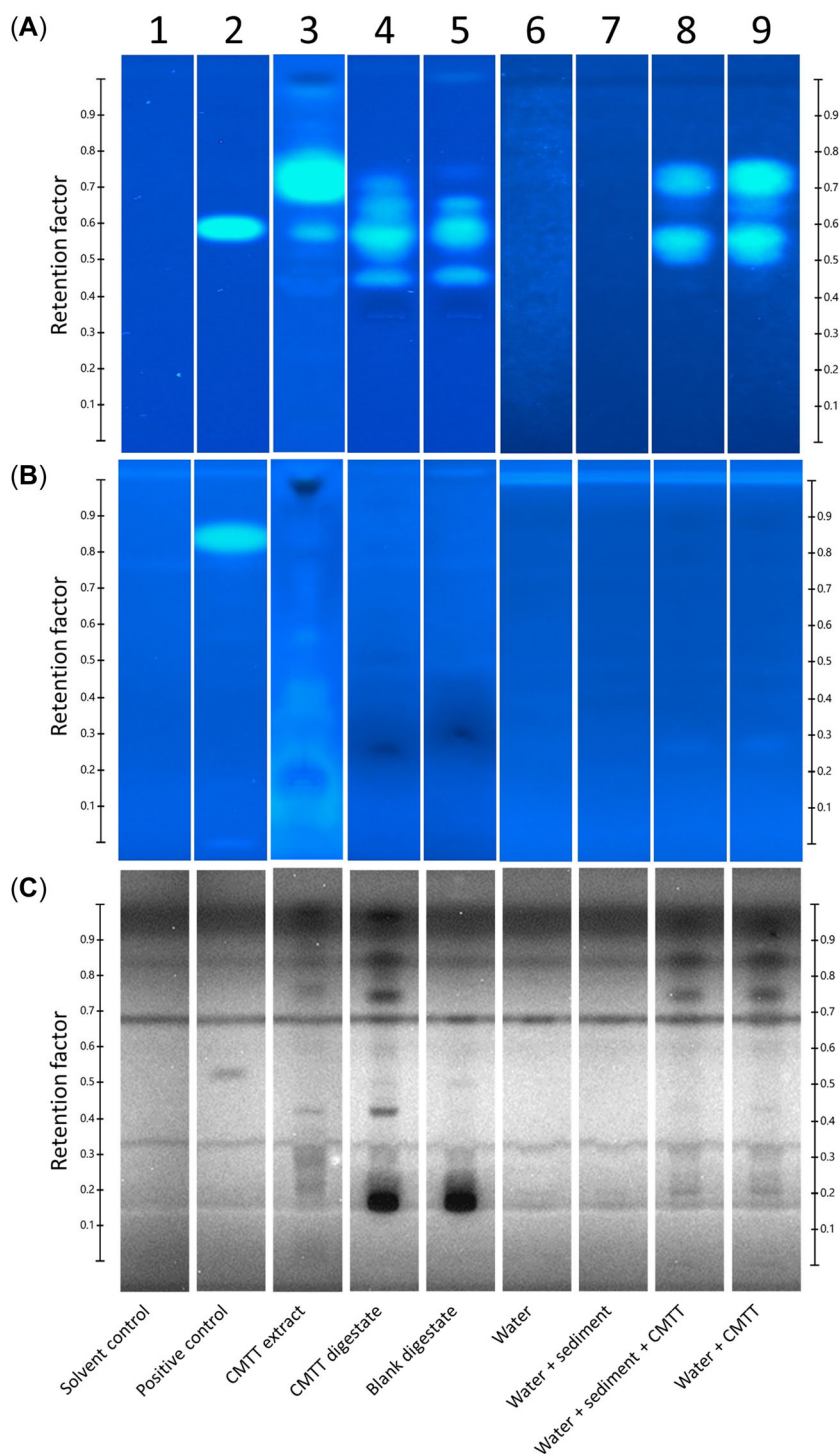
Native bioactivity of CMTT extracts, digestates, and leachates is shown in Figure 1. The HPTLC-YES assay detected at least two estrogenic zones in CMTT extracts at Rfs of 0.58 and 0.70 (Figure 1A, Track 3). Modification of the chromatographic method after initial screening lowered the Rfs of the estrogenic zones but did not further separate the bioactive zones. Native chemical fluorescence of the samples did not interfere with these bioassay signals (Supporting Information, Figure S4). Extracts of thermooxidized CMTT were similar in activity to unaged CMTT (Supporting Information, Figure S5).

Both CMTT and blank digestates were estrogenic with a similar band pattern (Tracks 4 and 5 in Figure 1A). A band unique to the digestate of CMTT can be seen as a shoulder band at Rf=0.70 and most closely matches the Rf of the large estrogenic zone of the CMTT extract. The estrogenic zones of CMTT and blank digestates at Rf=0.58 overlap with the lower zone of the CMTT extract (Track 3). Therefore, despite chromatography, estrogenicity in the digestate process control confounds complete interpretation of the CMTT digestate. By testing individual components of the digestate fluid, we determined that the blank estrogenicity was due to biologically sourced components of the digestates: porcine bile (likely containing endogenous steroids) and pancreatin (Supporting Information, Figure S6).

The process controls for water and sediment overlying water were both inactive in the HPTLC-YES (Figure 1A, Tracks 6 and 7). Two estrogenic zones were induced by CMTT leachates in water and sediment overlying water at Rfs 0.58 and 0.70 (Tracks 8 and 9). Overall, estrogenicity was observed in all CMTT-associated samples at Rf=0.58 and 0.70, although background estrogenicity may partially confound analysis of simulated digestates of CMTT.

## Genotoxicity

Several genotoxic zones were detected in extracts of CMTT tested with HPTLC-*umuC* (Figure 1B, Track 3). Three zones were above 10% of the intensity of the positive control. A diffuse zone at approximately Rf=0.2 was the strongest signal in the shape of a ring, which can form at high concentrations of a bioactive substance (Bergmann et al., 2020). However, native chemical fluorescence was observed at this Rf before the bioassay occurred (Supporting Information, Figure S7), which might have contributed to the final fluorescence signal. Zones at Rf=0.35 and Rf=0.40 were also above 10% of the positive control peak height, and native fluorescence did not match their band pattern. The native fluorescence was also not visible in the step between incubation with bacteria and signal detection with MUG. A possible genotoxic zone was apparent at Rf=0.55 but below 10% of the positive control, so it was not further



**FIGURE 1:** High-performance thin-layer chromatography (HPTLC) bioassay images of tire particle extracts, digestates, and water/sediment leachates. **(A)** Yeast estrogen screen (YES), positive control:  $17\beta$ -estradiol 4 pg. **(B)** *umuC*, positive control: 4-nitroquinoline-*N*-oxide 2.5 ng. **(C)** Bacterial luminescence inhibition test (BLIT), positive control: caffeine 625 ng. Cryogenically milled tire tread (CMTT) Soxhlet extract at 0.5 mg CMTT equivalent (YES), 1 mg (*umuC*), and 0.5 mg (BLIT). Aqueous samples (digestates, water, and sediment leachates and corresponding process controls) were extracted with liquid-liquid extraction into ethyl acetate:*n*-hexane prior to HPTLC bioassay testing and applied at 5 mg CMTT equivalent.

considered. Thermooxidized CMTT induced a similar response to unaged CMTT in HPTLC-*umuC*, with the same number and profile of bioactivity (Supporting Information, Figure S8).

No fluorescence induction was observed in aqueous leachates up to 5 mg CMTT equivalent. However, potential interferences, in the form of dark zones at  $R_f = 0.28$ , were observed

in the digestate samples. These might have obscured co-retained genotoxic chemicals. Sediment/water leachates showed minor fluorescence at  $R_f = 0.28$  but at levels below 10% of the positive control, so they were not considered bioactive. Overall, induction of genotoxicity was observed in CMTT extracts but not in aqueous leachates of CMTT.

## Bacterial luminescence inhibition

Bacterial luminescence was inhibited by every CMTT sample (Figure 1C). However, dark bands were seen across the plates, likely due to residual chemicals concentrating at the solvent front, possibly including chromatography solvents, to which the luminescent bacteria are sensitive. We attempted to adjust the plate conditioning with different solvents to remove the lines, which was unsuccessful. Others have observed similar background issues for which subtraction improved comparability between samples (Supporting Information, Figure S9; Schulz et al., 2017). After correction, the toxicity profile of CMTT extracts was dominated by a band at  $R_f=0.74$ , a weaker response at  $R_f=0.43$ , and uneven inhibition below  $R_f=0.3$ . Extracts of thermooxidized CMTT induced similar toxicity profiles to the unaged CMTT extracts (Supporting Information, Figure S10).

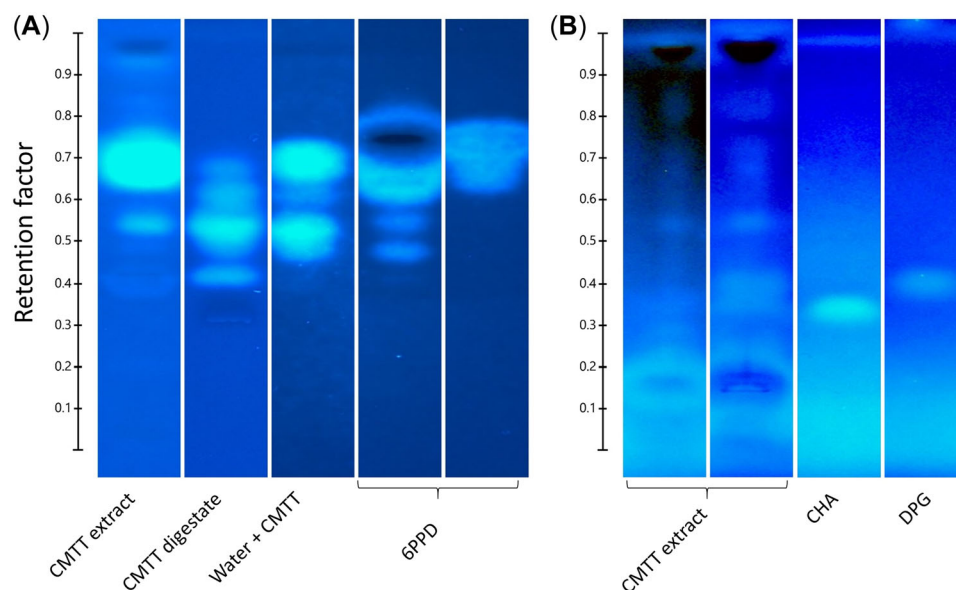
Digestates and leachates of CMTT also inhibited bacterial luminescence. Inhibition zones were observed at  $R_f=0.73$  and 0.41, matching those in CMTT extracts. The effective zone at  $R_f=0.41$  was strongest in CMTT digestates. There were also background effects of digestate blank apparent in the HPTLC-BLIT as an inhibition zone at approximately  $R_f=0.2$ . This background did not interfere with the zones of interest at  $R_f$ s 0.41 and 0.73 but could hinder interpretation of any effect of CMTT at lower  $R_f$ s. Although leachates showed only slight indication of inhibiting luminescence at  $R_f=0.41$ , a band at  $R_f=0.73$  was observed in every sample. Overall, similar profiles of toxicity to bacteria were observed in all CMTT-associated samples.

## Chemical testing

Chemicals preliminarily detected in CMTT extracts by Masset et al. (2022) are shown in Table 1 with the results of

testing in the HPTLC bioassays. Chemical analysis results for these chemicals in CMTT extracts and leachates are displayed in Supporting Information, Table S1. Two chemicals, *N*-(1,3-dimethylbutyl)-*N'*-phenyl-*p*-phenylenediamine (6PPD) and 2-2'-dithiobisbenzothiazole (MBTS), initially produced estrogenic responses in the HPTLC-YES. Because of the presence of multiple bands in the HPTLC-YES and reports of these chemicals' instability (Seiwert et al., 2022; ToxServices, 2021), we repeated estrogenicity testing of 6PPD and MBTS with freshly purchased and prepared stock solutions. Fresh solutions of MBTS were not bioactive, disconfirming estrogenicity. In contrast, freshly prepared 6PPD was still bioactive with multiple bands (e.g., Figure 2A). A now well-known ozonation transformation product of 6PPD, 6PPD-Q (Tian et al., 2021), was not estrogenic in the HPTLC-YES.

Two chemicals, diphenylguanidine (DPG) and cyclohexylamine (CHA), produced fluorescent signals in HPTLC-*umuC* (Figure 2B). Both chemicals also shared  $R_f$ s with unknown genotoxicants in CMTT extracts,  $R_f=0.40$  and 0.35, respectively, indicating that they were possibly responsible. In CMTT extracts DPG was detected at 440  $\mu\text{g}/\text{mL}$ . We applied 10  $\mu\text{L}$  of CMTT extract to HPTLC plates, equaling approximately 4.4  $\mu\text{g}$  DPG. This dose was more than 6 times greater than the lowest active dose of DPG (0.55  $\mu\text{g}$ ; Supporting Information, Figure S11) we observed in HPTLC-*umuC*. We can therefore expect DPG to likely be responsible for bioactivity in the CMTT extract at  $R_f=0.40$ . In contrast, CHA was active down to 0.22  $\mu\text{g}$  individually but only in the CMTT extract at 0.14  $\mu\text{g}$  on the plate, which is evidence against CHA as a responsible chemical. The doses of DPG in digestates and leachates were 0.27 and 0.12  $\mu\text{g}$ , respectively; therefore, it is not expected to be bioactive at the CMTT doses tested.



**FIGURE 2:** Individual chemicals possibly explain a part of estrogenicity and genotoxicity extracted and leached from cryogenically milled tire tread (CMTT). (A) Selected high-performance thin-layer chromatography (HPTLC)–yeast estrogen screen results of CMTT compared with *N*-(1,3-dimethylbutyl)-*N'*-phenyl-*p*-phenylenediamine tested at 11 and 1.1  $\mu\text{g}$ , left to right. (B) Selected HPTLC-*umuC* images of CMTT extracts (0.25 and 1 mg CMTT equivalents, left to right) compared with cyclohexylamine at 0.64  $\mu\text{g}$  and 1,3-diphenylguanidine at 0.65  $\mu\text{g}$ . See Figure 1 for representative negative and positive controls. 6PPD = *N*-(1,3-dimethylbutyl)-*N'*-phenyl-*p*-phenylenediamine; CHA = cyclohexylamine; DPG = diphenylguanidine.

All of the tested benzothiazoles and 6PPD were active in the HPTLC-BLIT (Figure 3). As with the estrogen results, 6PPD induced multiple bands of luminescence inhibition. Some benzothiazoles (2-mercaptobenzothiazole, MBTS) also had multiple bands, indicating potential impurities or transformation products. Some compound bands (e.g., benzothiazole, 6PPD) shared an Rf with the unknown band at 0.73 so were possibly responsible for activity in the CMTT samples. No tested chemical matched the luminescence inhibition of CMTT samples at Rf=0.41. Aniline seemed to diffuse on the plate, producing a large zone of slightly inhibited luminescence. It is therefore labeled “equivocal” (Table 1).

## DISCUSSION

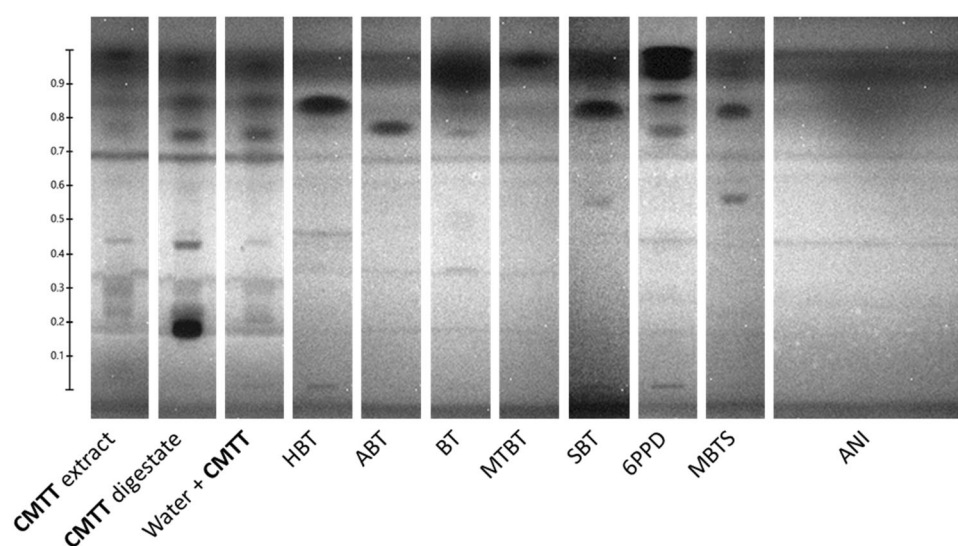
### CMTT extracts and leachates are bioactive

We evaluated estrogenicity, genotoxicity, and bacterial luminescence inhibition of chemicals extracted and leaching from CMTT. We observed that Soxhlet extracts of CMTT induced all three studied effects, indicating the presence of diverse bioactive chemicals in tire tread. However, only two bioactive zones were observed for any bioassay. Therefore, it is likely that only a few substances are responsible for the majority of the observed effects. Still, we cannot exclude coretenion of bioactive compounds at the same Rf, and some active compounds may not exceed our detection threshold. Therefore, the exact number of responsible chemicals remains unknown. The bioactive chemicals were also separated from natively fluorescent and colored chemicals, so we expect that only a minority of CMTT-originating chemicals were retained at bioactive zones. Further investigation of the bioactive zones could include more refined chromatography, through, for example, two-dimensional

chromatography. That CMTT extracts were bioactive means that estrogenic chemicals, genotoxicants, and antibacterial chemicals are present in tire tread. However, the bioavailability of these bioactive compounds in the environment is not addressed by the method applied in the present study, which relies on organic extracts.

Estrogenic and luminescence-inhibiting chemicals did leach from CMTT into the aqueous media: simulated digestive fluids and sediment/water. After subtracting for background effects of bioassay artifacts and digestate components, similar patterns were seen between sample types. Consequently, it may be that the same chemicals are responsible for estrogen activity in CMTT extracts, digestates, and leachates. (Xeno)Estrogens and antibacterial chemicals clearly have potential to be available to organisms that are in contact with very high concentrations of tire particles ambiently in water or after ingestion.

No genotoxicity was detected in aqueous leachates, suggesting that direct-acting DNA damage is not as likely to be a risk as estrogenicity. However, other studies have observed genotoxicity *in vitro* and *in vivo* from leachates of tire particles (Gualtieri, Andrioletti, Mantecca, et al., 2005; LaPlaca et al., 2022; Poma et al., 2019). We opted for *umuC* as a measure of genotoxicity because it captures a relatively broad spectrum of genotoxic endpoints and is established on HPTLC plates. One study observed similar results to ours, using a similar bioassay to the *umuC* SOS test, in that they did not see genotoxicity of tire leachates with or without metabolic activation (Day et al., 1993). Overall, we and others have shown that genotoxic chemicals can be found in tire particles. Differing study parameters, such as source, aging, and leaching of tire particles or bioassay protocol, could explain differences in detecting genotoxicity in aqueous leachates of tire particles.



**FIGURE 3:** High-performance thin-layer chromatography–bacterial luminescence inhibition test of active single chemicals compared with cryogenically milled tire tread samples. Chemical amounts are nominally 0.55  $\mu\text{g}$  (HBT), 0.50  $\mu\text{g}$  (ABT), 5.15  $\mu\text{g}$  (BT), 0.5  $\mu\text{g}$  (MTBT), 0.66  $\mu\text{g}$  (SBT), 0.7  $\mu\text{g}$  (6PPD), 0.14  $\mu\text{g}$  (MBTS), and 4.2  $\mu\text{g}$  (ANI). See Figure 1 for representative negative and positive controls. CMTT = cryogenically milled tire tread; HBT = 2-hydroxybenzothiazole; ABT = 2-aminobenzothiazole; BT = benzothiazole; MTBT = 2-(methylthio)benzothiazole; SBT = 2-mercaptobenzothiazole; 6PPD = *N*-(1,3-dimethylbutyl)-*N*'-phenyl-*p*-phenylenediamine; MBTS = 2,2'-dithiobisbenzothiazole; ANI = aniline.



## Thermooxidation did not affect bioactivity

Thermooxidation of CMTT did not produce or eliminate bioactive bands. Small degrees of transformation may have occurred and gone undetected in these bioassays, but the drivers of estrogenicity, genotoxicity, and bacterial luminescence inhibition seem to be unchanged. As reported previously and shown in Supporting Information, Table S1, thermooxidation of particles resulted in small differences in chemical leaching but did not result in significant differences in solubilization (Masset et al., 2022). Our methods targeted large changes in the drivers of effects, so smaller effects of aging on the solubilization of organic chemicals (Masset et al., 2022) may not be detected.

## Bioactivity is linked to individual chemicals

We linked known tire-associated chemicals with bioactivity in CMTT extracts and leachates. In fact, all but one chemical were active in at least one of three bioassays. The lone inactive chemical was 6PPD-Q, now well known for its toxicity to some salmonid species (Tian et al., 2021). So, although a concern for fish toxicity, 6PPD-Q does not drive the estrogenicity, genotoxicity, or bacterial luminescence inhibition observed in these HPTLC bioassays. Still, we observed multiple estrogenic bands when testing standard solutions of 6PPD-Q's parent compound, 6PPD, which is not expected to be estrogenic because it does not have hydroxyl or primary amine groups (ToxServices, 2021). However, many abiotic oxidation products of 6PPD besides 6PPD-Q have been identified or proposed, some, such as 4-aminodiphenylamine and 4-hydroxydiphenylamine, with moieties common among estrogenic chemicals (Seiwert et al., 2022; Zhao et al., 2023). That many tire-associated chemicals are inherently reactive to aid in producing tires or protect from oxidation (Tian et al., 2021) makes it plausible to find impurities or transformation products even in standards. The nature of HPTLC also means the chemicals have open contact with air between analysis steps, adding potential for transformation of test chemicals. The same transformation products may also occur in CMTT and TRWP during analysis and in the environment. Therefore, HPTLC reveals relevant hazards such as endocrine-disrupting potential of 6PPD transformation products.

In the HPTLC-*umuC*, DPG was active for genotoxicity at the same Rf as a bioactive zone of CMTT extracts. This, plus levels of DPG in CMTT extracts above bioassay detection limits, supports DPG as a suspect genotoxicant in CMTT extracts. It is not expected to be genotoxic according to the Organisation for Economic Co-operation and Development (2007), but DPG was weakly positive in trials with *Salmonella* mutagenicity assays, National Toxicology Program (2023) and records of mutagenicity in the presence of hamster S9 were reported to the European Chemicals Agency (2010). Although DPG is implicated as a responsible genotoxicant in CMTT extracts, it would need to be confirmed with future chemical analysis of active fractions. Additional genotoxic bands of the CMTT extracts remain unexplained and warrant investigation. However, no genotoxicity was detected in aqueous samples, so further

identification efforts of the genotoxic chemicals in CMTT extracts might not be deemed as important as toxicants that clearly leached to water, such as estrogens.

Benzothiazoles and 6PPD were able to inhibit bacterial luminescence. Benzothiazoles have been linked to antibacterial effects in previous studies with tires and industrial effluent (Day et al., 1993; Reemtsma et al., 1999). Because they are common and abundant in tires, hazard from benzothiazoles is relevant for the environment. When benzothiazoles leach to aquatic systems, their toxicity to bacteria may directly contribute to disruption of biofilm communities. Although the present study implicated several individual chemicals, unequivocal identification of the responsible chemicals will require further investigation. Target and nontarget chemical analyses of the bioactive zones, in an effect-directed analysis approach, could be successful in identifying remaining responsible chemicals (Brack et al., 2016).

## HPTLC elucidates complex samples in ecotoxicity evaluations

The HPTLC bioassays test that in a complex mixture, a minority of chemicals will drive particular toxic effects. Other studies have attempted to characterize the chemicals in tire particle extracts and leachates, finding many hundreds to thousands (Müller et al., 2022; Sørensen et al., 2023; Tian et al., 2021). Yet, we only observed a few bioactive bands in each HPTLC bioassay. So, without identifying the responsible substance(s), HPTLC already suggests that few chemicals are driving the toxicity. However, the precise number of chemicals causing bioactivity is unknown because chemicals may be co-retained in the active fractions. The HPTLC bioassay distinguished several bioactive fractions from each other and from background toxicity. This was demonstrated for CMTT digestates, which had interfering toxicity in the HPTLC-YES and HPTLC-BLIT. We observed that estrogenic chemicals and bacterial luminescence inhibitors in CMTT digestates were separated from the interferences and linked by retention factor to the CMTT extracts and sediment/water leachates. Also, HPTLC linked bioactivity to individual chemicals, although the definitive responsible toxicants are yet to be confirmed. Follow-up experiments with two-dimensional HPTLC or HPLC fractionation could further refine separation and possibly help further elucidate causative toxicants. The bioassay endpoints available on HPTLC are limited but growing (Klingelhöfer et al., 2021; Riegraf et al., 2019). Further development will allow insights to the chemicals causing other effects of tire particles, such as disruption of the aryl hydrocarbon receptor (Eriksson et al., 2022).

## Limitations

Our study used CMTT as a model of tire particles, which does not contain material from road surface, brakes, or natural matter, as TRWP do. Using CMTT helps our study isolate effects due to chemicals that originate in tires but does not address how those chemicals might interact with other components of TRWP.

Particles of CMTT have different shapes and sizes from TRWP, which might play a role in chemical leaching. We only addressed the effect of particle aging in a limited way with thermooxidation of CMTT. Additional aging processes, such as ultraviolet light exposure (Weyrauch et al., 2023), should be evaluated for toxic transformation products in future work. The aqueous samples in the present study were prepared at 100 g/L CMTT in water. This concentration of CMTT is much higher than recorded in surface water, sediment, or fish digestive systems. Therefore, although our results demonstrate that bioactive chemicals might leach to water from tire particles, we do not yet know the degree of leaching in more environmentally relevant scenarios. In addition, our detection methods may be blind to some bioactive chemicals because the open layer of HPTLC is not suited for testing volatile substances. By further monitoring the ecotoxicological effects of tire particles and identifying chemicals that are responsible, we can accurately develop risk models for chemicals leaching from tires.

## CONCLUSION

Cryogenically milled tire tread contains toxic substances. Specifically, we have seen multiple estrogenic, genotoxic, and antibacterial compounds in organic solvent extracts of CMTT. Effects were also observed in aqueous media after contact with CMTT. This suggests that estrogenic and antibacterial chemicals can become available to aquatic organisms ambiently or after ingestion. Through HPTLC profiles, we linked suspect chemicals to effects. Chemicals commonly associated with tire particles, such as DPG and benzothiazoles, likely contribute to toxic hazards from tires. Our study shows that estrogenicity is a concern alongside acute toxic effects of the transformation products of 6PPD. Further work will be needed to definitively identify all of the chemicals responsible for toxicity and evaluate their risk in natural aquatic systems.

**Supporting Information**—The Supporting Information is available on the Wiley Online Library at <https://doi.org/10.1002/etc.5934>.

**Acknowledgments**—The present study—including the production, handling, distribution, and information related to the CMTT—was funded by the World Business Council for Sustainable Development's Tire Industry Project, a global chief executive officer–led initiative undertaken by leading tire manufacturing companies that drives research on potential human health and environmental impacts of tires throughout their life cycle. The study design, execution, interpretation, and manuscript preparation were conducted solely by the authors. The authors declare they have no other conflict of interest. The authors gratefully acknowledge the assistance of A. Schifferli and N. Bramaz (Ecotox Centre, Dübendorf, Switzerland) for practical support with the HPTLC-BLIT assay and the research group of T. Reemtsma at UFZ–Leipzig for producing thermooxidized CMTT. Open access funding provided by ETH-Bereich Forschungsanstalten.

**Author Contribution Statement**—**Alan J. Bergmann**: Conceptualization; Formal analysis; Investigation; Methodology; Visualization; Writing—original draft. **T. Masset**: Conceptualization; Investigation; Methodology; Writing—review & editing. **F. Breider, K. Schirmer**: Conceptualization; Funding acquisition; Supervision; Writing—review & editing. **W. Dufefoi**: Conceptualization; Writing—review & editing. **B. J. D. Ferrari**: Conceptualization; Funding acquisition; Project administration; Supervision; Writing—review & editing. **E. L. E. Vermeirssen**: Conceptualization; Supervision; Writing—review & editing.

**Data Availability Statement**—Data will be made available on request to the corresponding author ([alanjames.bergmann@oekotozentrum.ch](mailto:alanjames.bergmann@oekotozentrum.ch)).

## REFERENCES

- Azadnia, E., & Morlock, G. E. (2019). Automated piezoelectric spraying of biological and enzymatic assays for effect-directed analysis of planar chromatograms. *Journal of Chromatography A*, 1602, 458–466.
- Beddows, D. C. S., & Harrison, R. M. (2021). PM10 and PM2.5 emission factors for non-exhaust particles from road vehicles: Dependence upon vehicle mass and implications for battery electric vehicles. *Atmospheric Environment*, 244, Article 117886.
- Bell, A. M., Baier, R., Kocher, B., Reifferscheid, G., Buchinger, S., & Ternes, T. (2020). Ecotoxicological characterization of emissions from steel coatings in contact with water. *Water Research*, 173, Article 115525.
- Bell, A. M., Keltch, N., Schweyen, P., Reifferscheid, G., Ternes, T., & Buchinger, S. (2021). UV aged epoxy coatings—Ecotoxicological effects and released compounds. *Water Research X*, 12, Article 100105.
- Bergmann, A. J., Breitenbach, M., Muñoz, C., Simon, E., McCombie, G., Biedermann, M., Schönborn, A., & Vermeirssen, E. L. M. (2023). Towards detecting genotoxic chemicals in food packaging at thresholds of toxicological concern using bioassays with high-performance thin-layer chromatography. *Food Packaging and Shelf Life*, 36, Article 101052.
- Bergmann, A. J., Simon, E., Schifferli, A., Schonborn, A., & Vermeirssen, E. L. M. (2020). Estrogenic activity of food contact materials—evaluation of 20 chemicals using a yeast estrogen screen on HPTLC or 96-well plates. *Analytical and Bioanalytical Chemistry*, 412(19), 4527–4536.
- Brack, W., Ait-Aissa, S., Burgess, R. M., Busch, W., Creusot, N., Di Paolo, C., Escher, B. I., Hewitt, L. M., Hilscherova, K., Hollender, J., Hollert, H., Jonker, W., Kool, J., Lamoree, M., Muschket, M., Neumann, S., Rostkowski, P., Ruttkies, C., Schollee, J., ... Krauss, M. (2016). Effect-directed analysis supporting monitoring of aquatic environments—An in-depth overview. *Science of the Total Environment*, 544, 1073–1118.
- Chow, M. I., Lundin, J. I., Mitchell, C. J., Davis, J. W., Young, G., Scholz, N. L., & McIntyre, J. K. (2019). An urban stormwater runoff mortality syndrome in juvenile coho salmon. *Aquatic Toxicology*, 214, Article 105231.
- Day, K. E., Holtze, K. E., Metcalfe-Smith, J. L., Bishop, C. T., & Dutka, B. J. (1993). Toxicity of leachate from automobile tires to aquatic biota. *Chemosphere*, 27(4), 665–675.
- Deutsches Institut für Bautechnik. (2011). *Grundsätze zur bewertung der auswirkungen von bauprodukten auf boden und grundwasser*.
- Eriksson, U., Titaley, I. A., Engwall, M., & Larsson, M. (2022). Examination of aryl hydrocarbon receptor (AhR), estrogenic and anti-androgenic activities, and levels of polyaromatic compounds (PACs) in tire granulates using in vitro bioassays and chemical analysis. *Chemosphere*, 298, Article 134362.
- European Chemicals Agency. (2010). 1,3-Diphenylguanidine. Retrieved September 11, 2023, from: <https://echa.europa.eu/da/registration-dossier/-/registered-dossier/14992/1/2>
- Gualtieri, M., Andrioletti, M., Mantecca, P., Vismara, C., & Camatini, M. (2005). Impact of tire debris on in vitro and in vivo systems. *Particle and Fibre Toxicology*, 2(1), Article 1.
- Gualtieri, M., Andrioletti, M., Vismara, C., Milani, M., & Camatini, M. (2005). Toxicity of tire debris leachates. *Environment International*, 31(5), 723–730.

- Halsband, C., Sørensen, L., Booth, A. M., & Herzke, D. (2020). Car tire crumb rubber: Does leaching produce a toxic chemical cocktail in coastal marine systems? *Frontiers in Environmental Science*, 8, Article 125.
- Hartwell, S. I., Jordahl, D. M., & Dawson, C. E. O. (2000). The effect of salinity on tire leachate toxicity. *Water, Air, and Soil Pollution*, 121(1), 119–131.
- Karlsson, H. L., Ljungman, A. G., Lindbom, J., & Moller, L. (2006). Comparison of genotoxic and inflammatory effects of particles generated by wood combustion, a road simulator and collected from street and subway. *Toxicology Letters*, 165(3), 203–211.
- Klingelhöfer, I., Ngoc, L. P., van der Burg, B., & Morlock, G. E. (2021). A bioimaging system combining human cultured reporter cells and planar chromatography to identify novel bioactive molecules. *Analytica Chimica Acta*, 1183, Article 338956.
- Klöckner, P., Seiwert, B., Wagner, S., & Reemtsma, T. (2021). Organic markers of tire and road wear particles in sediments and soils: Transformation products of major antiozonants as promising candidates. *Environmental Science & Technology*, 55(17), 11723–11732.
- LaPlaca, S. B., Rice, C. D., & van den Hurk, P. (2022). Chronic toxicity of tire crumb rubber particles to mummichog (*Fundulus heteroclitus*) in episodic exposures. *Science of the Total Environment*, 846, Article 157447.
- Li, J., Xu, J., & Jiang, X. (2023). Urban runoff mortality syndrome in zooplankton caused by tire wear particles. *Environmental Pollution*, 329, Article 121721.
- Li, W., Seiffert, M., Xu, Y., & Hock, E. (2006). Assessment of estrogenic activity of leachate from automobile tires with two in vitro bioassays. *Fresenius Environmental Bulletin*, 15(1), 74–79.
- Masset, T., Ferrari, B. J. D., Dudefoi, W., Schirmer, K., Bergmann, A., Vermeirssen, E., Grandjean, D., Harris, L. C., & Breider, F. (2022). Bioaccessibility of organic compounds associated with tire particles using a fish in vitro digestive model: Solubilization kinetics and effects of food coingestion. *Environmental Science & Technology*, 56(22), 15607–15616.
- Masset, T., Ferrari, B. J. D., Oldham, D., Dudefoi, W., Minghetti, M., Schirmer, K., Bergmann, A., Vermeirssen, E., & Breider, F. (2021). In vitro digestion of tire particles in a fish model (*Oncorhynchus mykiss*): Solubilization kinetics of heavy metals and effects of food coingestion. *Environmental Science & Technology*, 55(23), 15788–15796.
- McDonnell, D. P., Nawaz, Z., Densmore, C., Weigel, N. L., Pham, T. A., Clark, J. H., & O'Malley, B. W. (1991). High level expression of biologically active estrogen receptor in *Saccharomyces cerevisiae*. *Journal of Steroid Biochemistry and Molecular Biology*, 39(3), 291–297.
- Meyer, D., Marin-Kuan, M., Debon, E., Serrant, P., Cottet-Fontannaz, C., Schilter, B., & Morlock, G. E. (2021). Detection of low levels of genotoxic compounds in food contact materials using an alternative HPTLC-SOS-Umu-C assay. *ALTEX*, 38(3), 387–397.
- Meyer, D., Marin-Kuan, M., Mayrhofer, E., Kirchnawy, C., Debon, E., Latado, H., Patin, A., Schilter, B., & Morlock, G. (2023). Effect-detection by planar SOS-Umu-C genotoxicity bioassay and chemical identification of genotoxins in packaging migrates, proven by microtiter plate assays SOS-Umu-C and Ames-MPF. *Food Control*, 147, Article 109546.
- Microsoft Office. (2016). Computer software. Microsoft.
- Müller, K., Hübner, D., Huppertsberg, S., Knepper, T. P., & Zahn, D. (2022). Probing the chemical complexity of tires: Identification of potential tire-borne water contaminants with high-resolution mass spectrometry. *Science of the Total Environment*, 802, Article 149799.
- National Toxicology Program. (2023). Ames Conclusions. Retrieved October 5, 2023, from <https://doi.org/10.22427/NTP-DATA-022-00002-0001-000-7>
- Organisation for Economic Co-operation and Development. (2007). *OECD existing chemicals database: 1,3-Diphenylguanidine*. [https://hpvchemicals.oecd.org/ui/SIDS\\_Details.aspx?id=9a361ac3-b9ec-48d1-a803-0844b703e8c2](https://hpvchemicals.oecd.org/ui/SIDS_Details.aspx?id=9a361ac3-b9ec-48d1-a803-0844b703e8c2)
- Poma, A., Vecchiotti, G., Colafarina, S., Zarivi, O., Arrizza, L., Di Carlo, P., & Di Cola, A. (2019). Exposure to particle debris generated from passenger and truck tires induces different genotoxicity and inflammatory responses in the RAW 264.7 cell line. *PLOS ONE*, 14(9), Article e0222044.
- Reemtsma, T., Putschew, A., & Jekel, M. (1999). Industrial wastewater analysis: A toxicity-directed approach. *Waste Management*, 19(2), 181–188.
- Riegraf, C., Reifferscheid, G., Belkin, S., Moscovici, L., Shakibai, D., Hollert, H., & Buchinger, S. (2019). Combination of yeast-based in vitro screens with high-performance thin-layer chromatography as a novel tool for the detection of hormonal and dioxin-like compounds. *Analytica Chimica Acta*, 1081, 218–230.
- Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH image to ImageJ: 25 years of image analysis. *Nature Methods*, 9(7), 671–675. <https://doi.org/10.1038/nmeth.2089>
- Schulz, W., Weiss, S. C., Weber, W. H., & Winzenbacher, R. (2017). The reciprocal iso-inhibition volume concept: A procedure for the evaluation in effect-directed analysis with thin-layer chromatography—Using the thin-layer chromatography-luminescent bacteria assay as an example. *Journal of Chromatography A*, 1519, 121–130.
- Seiwert, B., Nihemaiti, M., Troussier, M., Weyrauch, S., & Reemtsma, T. (2022). Abiotic oxidative transformation of 6-PPD and 6-PPD-quinone from tires and occurrence of their products in snow from urban roads and in municipal wastewater. *Water Research*, 212, Article 118122.
- Sieber, R., Kawecki, D., & Nowack, B. (2020). Dynamic probabilistic material flow analysis of rubber release from tires into the environment. *Environmental Pollution*, 258, Article 113573.
- Sørensen, L., Gomes, T., Igartua, A., Lyngstad, I. L., Almeida, A. C., Wagner, M., & Booth, A. M. (2023). Organic chemicals associated with rubber are more toxic to marine algae and bacteria than those of thermoplastics. *Journal of Hazardous Materials*, 458, Article 131810.
- Tian, Z., Zhao, H., Peter, K. T., Gonzalez, M., Wetzell, J., Wu, C., Hu, X., Prat, J., Mudrock, E., Hettlinger, R., Cortina, A. E., Biswas, R. G., Kock, F. V. C., Soong, R., Jenne, A., Du, B., Hou, F., He, H., Lundeen, R., ... Kolodziej, E. P. (2021). A ubiquitous tire rubber-derived chemical induces acute mortality in coho salmon. *Science*, 371(6525), 185–189.
- ToxServices. (2021). N-(1,3-Dimethylbutyl)phenyl-p-phenylenediamine (6PPD) (CAS #793-24-8) Greenscreen for Safer Chemicals (Greenscreen®) assessment-N<sup>o</sup>.
- Wang, Y., Fu, R., Li, X., Zhao, W., Liu, M., & Li, Y. (2023). Potential thyroid hormone disorder risks of tire antioxidants to aquatic food chain organisms after absorbing free radicals in marine and freshwater environments. *Aquatic Toxicology*, 260, Article 106587.
- Weyrauch, S., Seiwert, B., Voll, M., Wagner, S., & Reemtsma, T. (2023). Accelerated aging of tire and road wear particles by elevated temperature, artificial sunlight and mechanical stress—A laboratory study on particle properties, extractables and leachables. *Science of the Total Environment*, 904, Article 166679.
- Wik, A., & Dave, G. (2009). Occurrence and effects of tire wear particles in the environment—A critical review and an initial risk assessment. *Environmental Pollution*, 157(1), 1–11.
- Zeng, F., Sherry, J. P., & Bols, N. C. (2016). Evaluating the toxic potential of benzothiazoles with the rainbow trout cell lines, RTgill-W1 and RTL-W1. *Chemosphere*, 155, 308–318.
- Zhang, Q. H., Xu, Y., Schramm, K. W., Jiang, G. B., & Kettrup, A. (2002). Antiestrogenic and antiprogesterone activity of tire extracts with yeast-based steroid hormone receptor gene transcription assay. *Bulletin of Environmental Contamination and Toxicology*, 69(6), 863–868.
- Zhao, H. N., Hu, X., Tian, Z., Gonzalez, M., Rideout, C. A., Peter, K. T., Dodd, M. C., & Kolodziej, E. P. (2023). Transformation products of tire rubber antioxidant 6PPD in heterogeneous gas-phase ozonation: Identification and environmental occurrence. *Environmental Science & Technology*, 57(14), 5621–5632.