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Article

Bioaccessibility of Organic Compounds Associated with Tire Particles Using a Fish *In Vitro* Digestive Model: Solubilization Kinetics and Effects of Food Coingestion

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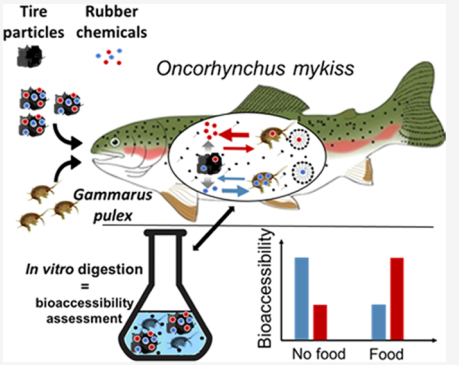
ABSTRACT: Tire and road wear particles (TRWP) account for an important part of the polymer particles released into the environment. There are scientific knowledge gaps as to the potential bioaccessibility of chemicals associated with TRWP to aquatic organisms. This study investigated the solubilization and bioaccessibility of seven of the most widely used tire-associated organic chemicals and four of their degradation products from cryogenically milled tire tread (CMTT) into fish digestive fluids using an *in vitro* digestion model based on *Oncorhynchus mykiss*. Our results showed that 0.06−44.1% of the selected compounds were rapidly solubilized into simulated gastric and intestinal fluids within a typical gut transit time for fish (3 h in gastric and 24 h in intestinal fluids). The environmentally realistic scenario of coingestion of CMTT and fish prey was explored using ground *Gammarus pulex*. Coingestion caused compound-specific changes in solubilization, either increasing or decreasing the compounds' bioaccessibility in simulated gut fluids compared to CMTT alone. Our results emphasize that tire-associated compounds become accessible in a digestive milieu and should be studied further with respect to their bioaccumulation and toxicological effects upon passage of intestinal epithelial cells.



KEYWORDS: *tire, tyre, TRWP, additives, microplastics, digestive fluids, chemical leaching 6PPD(Q)*

INTRODUCTION

Tire and road wear particles (TRWP) are produced during abrasion of tires on road pavement. Low amounts of small-sized TRWP (<10 μm) enter the atmosphere during use but between 95 and 99% of total emitted TRWP are expected to be deposited on the road side[1] and be transferred into the nearby soil from which a fraction will eventually enter surface water.[2] A modeling study estimated that 49% of TRWP emitted on the road would reach the freshwater system in the French Seine basin.[3] Field measurements suggest that the levels of TRWP decrease between its emission source and the aquatic environment with concentrations of 0.1−100 g kg^{-1} on the road side, 0.5−1.2 g kg^{-1} in river sediment, and 0.5−5 mg L^{-1} in river water.[4] TRWP are heterogeneous particles composed of rubber polymer, minerals, bitumen, and various chemicals originating from the road environment or from the rubber itself.[5,6] They are susceptible to environmental weathering, leading to changes in physical properties and chemical composition of the particles.[4,7] For instance, metals, such as Pb, Mn, Co, Cr, Ba, and Ni, were measured as traces in the tire rubber and also in higher concentration in TRWP, revealing the contribution of the road constituents to the overall metal burden of TRWP.[5,6,8]

Several organic chemicals are added to tire rubber to facilitate polymerization during manufacturing or to increase the performance and longevity of the tires during use. Among many other compounds, 2-mercaptobenzothiazole (MBT) and 1,3-diphenylguanidine (DPG) are intensively used as vulcanization agents; they can represent up to 0.5% of the tire rubber.[9] Phenylenediamine compounds, such as *N*-(1,3-dimethylbutyl)-*N*′-phenyl-1,4-phenylenediamine (6PPD), are also commonly used as antioxidants and antiozonants (about 0.9% of the tire tread) in the final product to prevent cracking and degradation of the rubber during wear.[10] Highly aromatic oils used in rubber manufacturing commonly include polycyclic aromatic hydrocarbons (PAHs), some of which are classified as carcinogenic. The use of PAHs by the tire industry has been regulated by the EU Directive 2005/69/EC since January 2010. Accordingly, tire tread may no longer contain more than 10 μg g^{-1} $\sum$8PAHs.[11] However, lower

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levels of regulated PAHs as well as unregulated PAHs could still be present in tire tread. Finally, zinc oxide is commonly used as a sulfur vulcanization catalyst during the curing process of rubber and represents up to 2.5% mass of the final tire composition.[8,12]

The potential toxic impact of these tire-associated chemicals for aquatic biota has been mainly assessed by laboratory experiments with organisms exposed to aqueous leachates of unaltered tire particles.[13−18] A few studies explored the toxicity of aged tire particle leachates.[19,20] These studies were conducted with heterogeneous experimental conditions (temperature of leaching, pH of leaching solution, and salinity), which could impact the solubilization and further bioaccessibility of tire-associated compounds. Overall, the studies investigating the toxicity of tire particle leachates led to contrasting results. These could originate from the variability of the exposure conditions but may also suggest species-specific sensitivity.[14,17,18,21−24] A recent study incriminated a 6PPD oxidation product, namely, 2-((4-methylpentan-2-yl)-amino)-5-(phenylamino)cyclohexa-2,5-diene-1,4-dione (6PPD-Q), as responsible for acute toxicity to Coho salmon (*Oncorhynchus kisutch*), threatening the local population of this fish species in urban creeks of Seattle.[25,26] 6PPD-Q was also found to be highly toxic for two other salmonid species (Brook trout (*Salvelinus fontinalis*) and Rainbow trout (*Oncorhynchus mykiss*)) but not for five other fish species and two crustacean species,[27−30] suggesting a species-specific mode of action for this chemical.

A recent study found microplastics, including tire particles, in the stomach content of several wild fish species, showing that fish can also ingest TRWP.[31] Studies investigating direct effects of TRWP remain scarce,[21,32,33] which highlights the need to investigate the effects of the particles themselves in addition to the effects of leachates.[34] Indeed, several studies showed that the solubilization of polymer-bound chemicals was enhanced in fish gut fluids compared to water and could promote bioaccessibility of the chemicals for uptake into the circulatory system.[35−40] More specifically, we demonstrated in a previous article that bioaccessibility of Zn from tire particles was enhanced by the organic components of the fish gut fluids and assessed the effects of coingestion of food organic matter on Zn bioaccessibility in fish gut.[8] Nonetheless, the bioaccessibility of organic compounds associated with tire particles, which could contribute to the toxic effects observed in several studies, remains poorly investigated. Therefore, this study used a fish (Rainbow trout) *in vitro* digestion model and cryogenically milled tire tread (CMTT) as a surrogate material for environmental TRWP to (i) determine the solubilization kinetics of several commonly used antioxidants, vulcanization aids, and transformation products from unaltered and artificially aged CMTT into simulated gastrointestinal fluids of fish and (ii) assess the overall bioaccessibility (defined as the soluble fraction of the chemical available for uptake) of these organic compounds in fish gut with and without coingestion of food organic matter.

MATERIALS AND METHODS

Materials. The generation of CMTT was previously described by Masset et al.[8] 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 cut into small pieces of 1 cm^3 using industrial scissors and a water jet machine and cryogenically milled using a model A Hammer Mill (Pulva). The cutting process using a water jet was very brief and performed on large tread particles. Therefore, significant leaching of chemicals during this step was not expected. The particles were collected and stored in amber glass vials in dark at room temperature. Artificially aged CMTT were generated by thermooxidation, following the protocol of Klöckner et al.[41] More details regarding the physicochemical characteristics of CMTT (size distribution, electron microscopy images) are presented in Figures S1 and S2.

The composition of the fish simulated gastric fluid ($SF_{GASTRIC}$) and simulated intestinal fluid ($SF_{INTESTINAL}$) used in this study was the same as of Masset et al.[8] (Table S1). 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. The digestive fluids were designed to be used in combination with a cell line isolated from Rainbow trout intestine, the RTgutGC, which is cultured using L-15 medium.[42] Purified pepsin (Sigma-Aldrich) was added to the luminal buffer at a concentration of 12.5 U mg^{-1} of protein and pH was adjusted to 2 with 32% HCl to obtain $SF_{GASTRIC}$. A concentration of 4 mg mL^{-1} of porcine bile extract (Sigma-Aldrich) and 2 mg mL^{-1} of pancreatin (Sigma-Aldrich) was added to the luminal buffer to obtain $SF_{INTESTINAL}$ with a pH of 7.4. Control experiments were performed in mineral water (MW) (Evian) (composition in Table S2) for comparison with digestive fluids.

Determination of Particle and Digestive Fluids Characteristics. Qualitative analysis of particle morphology was performed on CMTT and aged CMTT before and after *in vitro* digestion using scanning electron microscopy (SEM) (GeminiSEM 300, Zeiss). This was done to assess the general morphology of the particles and to investigate any morphological changes at the surface of the particles that might result from the aging process or from *in vitro* digestion. Surface tension of the $SF_{INTESTINAL}$ was measured with a goniometer (EasyDrop, Kruss) and the presence, size, and stability of micelles in the digestive fluids were assessed by dynamic light scattering and measurements of the ζ potential of the solutions using a Zetasizer ZS. The concentration of dissolved organic carbon (DOC) was measured in the digestive fluids using an organic carbon analyzer (vario TOC cube, Elementar).

CMTT Organic Chemical Composition Determination. To determine the total concentration of selected antioxidants and vulcanization agents in CMTT, preliminary tests showed that ultrasound-assisted extraction resulted in poor recovery as some compounds were strongly bound to the rubber matrix and required harsher extraction conditions. Therefore, CMTT spiked with deuterated internal standards (benzothiazole-d_4, aniline-d_5, diphenylurea-d_{10}, and 6PPD-Q-d_5 and a mix of 16 deuterated PAHs) was Soxhlet-extracted with 150 mL of methanol for 16 h, followed by 150 mL of dichloromethane for another 16 h. Both the fractions were combined and evaporated to 2 mL using a rotavapor (Büchi) and passed through a 0.45 μm glass fiber filter. An aliquot of 1 mL was prepared without further cleanup for direct analysis with ultra performance liquid chromatography coupled with a tandem mass spectrometer (UPLC-MSMS). Another aliquot for PAH analysis was passed through a chromatographic column filled with 3 g of silica gel previously activated at 180 °C for 8 h, eluted with 50 mL of hexane, and concentrated with a rotavapor to a volume of 2 mL. Finally, the extracts were

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Table 1. Concentration of Tire-Associated Chemicals, Their Transformation Products, and PAHs in CMTT and Aged CMTT (in $\mu g\ g^{-1}$)[a]

conc. ($\mu g\ g^{-1}$)	ANI	BT	HBT	MBT	DPG	6PPD	6PPD-Q	PHE	FLT	PYR	BPY
CMTT	52	630	194	1363	4427	31 008	14	4.3	6.6	21.7	8.1
	(6)	(42)	(8)	(42)	(153)	(1991)	(3)	(0.5)	(0.6)	(2.1)	(1.7)
aged CMTT	35	247	332	399	3197	13 073	30	3.9	6.7	24.2	7.9
	(6)	(24)	(30)	(71)	(213)	(1516)	(1)	(0.2)	(0.2)	(1.2)	(0.5)

[a](sd) = standard deviation of n = 3 replicates. ANI = aniline, BT = benzothiazole, HBT = 2-hydroxybenzothiazole, MBT = 2-mercaptobenzothiazole, DPG = 1,3-diphenylguanidine, 6PPD = *N*-(1,3-dimethylbutyl)-*N*′-phenyl-1,4-phenylenediamine, 6PPD-Q = 2-((4-methylpentan-2-yl)amino)-5-(phenylamino)cyclohexa-2,5-diene-1,4-dione, PHE = phenanthrene, FLT = fluoranthene, PYR = pyrene, BPY = benzo(*g*,*h*,*i*)perylene.

concentrated near dryness under a gentle stream of nitrogen and solvent exchanged to 500 μL of isooctane for analyses with gas chromatography coupled with a tandem mass spectrometer (GC-MSMS) (more details in Section "Chemical Analyses").

***In Vitro* Digestion Experiment.** *Solubilization Kinetics.* Solubilization kinetics of organic chemicals were investigated with both CMTT and aged CMTT in $SF_{GASTRIC}$ and $SF_{INTESTINAL}$ separately to investigate the effects of pH and composition of the fluids on the kinetics. *In vitro* digestion was performed at 10 g of CMTT L^{-1} of digestive fluid by introducing 150 mg of CMTT in amber glass vessels containing 15 mL of $SF_{GASTRIC}$ or $SF_{INTESTINAL}$. The digestion was carried out at 20 °C under gentle agitation for 3 h ($SF_{GASTRIC}$) and 24 h ($SF_{INTESTINAL}$). To maximize the coating of the tire particles with the fluids, the volume of the digestion vessels was chosen to lower the headspace filled with air as much as possible while still allowing for efficient agitation. At repeated times within 3 h (digestion in $SF_{gastric}$) or 24 h (digestion in $SF_{intestinal}$), a 15 mL sample was 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 glass fiber filters. All experiments were conducted in triplicates and control experiments consisting of leaching of 10 g of CMTT L^{-1} for 24 h were performed in mineral water (Evian) for comparison with digestive fluids. Experimental blanks with digestives fluids and mineral water without CMTT were also prepared and analyzed.

Coingestion Experiments. The environmentally realistic scenario of coingestion with food was explored with unaltered CMTT as unaltered and aged CMTT exhibited close composition and behavior in digestive fluids (see Results and Discussion). In this experiment, sequential *in vitro* digestion was chosen. It consisted of a 3 h incubation in $SF_{GASTRIC}$ to mimic the transit time in the fish stomach followed with 24 h digestion in $SF_{GASTRIC}$ + $SF_{INTESTINAL}$ estimated as an average transit time in the fish small intestine.[43] Four grams of *Gammarus pulex*, used as a surrogate for fish prey, was ground with a mortar and pestle and 0.4 g of CMTT (food/CMTT ratio = 10) was placed in digestion vessels. Twenty milliliters of $SF_{GASTRIC}$ was added in the vessels and the digestion was performed at 20 °C under gentle agitation. After 3 h, 20 mL of $SF_{INTESTINAL}$ was added to the vessel and the pH (pH meter, FiveEasy F20, Mettler Toledo) was adjusted to 7.4 with stepwise addition of NaOH. The digestion was stopped after 27 h in total (3 h in $SF_{GASTRIC}$ and 24 h in $SF_{GASTRIC}$ + $SF_{INTESTINAL}$), and all samples were centrifuged at 950*g* force for 5 min and passed through 0.45 μm glass fiber filters. Control experiments were performed with ground *G. pulex* alone and with CMTT only for comparison. All experiments were performed in triplicates.

Chemical Analyses. For each digestate sample, a subsample (1 mL) was spiked with deuterated internal standards (benzothiazole-d_4, aniline-d_5, diphenylurea-d_{10}, and 6PPD-quinone-d_5) and analyzed without further cleanup with UPLC-MSMS. A second subsample (12 mL) was collected for PAH analysis and was spiked with deuterated internal standards (mix of 16 deuterated PAHs) and liquid/liquid extracted twice with 10 mL of dichloromethane. Then, the extracts were concentrated with a rotavapor to 2 mL and followed a similar purification and preparation protocol as described for the CMTT extracts (see Section "CMTT Organic Chemical Composition Determination").

The following tire-associated compounds in the CMTT extracts as well as in the *in vitro* CMTT digestates were analyzed with a UPLC-MSMS (Xevo TQ MS, Waters): benzothiazole (BT), 2-hydroxybenzothiazole (HBT), 2-mercaptobenzothiazole (MBT), aniline (ANI), 1,3-diphenylguanidine (DPG), *N*-(1,3-dimethylbutyl)-*N*′-phenyl-1,4-phenylenediamine (6PPD), and 2-((4-methylpentan-2-yl)amino)-5-(phenylamino)cyclohexa-2,5-diene-1,4-dione (6PPD-Q). The EPAs 16 priority pollutant PAHs were analyzed with a GC-MSMS (TSQ Quantum XLS Ultra, Thermo Scientific). Six calibration standards were analyzed for each batch of samples (1−500 ng mL^{-1}, linearity R^2 > 0.99). Details regarding the chemicals used, UPLC-MSMS, GC-MSMS methods, and QA/QC for chemical analyses of CMTT particles and simulated gastrointestinal extracts are provided in Text S1. Details regarding the synthesis and quality control of 6PPD-Q produced in-house are provided in Text S2.

Statistical Analyses. All statistical tests were performed using R ver. 3.5.0. Tentative fittings of four models (logarithmic kinetic, diffusion-controlled kinetic, zeroth- and first-order kinetics) were performed for each compound. Differences in chemical concentrations following ingestion with or without coingestion of food were tested using *t*-tests or Kruskal−Wallis test for nonnormally distributed data.

RESULTS AND DISCUSSION

CMTT Organic Chemical Composition. Eleven compounds were quantified in unaltered CMTT and aged CMTT extracts (Table 1). In unaltered CMTT, 6PPD represented 31.0 mg g^{-1} (3.1% of the CMTT mass), a higher amount that was previously reported in the literature (up to 0.9%[10]). One of its oxidation byproducts, 6PPD-Q, was detected in much lower amount (14 $\mu g\ g^{-1}$, 0.0014%), which can be explained by the fact that CMTT was not exposed to oxidative conditions prior to analyses. However, in aged CMTT, the concentration of 6PPD was reduced (13.1 mg g^{-1}, 1.3%) and that of 6PPD-Q increased (30 $\mu g\ g^{-1}$, 0.0030%). Similarly, MBT and BT concentrations decreased by 71 and 61%,

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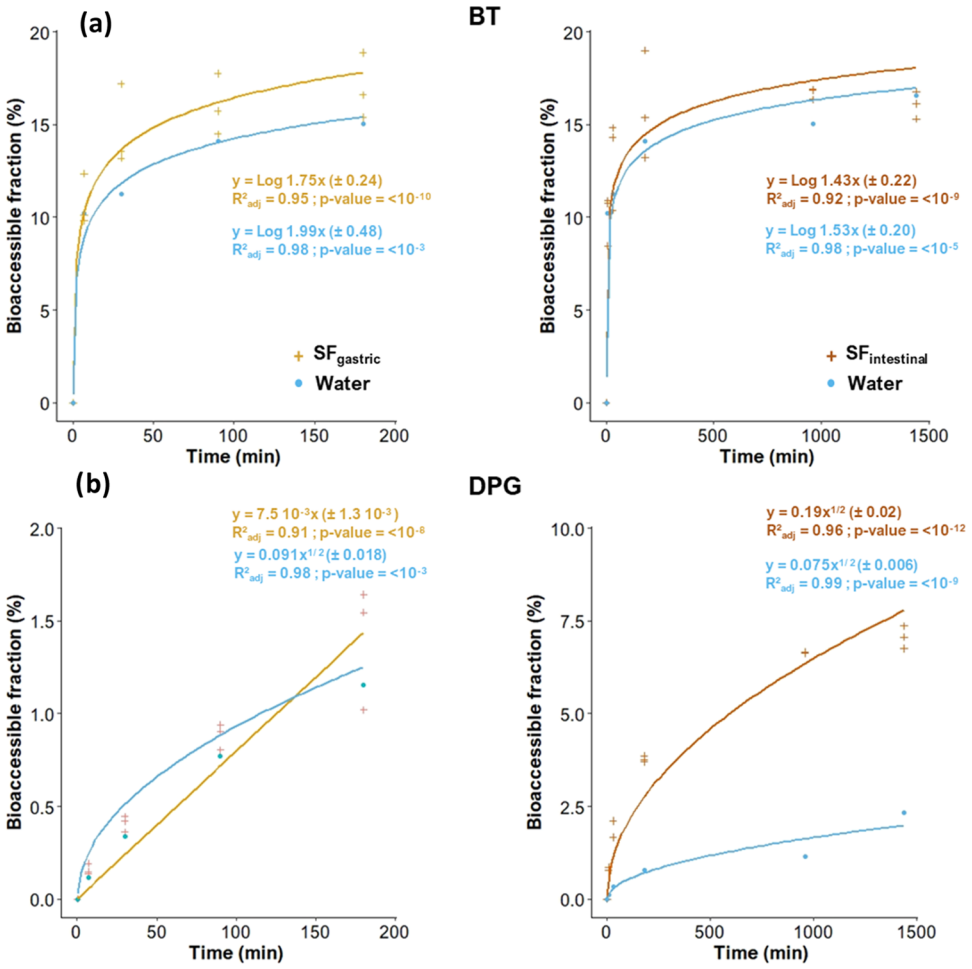

Figure 1. Solubilization kinetics and best fit models of (a) benzothiazole (BT) and (b) 1,3-diphenylguanidine (DPG) from CMTT in $SF_{GASTRIC}$, $SF_{INTESTINAL}$, and water (water data result from the same experiment but are plotted along 3 and 24 h on the left and right panels, respectively, for easier comparison). Logarithmic model: $y = \log a \times x$; diffusion-controlled model: $y = a \times x^{1/2}$.

respectively, whereas HBT concentration increased by 71%. These results show that the artificial aging of CMTT led to chemical modification of the particles *via* oxidation processes, as has previously been described.[41,44,45] They also reveal the high degradability of 6PPD resulting in the production of several transformation products, including 6PPD-Q. In contrast, the PAH content in unaltered CMTT and aged CMTT was similar, likely due to the low vapor pressure of these congeners preventing volatilization from the surface of the CMTT and to their weak oxidation under controlled atmospheric conditions. The PAH profile was dominated by 4 PAHs, namely, phenanthrene (PHE), fluoranthene (FLT), pyrene (PYR), and benzo(*g*,*h*,*i*)perylene (BPY) (Table 1). These four PAHs represented more than 80% of the total measured PAH content of the particles (the full 16 PAHs profile analyzed is provided in Figure S3). A qualitative observation of the CMTT by electron microscopy did not reveal any visible alteration of the surface of the particles or formation of cracks from the aging process (Figure S1). However, it has been shown that exposure to oxidative conditions of microplastics could lead to modification of the particle surface microstructure,[46] possibly not detectable with electron microscopy. Therefore, we investigated whether the desorption of tire-associated chemicals in simulated digestive fluids was impacted by aging.

***In Vitro* Digestion Experiment.** *Solubilization Kinetics.* *In vitro* digestions of unaltered CMTT and aged CMTT in $SF_{GASTRIC}$ and $SF_{INTESTINAL}$ were performed in both types of fluids separately to reveal the underlying mechanisms responsible for the solubilization of tire-associated compounds. The solubilization kinetics from unaltered CMTT for all compounds are presented in Figure S4. To facilitate comparison between compounds present in different concentrations in CMTT (Table 1), the results were expressed as the bioaccessible fraction (%), which was calculated as follows

$$\text{bioaccessible fraction (\%)} = \frac{m_{ff}}{m_{CMTT}} \times 100$$

where m_{ff} is the mass of the compounds solubilized in the digestive fluid at the end of the digestion (μg) and m_{CMTT} is the nominal total mass of chemicals based on measuring extracts of CMTT and the mass of CMTT in the digestion vessel (μg).

All compounds except PAHs were rapidly solubilized in $SF_{GASTRIC}$ within the 3 h digestion time (Figures 1 and S3 and Table S3). The compounds' solubilization kinetics were best fitted by a logarithmic or a diffusion-controlled model,[35,47] suggesting that at least two mechanisms were involved in the solubilization from the rubber matrix (Figure 1). With one exception, the data revealed a fast solubilization within the first

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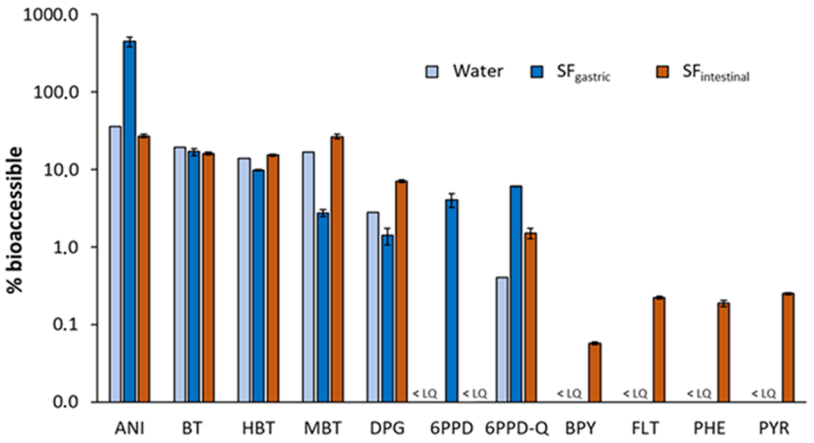

Figure 2. Bioaccessibility of the tire-associated compounds measured in CMTT (relative to the total CMTT content) in $SF_{GASTRIC}$, $SF_{INTESTINAL}$, and water at the end of the digestion (3 h for $SF_{GASTRIC}$, 24 h for $SF_{INTESTINAL}$, and 27 h for water). Error bars represent the standard deviation of n = 3 measurements. <LQ = below the limit of quantitation.

hour of digestion before a pseudo-equilibrium was reached. The exception was DPG, for which a constant solubilization rate was observed during the 3 h digestion time (Figure 1b). PAHs were not detected in $SF_{GASTRIC}$, indicating very poor solubilization potential for these hydrophobic molecules. Concentration of ANI at the end of the digestion in $SF_{gastric}$ was very high (2.5 mg mL^{-1}), *i.e.*, at 449% of the total ANI content in CMTT introduced in the digestion vessel, suggesting that ANI was formed during the digestion. Indeed, as nitrobenzene is widely used within the rubber industry[48−50] and is a recognized precursor for ANI production under acidic conditions,[51,52] it is possible that the excess of ANI measured in $SF_{gastric}$ was formed *via* reduction of nitrobenzene at pH = 2 (Figure S5). An alternative explanation could be that ANI was formed due to degradation of DPG.[9]

In $SF_{intestinal}$, all compounds were rapidly solubilized during the *in vitro* digestion time of 24 h. All solubilization kinetics were best fitted by a logarithmic model except for DPG for which the solubilization was best fitted by a diffusion-controlled model (Figure 1b). PAHs were poorly solubilized in $SF_{intestinal}$ (solubilization rate k = $(1.1 \times 10^{-2})-(5.4 \times 10^{-2})$) (Table S3). However, tentative fitting with the four models tested in this work was not satisfactory as a peak of concentration in the $SF_{intestinal}$ was reached after 3 h and concentrations decreased afterward until the end of the digestion (24 h) (Figure S4). One possible explanation could be the high affinity of the PAHs for organic particulates; hence, the compounds would adsorb on bile particulates, which were removed by filtration before analysis. This could also explain the decreasing trend of the solubilization of 6PPD in $SF_{intestinal}$ (Figure S4). Moreover, as this chemical is known to be poorly stable in aqueous solution[53,54] (half-life = 8 h at pH 7), it is possible that most of the 6PPD solubilized within the 24 h experiment was degraded before analysis. The low pH = 2 of $SF_{gastric}$ could explain why 6PPD did not show a decreasing trend during the 3 h experiment as the chemical was shown to be more stable at pH = 2.[54,55]

The solubilization kinetics of chemicals from aged CMTT are presented in Table S3. The aging treatment did not lead to significant changes in the solubilization rates of most compounds in the three solutions (water, $SF_{gastric}$, and $SF_{intestinal}$). The solubilization rate only increased slightly for HBT in $SF_{gastric}$ and $SF_{intestinal}$, whereas it decreased for DPG in water and $SF_{gastric}$ and for 6PPD in $SF_{gastric}$ (Table S3). These results suggest that only a minor alteration of the polymer matrix occurs during the aging process and confirms the lack of visible physical changes observed by electron microscopy. Indeed, the artificial aging treatment by thermooxidation did not result in significant modification of the solubilization potential of the tire-associated compounds. In contrast, a strong oxidative treatment using potassium persulfate as a surrogate for aging treatment of tire particles led to morphological modifications of tire particles, and solubilization of antibiotics adsorbed on these tire particles was reduced.[56] Chemical aging with potassium persulfate is very harsh and likely poorly representative of environmental aging, whereas thermooxidation is only one of the weathering processes that could occur in the environment. More research on the impact of environmentally representative aging on solubilization of tire-associated chemicals is needed.

Overall, for the more polar compounds, such as ANI, benzothiazoles, and DPG, the bioaccessible fraction was higher than that for the more hydrophobic PAHs (Figure 2). This difference could be explained by the lower hydrophobicity of the former chemicals. Furthermore, the solubilization potential of the polar compounds was similar in water, $SF_{GASTRIC}$, and $SF_{INTESTINAL}$, meaning that solubilization was hardly affected by the presence of enzymes and by the low pH of the $SF_{gastric}$ or by the presence of bile constituents in $SF_{intestinal}$ compared to mineral water. In contrast, the solubilization of PAHs was strongly affected by the nature of the digestion fluids. PAH concentrations were below LQ in the $SF_{gastric}$ and in water but were quantifiable in $SF_{intestinal}$. Nonetheless, only a small fraction of the total PAH content of CMTT (0.06−0.25%) was bioaccessible compared to the more polar compounds (>1%).

The critical micelle concentration in $SF_{INTESTINAL}$ calculated from contact angle measurements was approximately equal to 2000 mg_{bile} L^{-1} (Figure S6). The $SF_{INTESTINAL}$ used in this study contained 5000 mg_{bile} L^{-1}, thus well above the critical micelle concentration, indicating that $SF_{INTESTINAL}$ was a micellar solution. The presence of stable micelles was confirmed by dynamic light scattering, which revealed a high concentration of micelles with an average size of 154.8 nm and a ζ potential of −10 mV. Micelle-mediated solubilization has been demonstrated for bisphenol A[26] and polychlorinated biphenyls (PCBs)[41] and is also likely responsible for the

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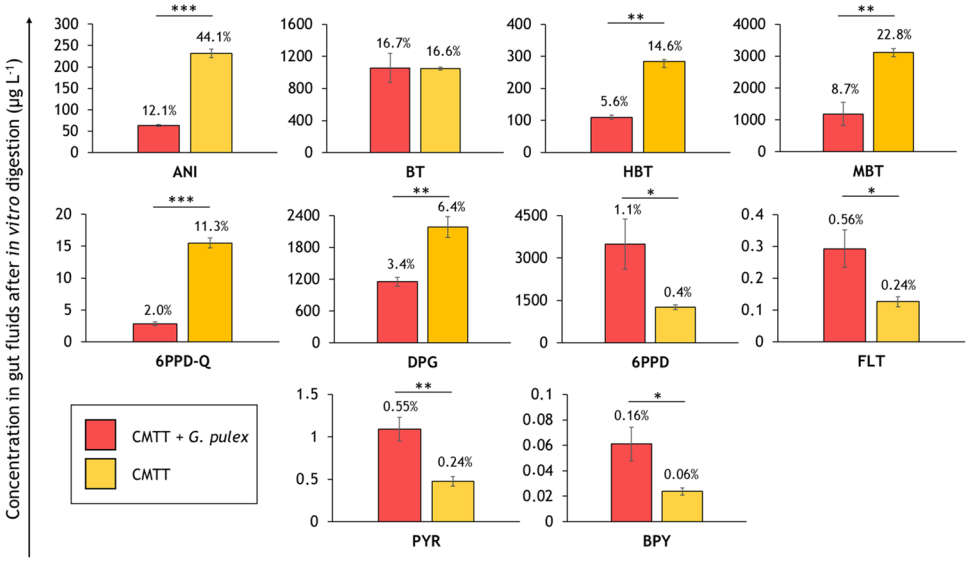

Figure 3. Concentration of tire-associated compounds solubilized into digestive fluids with or without coingestion of food prey surrogate (*G. pulex*). Values (expressed as %) represent the fraction of the chemical content solubilized in the fluids from the total CMTT digested (bioaccessible fraction %). The *in vitro* digestion simulating a coingestion of CMTT was conducted with a food/CMTT ratio of 10:1. Error bars represent the standard deviation of the replicates ($n = 3$). Statistical analyses conducted with *t*-tests or Kruskal–Wallis tests for nonnormally distributed data: *, **, *** = $p_{value} < 0.05$, 0.01, and 0.001, respectively.

solubilization of hydrophobic organic compounds such as PAHs. These results are consistent with previous studies that showed greater solubilization of hydrophobic contaminants, such as PAHs and PCBs, from sediment in gut fluids compared to water, both *in vitro* and *in vivo*[57,58] and also from various types of microplastics.[59,60] Micelle-mediated processes along with the hydrophobic nature of the gut fluids were also pointed out in these prior studies as drivers of the solubilization. In contrast, solubilization of only one (butyl benzyl phthalate) of 12 estrogenic compounds was enhanced under simulated fish gut conditions compared to water,[39,61] indicating that the mechanisms facilitating solubilization of organic chemicals in digestive fluids might be compound-specific.

Coingestion Experiments. *In vitro* digestion of CMTT along with surrogate fish prey (*G. pulex*) affected the solubilization of the tire-associated compounds. When comparing the digestion of CMTT alone to the coingestion scenario, only the solubilization of BT was not impacted by the addition of food (Figure 3). The solubilization of ANI, HBT, MBT, 6PPD-Q, and DPG was reduced by a factor of 1.8–5.6 in the coingestion scenario. In contrast, the solubilization of 6PPD, FLT, PYR, and BPY was enhanced by a factor of 2.3–2.7 with the addition of food. The DOC of both fluids reached 12 700 mg L^{-1} in the coingestion scenario but only 1250 mg L^{-1} without coingestion of food organic matter. Reduced solubilization of the most polar compounds in the presence of food could be due to the increased hydrophobic properties of the gut fluids due to the solubilization of organic matter from food particles, preventing solubilization of the hydrophilic compounds. In opposition, the enhanced solubilization of PAHs and 6PPD was related to the more hydrophobic nature of the fluids in the coingestion scenario and to the presence of higher DOC concentration. The effect of DOC or dissolved organic matter (DOM) on the solubilization of PAHs from microplastic particles has been studied and the dissolution of phenanthrene was enhanced by 3.7-fold when DOM increased from 0 to 1000 mg L^{-1}.[62] From a broader standpoint, the presence of increasing levels of DOC in aqueous solution favors the transfer of hydrophobic organic compounds from polymers[63,64] and nonaqueous phase liquids[65] to water. Finally, lower solubility of polar drugs and enhanced solubility of nonpolar drugs was observed in the fed state compared to the fasted state in simulated human intestinal fluids.[66] This corroborates our findings and highlights that solubilization of hydrophobic compounds in simulated gut fluids is not only controlled by the intraparticle diffusion but can also be impacted by external mass transfer as a function of the fluid's characteristics. As a consequence, a strong exponential relationship between the bioaccessible fraction and the octanol–water partition coefficient (K_{ow}) of the compounds was found for both scenarios (with or without coingestion of *G. pulex*) (Figure 4). The decreased solubilization of the more polar compounds and the increased solubilization of the apolar compounds in the coingestion scenario is emphasized by the lower slope of the regression line compared to CMTT digestion only (slope = −0.34 with $CI_{95\%}$: [−0.39; −0.30] and slope = −0.52 with $CI_{95\%}$: [−0.59; −0.45], respectively). This highlights that both the compound properties ($\log K_{ow}$) and the fluid's composition drive the solubilization of the tire-associated compounds in our model fish digestive fluids.

It should be noted that 6PPD-Q was highly solubilized in the treatment without food coingestion with regard to its hydrophobic properties ($\log K_{ow} = 3.96$[67]) (Figures 3 and 4). As 6PPD-Q is likely mainly formed at the surface of the CMTT particles by oxidation of 6PPD, its solubilization into the gut fluids was probably facilitated compared to other compounds that are distributed homogeneously in the CMTT particles and for which bioaccessibility was lower.

Limitations and Environmental Implications. In our *in vitro* digestion experiments, only a small to moderate percentage of tire-associated compounds (between 0.4 and 11.3%) was found to be solubilized and bioaccessible in the simulated gastrointestinal fluids within a representative gut transit time for fish. These values are low compared to studies

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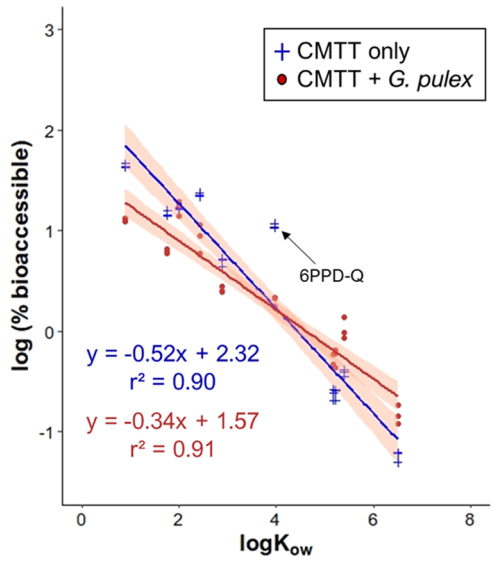

Figure 4. Relationship between the solubilization of chemicals (% bioaccessible) from CMTT into simulated gut fluids and the hydrophobic properties of the chemicals (K_{ow}) without (blue dots) or with coingestion of *G. pulex* (red dots). The lines represent the linear regression model and the shaded area represents the 95% confidence interval.

investigating the bioaccessibility of PAHs from microplastics where this parameter was assessed following loading of exogenous chemicals on the test material by an adsorption step.[68] Desorption of compounds adsorbed on the surface and micropores of a polymeric matrix is likely to be faster compared to tire-related compounds. Indeed, tire-related compounds are part of the blend of the polymer matrix, are homogeneously distributed within the particles, and are more strongly bound to the matrix. Nonetheless, the *in vitro* digestion of 10 g of CMTT L^{-1} of digestive fluids without coingestion of *G. pulex* resulted in marked concentrations of tire-associated compounds, such that they can be compared with LC_{50} available in the literature. Concentrations of 6PPD, DPG, 6PPD-Q, and MBT approached or were above LC_{50} values determined for salmonid species (Table 2). It should be

Table 2. Concentrations of Four Main Tire-Associated Chemicals after an *In Vitro* Digestion of 10 g L^{-1} of CMTT Compared to the Corresponding LC_{50} Determined for Salmonid Species (Coho Salmon (6PPD) and Rainbow Trout (MBT, DPG, and 6PPD-Q))

	MBT	DPG	6PPD	6PPD-Q
in vitro digestate (μg L^{-1})	3111	2184	1262	15
LC_{50} (μg L^{-1}) (ref)	1300−6200	11 000	250	1.96
	LC_{50} (96 h)	LC_{50} (96 h)	LC_{50} (24 h)	LC_{50} (24 h)

noted that LC_{50} values were determined *in vivo* and account for all exposure routes, whereas our study focused only on the exposure *via* the digestive track. The LC_{50} for 6PPD-Q was determined for rainbow trout and recent studies showed contrasting toxicity of this compound for other fish species and crustaceans (LC50 from 0.095 to 309 μg L^{-1}[25−28,30]) suggesting species-specific mode of action for this compound.[29]

One limitation of our study is the use of a high concentration of CMTT in the *in vitro* digestion experiments (10 g L^{-1}) and that a scenario with a low food/CMTT ratio of 10 was tested. Although ingestion of TRWP by aquatic organisms, including fish, has been demonstrated,[31] the level of exposure of fish to TRWP remains poorly documented and the concentration of CMTT and the food/CMTT ratio used in our study are likely overestimated compared to an environmentally realistic scenario. Moreover, we used a static *in vitro* digestion setup mimicking a finite bath digestion scenario. This type of experiment does not account for the passive diffusion of the compounds across the small intestine, which will cause a disequilibrium between the CMTT and the digestive fluid and create a concentration gradient for further solubilization of contaminants from CMTT[69−73] and for compounds that would be adsorbed on bile particulates. Another limitation of this study is that it relies on the use of CMTT as a surrogate 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.[5,8] The different surface areas of CMTT and TRWP could impact the solubilization kinetics of the associated compounds as well as their overall bioaccessibility. Furthermore, TRWP will undergo various types of weathering (thermooxidation, photodegradation, mechanical shear stress, biodegradation) once released in the environment that may affect its chemical and physical properties.[7] The effect of aging on tire particles was addressed in this study *via* exposure of CMTT to thermooxidative conditions, which led to a small fraction of 6PPD being converted into 6PPD-Q (0.09%) (Table 1). It is likely that other yet unknown transformation products of tire-associated chemicals were formed. Nevertheless, the bioaccessibility of tire-associated compounds did not vary significantly in artificially aged CMTT and unaltered CMTT. As in our case, the artificial aging of CMTT only consisted of thermooxidative conditions, and other weathering mechanisms (photooxidation, biodegradation) could come into play. Further studies should take these other aging processes into account and investigate the bioaccessibility of organic compounds from such aged tire particles and TRWP.

Overall, our study shows that the ingestion of CMTT by fish, as a surrogate for environmental TRWP, could lead to exposure of a cocktail of identified and probably other still unknown chemicals. The coingestion of food organic matters impacted the bioaccessible fraction of the chemicals in the fish digestive fluids. Thus, the bioaccessibility and further uptake of tire-associated compounds by the epithelial cells and related toxicity to fish based on refined environmental concentrations should be investigated. Finally, the bioaccumulation potential of the more hydrophobic tire-associated chemicals (6PPD-Q, DPG) needs to be determined.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c04291.

Details regarding the chemical analyses of the tire-associated compounds, the physical and chemical characteristics of cryogenically milled tire tread (CMTT) and of the simulated gastrointestinal fluids, surface tension of simulated intestinal fluid

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($SF_{INTESTINAL}$), solubilization kinetics of tire-associated compounds from unaltered and artificially aged CMTT in water, $SF_{GASTRIC}$, and $SF_{INTESTINAL}$, and the profile of the 16 measured PAHs in unaltered CMTT and aged CMTT (PDF)

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Notes

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