# Bioaccessibility of organic compounds associated with tire particles using a fish *in vitro* digestive model: solubilization kinetics and effects of food co-ingestion

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# 22 Abstract:

23 Tire and road wear particles (TRWP) account for an important part of the polymer particles released 24 into the environment. There are scientific knowledge gaps as to the potential bioaccessibility of 25 chemicals associated with TRWP to aquatic organisms. This study investigated the solubilization and 26 bioaccessibility of seven of the most widely used tire-associated organic chemicals and four of their 27 degradation products from cryogenically milled tire tread (CMTT) into fish digestive fluids using an in 28 vitro digestion model based on Oncorhynchus mykiss. Our results showed that 0.06% to 44.1% of the 29 selected compounds were rapidly solubilized into simulated gastric and intestinal fluids within a 30 typical gut transit time for fish (3 h in gastric and 24 h in intestinal fluids). The environmentally realistic 31 scenario of coingestion of CMTT and fish prey was explored using ground Gammarus pulex. 32 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 33 34 that tire-associated compounds become accessible in a digestive milieu and should be studied further 35 with respect to their bioaccumulation and toxicological effects upon passage of intestinal epithelial 36 cells.

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38 Keywords: tire; tyre; TRWP; additives; microplastics; digestive fluids; chemical leaching 6PPD(Q)

<u>Synopsis:</u> This work investigated the bioaccessibility of tire-associated organic compounds to rainbow
 trout after ingestion.

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# 44 Introduction:

45 Tire and road wear particles (TRWP) are produced during abrasion of tires on road pavement. Low 46 amounts of small-sized TRWP (<10  $\mu$ m) enter the atmosphere during use but between 95 – 99% of 47 total emitted TRWP are expected to be deposited on the road side<sup>1</sup> and be transferred into the nearby soil, from which a fraction will eventually enter the water streams<sup>2</sup>. A modelling study estimated that 48 49 49% of TRWP emitted on the road would reach the freshwater system in the French Seine basin<sup>3</sup>. Field 50 measurements suggest that levels of TRWP decrease between its emission source and the aquatic 51 environment with concentrations of 0.1 - 100 g kg<sup>-1</sup> on the road side, 0.5 - 1.2 g kg<sup>-1</sup> in river sediment 52 and  $0.5 - 5 \text{ mg L}^{-1}$  in river water<sup>4</sup>. TRWP are heterogeneous particles composed of rubber polymer, 53 minerals, bitumen and various chemicals originating from the road environment or from the rubber 54 itself<sup>5,6</sup>. They are susceptible to environmental weathering, leading to changes in physical properties and chemical composition of the particles<sup>4,7</sup>. For instance, metals, such as Pb, Mn, Co, Cr, Ba, and Ni, 55 were measured as traces in the tire rubber but also in higher concentration in TRWP, revealing the 56 57 contribution of the road constituents to the overall metal burden of TRWP<sup>5,6,8</sup>.

58 Several organic chemicals are added to tire rubber to facilitate polymerization during manufacturing 59 or to increase the performance and longevity of the tires during use. Among many other compounds, 60 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<sup>9</sup>. Phenylenediamine 61 62 compounds, such as N-isopropyl-N'-phenyl-p-phenylenediamine (IPPD) and N-(1,3-dimethylbutyl)-N'-63 phenyl-1,4-phenylenediamine (6PPD), are also commonly used as antioxidants and antiozonants (up 64 to 4% of the tire tread) in the final product to prevent cracking and degradation of the rubber during 65 wear<sup>10</sup>. Highly aromatic oils used in rubber manufacturing commonly include polycyclic aromatic 66 hydrocarbons (PAHs), some of which are classified as carcinogenic. The use of PAHs by the tire industry 67 has been regulated by the EU Directive 2005/69/EC since January 2010. Accordingly, tire tread may no longer contain more than 10  $\mu$ g $\Sigma$ 8PAHs g<sup>-111</sup>. However, lower levels of regulated PAHs as well as 68 unregulated PAHs could still be present in tire tread. Finally, Zinc oxide is commonly used as a sulphur 69 70 vulcanization catalyst during the curing process of rubber and represents up to 2.5 mass% of the final tire composition<sup>12,8</sup>. 71

72 The potential toxic impact of these tire-associated chemicals for aquatic biota has been mainly 73 assessed by laboratory experiments with organisms exposed to aqueous leachates of unaltered tire particles<sup>13–18</sup>. A few studies explored the toxicity of aged tire particle leachates<sup>19,20</sup>. These studies were 74 75 conducted with heterogeneous experimental conditions (temperature of leaching, pH of leaching 76 solution and salinity), which could impact the solubilization and further bioaccessibility of the tire-77 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 78 79 suggests species-specific sensitivity<sup>14,17,18,21–24</sup>. A recent study incriminated a 6PPD oxidation product, 80 namely 2-((4-methylpentan-2-yl)amino)-5-(phenylamino)cyclohexa-2,5-diene-1,4-dione (6PPD-Q), as 81 responsible for acute toxicity to Coho salmon (Oncorhynchus kisutch), threatening the local population of this fish species in urban creeks of Seattle (United States of America)<sup>25,26</sup>. 6PPD-Q was also found to 82 be highly toxic for two other salmonid species (Brook trout (Salvelinus fontinalis) and Rainbow trout 83 84 (Oncorhynchus mykiss) but not for five other fish species and two crustacean species<sup>27–30</sup>, suggesting 85 a species-specific mode of action for this chemical.

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86 A recent study found microplastics, including tire particles, in the stomach content of several wild fish species, showing that fish can also ingest TRWP<sup>31</sup>. Studies investigating direct effects of TRWP remain 87 scarce<sup>21,32,33</sup> which highlights the need to investigate the effects of the particles themselves in addition 88 to the effects of leachates<sup>34</sup>. Indeed, several studies showed that the solubilization of polymer-bound 89 90 chemicals was enhanced in fish gut fluids compared to water and could promote bioaccessibility of 91 the chemicals for uptake into the circulatory system<sup>35–40</sup>. More specifically, we demonstrated in a 92 previous article that bioaccessibility of Zn from tire particles was enhanced by the organic components 93 of the fish gut fluids and assessed the effects of coingestion of food organic matter on Zn 94 bioaccessibility in fish gut<sup>8</sup>. Nonetheless, the bioaccessibility of organic compounds associated with 95 tire particles, which could contribute to the toxic effects observed in several studies, remains poorly 96 investigated. Therefore, this study used a fish (Rainbow trout) in vitro digestion model and 97 cryogenically milled tire tread (CMTT) as a surrogate material for environmental TRWP in order to (i) 98 determine the solubilization kinetics of several commonly used antioxidants, vulcanization aids and 99 transformation products from unaltered and artificially aged CMTT into simulated gastrointestinal 100 fluids of fish and (ii) assess the overall bioaccessibility (defined as the soluble fraction of the chemical 101 available for uptake) of these organic compounds in fish gut with and without coingestion of food 102 organic matter.

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# 104 Material and Methods

# 105 Materials

106 The generation of CMTT was previously described by Masset et al. (2021)<sup>8</sup>. Briefly, the upper layer of 107 the tire tread from Pirelli<sup>®</sup> (Sottozero 3), Michelin<sup>®</sup> (Primacy 3) and Bridgestone<sup>®</sup> (Saetta Touring 2) 108 tires (ratio 1:1:2, respectively) were cut into small pieces of 1 cm<sup>3</sup> using industrial scissors and a water 109 jet machine and cryogenically milled using a model A Hammer Mill (Pulva®). The particles were 110 collected and stored in amber glass vials in darkness at room temperature. Artificially aged CMTT were generated by thermooxidation, following the protocol of Klöckner et al. (2021)<sup>41</sup>. More details 111 112 regarding the physicochemical characteristics of CMTT (size distribution, electron microscopy images) 113 are presented in figures S1 and S2.

The composition of the fish simulated gastric fluid (SF<sub>GASTRIC</sub>) and simulated intestinal fluid (SF<sub>INTESTINAL</sub>) 114 used in this study was the same as in Masset et al. 2021<sup>8</sup> (table S1). Briefly, both SF<sub>GASTRIC</sub> and SF<sub>INTESTINAL</sub> 115 116 consisted of a luminal buffer adapted from Leibovitz's L-15 cell culture medium to mimic the 117 composition of the lumen of fish intestine. The digestive fluids were designed to be used in 118 combination with a cell line isolated from Rainbow trout intestine, the RTgutGC, which is cultured using L-15 medium<sup>42</sup>. Purified pepsin (Sigma-Aldrich®) was added to the luminal buffer at a 119 120 concentration of 12.5 U mg<sup>-1</sup> of protein and pH was adjusted to 2 with 32 % HCl to obtain SF<sub>GASTRIC</sub>. A 121 concentration of 4 mg mL<sup>-1</sup> of porcine bile extract (Sigma-Aldrich®) and 2 mg mL<sup>-1</sup> of pancreatin (Sigma-122 Aldrich<sup>®</sup>) was added to the luminal buffer to obtain SF<sub>INTESTINAL</sub> with a pH of 7.4. Control experiments 123 were performed in mineral water (MW) (Evian®) (composition in table S2) for comparison with 124 digestive fluids.

# 125 Determination of particle and digestive fluids characteristics

126 A 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<sup>®</sup>). This was done

- in order to assess the general morphology of the particles and to investigate any morphological
- 129 changes at the surface of the particles that might result from the aging process or from the *in vitro*

digestion. Surface tension of the SF<sub>INTESTINAL</sub> was measured with a goniometer (EasyDrop, Kruss<sup>®</sup>) and the presence, size and stability of micelles in the digestive fluids was assessed by dynamic light scattering and measurements of the zeta potential of the solutions using a Zetasizer ZS<sup>®</sup>. The concentration of dissolved organic carbon (DOC) was measured in the digestive fluids using an organic carbon analyser (vario TOC cube, Elementar<sup>®</sup>).

# 135 CMTT organic chemical composition determination

136 In order to determine the total concentration of selected antioxidants and vulcanization agents in 137 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. 138 139 Therefore, CMTT spiked with deuterated internal standards (benzothiazole-d4, aniline-d5, 140 diphenylurea-d10 and 6PPD-Q-d5 and a mix of 16 deuterated PAHs) were Soxhlet-extracted with 150 141 mL of methanol for 16 h, followed by 150 mL of dichloromethane for another 16 h. Both fractions were combined and evaporated to 2 mL using a rotavapor (Büchi®) and passed through a 0.45 µm 142 143 Glass Fiber Filter (GFF). An aliquot of 1 mL was prepared without further clean-up for direct analysis 144 with Ultra Performance Liquid Chromatography coupled with a tandem mass spectrometer (UPLC-145 MSMS). Another aliquot was passed through a chromatographic column filled with 3 g of silica-gel 146 previously activated at 180°C for 8 h, eluted with 50 mL of hexane and concentrated with a rotavapor 147 to a volume of 2 mL. Finally, the extracts were concentrated near dryness under a gentle stream of 148 nitrogen and solvent exchanged to 500  $\mu$ L of isooctane for analyses of PAHs with Gas Chromatography 149 coupled with a tandem mass spectrometer (GC-MSMS) (more details in section "Chemical analyses").

## 150 In vitro digestion experiment

Solubilization kinetics. Solubilization kinetics of organic chemicals were investigated with both CMTT 151 152 and aged CMTT in SFGASTRIC and SFINTESTINAL separately to investigate the effects of pH and of the 153 composition of the fluids on the kinetics. The *in vitro* digestion was performed at 10 g of CMTT L<sup>-1</sup> of 154 digestive fluid by introducing 150 mg of CMTT in amber glass vessels containing 15 mL of SFGASTRIC or 155 SFINTESTINAL. The digestion was carried out at 20°C under gentle agitation for 3 h (SFGASTRIC) and 24 h 156 (SF<sub>INTESTINAL</sub>). At regular time, a 15 mL sample was collected and centrifuged at 950 g-force for 5 min 157 to remove large CMTT particles and bile aggregates and the supernatant was filtered through 0.45  $\mu$ m 158 GFF filters. All experiments were conducted in triplicates and control experiments consisting of 159 leaching of 10 g of CMTT L<sup>-1</sup> for 24 h were performed in mineral water (Evian<sup>®</sup>) for comparison with 160 digestive fluids. Experimental blanks with digestives fluids and mineral water without CMTT were also 161 prepared and analysed.

162 Coingestion experiments. The environmentally realistic scenario of coingestion with food was 163 explored with unaltered CMTT as unaltered and aged CMTT exhibited close composition and 164 behaviour in digestive fluids (see Results and Discussion). In this experiment, a sequential in vitro digestion was chosen. It consisted of a 3 h incubation in SFGASTRIC to mimic the transit time in the fish 165 166 stomach followed with a 24 h digestion in SFGASTRIC + SFINTESTINAL estimated as an average transit time in the fish small intestine<sup>43</sup>. Four g of *Gammarus pulex* (*G. pulex*), used as a surrogate for fish prey, were 167 ground with a mortar and pestle and 0.4 g of CMTT (food/CMTT ratio = 10) were placed in digestion 168 vessels. Twenty mL of SF<sub>GASTRIC</sub> was added in the vessels and the digestion was performed at 20°C under 169 170 gentle agitation. After 3 h, 20 mL of SFINTESTINAL was added to the vessel and the pH was adjusted to 7.4 171 with stepwise addition of NaOH. The digestion was stopped after 27 h in total (3 h in SFGASTRIC and 24 172 h in SFGASTRIC + SFINTESTINAL) and all samples were centrifuged at 3000 rpm for 5 min and passed through 173 0.45 µm GFF filters. Control experiments were performed with ground G. pulex alone and with CMTT 174 only for comparison. All experiments were performed in triplicates.

#### 175 Chemical analyses

- For each digestate sample, a sub-sample (1 mL) was spiked with deuterated internal standards (benzothiazole-d4, aniline-d5, diphenylurea-d10 and 6PPD-quinone-d5) and analysed without further clean-up with UPLC-MSMS. A second sub-sample (12 mL) was collected for PAHs 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
- 182 (section "CMTT organic chemical composition determination").
- The following tire-associated compounds in the CMTT extracts as well as in the in vitro CMTT 183 184 digestates were analysed with an UPLC-MSMS (Xevo TQ MS, Waters®): Benzothiazole (BT), 2-185 hydroxybenzothiazole (HBT), 2-mercaptobenzothiazole (MBT), aniline (ANI), 1,3-diphenylguanidine 186 (DPG), N-(1,3-dimethylbutyl)-N'-phenyl-1,4-phenylenediamine (6PPD) and 2-((4-methylpentan-2-187 yl)amino)-5-(phenylamino)cyclohexa-2,5-diene-1,4-dione (6PPD-Q). The EPA's 16 priority pollutant 188 PAHs were analysed with a GC-MSMS (TSQ Quantum XLS Ultra, Thermo Scientific®). Six calibration standards were analysed for each batch of samples (1 ng mL<sup>-1</sup> to 500 ng mL<sup>-1</sup>, linearity R<sup>2</sup> >0.99). Details 189 190 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
- 191 CMTT particles and simulated gastrointestinal extracts are provided in text S1. Details reg 192 synthesis and quality control of the 6PPD-Q produced in-house are provided in text S2.

# 193 Statistical analyses

- 194 All statistical tests were performed using R ver. 3.5.0. Tentative fitting of four models (logarithmic
- 195 kinetic, diffusion-controlled kinetic, 0<sup>th</sup> and 1<sup>st</sup> order kinetics) were performed for each compound.
- 196 Differences in chemical concentrations following ingestion with or without coingestion of food were
- 197 tested using t-tests or Kruskal-Wallis test for non-normally distributed data.

# 198 **Results and discussion**

# 199 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<sup>-1</sup> (3.1% of the CMTT mass). One of its oxidation by-products, 6PPD-Q was detected in much lower amount (14  $\mu$ g g<sup>-1</sup>, 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<sup>-1</sup>, 1.3%) and that of 6PPD-Q increased (30  $\mu$ g g<sup>-1</sup>, 0.0030%). Similarly, MBT and BT concentrations decreased by 71 and 61% respectively, whereas HBT concentration increased by 71%. These results show that the artificial

207Table 1: Concentration of tire-associated chemicals, their transformation products and PAHs in CMTT and aged CMTT (in208 $\mu g g^{-1}$ ). (sd) = Standard deviation of n = 3 replicates. ANI = Aniline, BT = Benzothiazole, HBT = 2-hydroxybenzothiazole, MBT209= 2-mercaptobenzothiazole, DPG = 1,3-diphenylguanidine, 6PPD = N-(1,3-dimethylbutyl)-N'-phenyl-1,4-210phenylenediamine, 6PPD-Q = 2-((4-methylpentan-2-yl)amino)-5-(phenylamino)cyclohexa-2,5-diene-1,4-dione, PHE =211Phenanthrene, FLT = Fluoranthene, PYR = Pyrene, BPY = Benzo(g,h,i)perylene

Conc. (µg g⁻¹)	ANI	ВТ	НВТ	MBT	DPG	6PPD	6PPD-Q	PHE	FLT	PYR	ВРҮ
СМТТ	52	630	194	1363	4427	31008	14	4.3	6.6	21.7	8.1
	(6)	(42)	<i>(8)</i>	<i>(42)</i>	(153)	(1991)	(3)	(0.5)	(0.6)	(2.1)	(1.7)
Aged CMTT	35	247	332	399	3197	13073	30	3.9	6.7	24.2	7.9
	<b>(6)</b>	(24)	(30)	(71)	<i>(213)</i>	<i>(1516)</i>	(1)	(0.2)	(0.2)	(1.2)	(0.5)

aging of CMTT led to chemical modification of the particles via oxidation processes, as has previously 213 been described<sup>41,44,45</sup>. They also reveal the high degradability of 6PPD resulting in the production of 214 215 several transformation products, among which 6PPD-Q. Contrastingly, the PAH content in unaltered CMTT and aged CMTT was similar, likely due to the low vapor pressure of these congeners preventing 216 volatilization from the surface of the CMTT and to their weak oxidation under controlled atmospheric 217 218 conditions. The PAH profile was dominated by 4 PAHs, namely phenanthrene (PHE), fluoranthene 219 (FLT), pyrene (PYR) and benzo(g,h,i)perylene (BPY) (table 1). These 4 PAHs represented more than 80% 220 of the total measured PAH content of the particles (the full 16 PAHs profile analysed is provided in 221 figure S3). A qualitative observation of the CMTT by electronic microscopy did not reveal any visible 222 alteration of the surface of the particles or formation of cracks from the aging process (figure S1). 223 However, it has been shown that exposure to oxidative conditions of microplastics could lead to modification of the particles surface microstructure<sup>46</sup>, possibly not detectable with electron 224 225 microscopy. Therefore, we investigated whether the desorption of tire-associated chemicals in 226 simulated digestive fluids was impacted by aging.

#### 227 In vitro digestion experiment

## 228 Solubilization kinetics

In vitro digestions of unaltered CMTT and aged CMTT in SF<sub>GASTRIC</sub> and SF<sub>INTESTINAL</sub> were performed in both types of fluids separately to reveal the underlying mechanisms responsible for the solubilization of the 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:

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

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

All compounds except PAHs were rapidly solubilized in SFGASTRIC within the 3 h digestion time (figure 1, 239 240 figure S3, table S3). The compounds' solubilization kinetics were best fitted by a logarithmic or a diffusion-controlled model<sup>47,48</sup>, suggesting that at least two mechanisms were involved in the 241 242 solubilization from the rubber matrix (figure 1). With one exception, the data revealed a fast solubilization within the first hour of digestion before a pseudo-equilibrium was reached. The 243 244 exception was DPG, for which a constant solubilization was observed during the 3 h digestion time 245 (figure 1b). PAHs were not detected in SFGASTRIC, indicating very poor solubilization potential for these hydrophobic molecules. Concentration of ANI at the end of the digestion in SF<sub>gastric</sub> was very high (2.5 246 247 mg mL<sup>-1</sup>), i.e. at 449% of the total ANI content in CMTT introduced in the digestion vessel, suggesting 248 that ANI was formed during the digestion. Indeed, as nitrobenzene is widely used within the rubber 249 industry<sup>49-51</sup> and is a recognized precursor for ANI production under acidic condition<sup>52,53</sup>, it is possible 250 that the excess of ANI measured in SF<sub>gastric</sub> was formed via reduction of nitrobenzene at pH = 2 (figure 251 S5). An alternative explanation could be that ANI was formed due to degradation of DPG<sup>9</sup>.

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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<sub>GASTRIC</sub>, SF<sub>INTESTINAL</sub> and water. Logarithmic model:  $y = \log a^*x$ , diffusion-controlled model:  $y = a^*x^{1/2}$ .

256 In SF<sub>intestinal</sub> all compounds were rapidly solubilized during the *in vitro* digestion time of 24 h. All 257 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 258 in SF<sub>intestinal</sub> (solubilization rate  $k = 1.1 \times 10^{-2} - 5.4 \times 10^{-2}$ ) (table S3). However, tentative fitting with the 259 4 models tested in this work was not satisfactory as a peak of concentration in the SF<sub>intestinal</sub> was 260 reached after 3 h and concentrations decreased afterward until the end of the digestion (24 h) (figure 261 262 S3). 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. 263

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

271 The solubilization kinetics of chemicals from aged CMTT are presented in table S3. The aging treatment 272 did not lead to significant changes in the solubilization rates of most compounds in the three solutions 273 (water, SFgastric and SFintestinal). The solubilization rate only increased slightly for HBT in SFgastric and 274 SF<sub>intestinal</sub>, whereas it decreased for DPG in water and SF<sub>gastric</sub> and for 6PPD in SF<sub>gastric</sub> (table S3). These 275 results suggest that only a minor alteration of the polymer matrix occurs during the aging process and 276 confirms the lack of visible physical changes observed by electron microscopy. Indeed, the artificial 277 aging treatment by thermooxidation did not result in significant modification of the solubilization 278 potential of the tire-associated compounds. In contrast, a strong oxidative treatment using potassium 279 persulfate as a surrogate for aging treatment of tire particles led to morphological modifications of 280 tire particles and solubilization of antibiotics adsorbed on these tire particles was reduced<sup>54</sup>. Chemical 281 aging with potassium persulfate is very harsh and likely poorly representative of environmental aging 282 whereas thermooxidation is only one of the weathering processes that could occur in the 283 environment. More research on the impact of environmentally representative aging on solubilization 284 of tire-associated chemicals is needed.

285 Overall, for the more polar compounds, such as ANI, benzothiazoles and DPG, the bioaccessible fraction was higher than for the more hydrophobic PAHs (figure 2). This 286 287 difference could be explained by the lower hydrophobicity of the former chemicals. Furthermore, the 288 solubilization potential of the polar compounds was similar in water, SFGASTRIC and SFINTESTINAL, meaning 289 that solubilization was hardly affected by the presence of enzymes and by the low pH of the SF<sub>gastric</sub> or 290 by the presence of bile constituents in SF<sub>intestinal</sub> compared to mineral water. Contrastingly, the 291 solubilization of PAHs was strongly affected by the nature of the digestion fluids. PAH concentrations 292 were below LQ in the SF<sub>gastric</sub> and in water but were quantifiable in SF<sub>intestinal</sub>. Nonetheless, only a small 293 fraction of the total PAH content of CMTT (0.06 - 0.25%) was bioaccessible compared to the more 294 polar compounds (> 1%).

The critical micelle concentration in SF<sub>INTESTINAL</sub> calculated from contact angle measurements was approximately equal to 2000 mg <sub>bile</sub>  $L^{-1}$  (figure S6). The SF<sub>INTESTINAL</sub> used in this study contained 5000 mg <sub>bile</sub>  $L^{-1}$ , thus well above the critical micelle concentration, indicating that SF<sub>INTESTINAL</sub> 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 zeta potential of -10 mV. Micelles-mediated solubilization has been demonstrated for bisphenol A<sup>26</sup> and PCBs<sup>41</sup> and is also likely 301 responsible for the solubilization of hydrophobic organic compounds such as PAHs. These results are 302 consistent with previous studies that showed greater solubilization of hydrophobic contaminants, 303 such as PAHs and PCBs, from sediment in gut fluids compared to water, both in vitro and in vivo 56,57 but also from various types of microplastics<sup>58,59</sup>. Micelles-mediated processes along with the 304 hydrophobic nature of the gut fluids were also pointed out in these prior studies as drivers of the 305 306 solubilization. In contrast, solubilization of only one (Butyl benzyl phthalate) of 12 estrogenic 307 compounds was enhanced under simulated fish gut conditions compared to water <sup>60,61</sup>, indicating that 308 the mechanisms facilitating solubilization of organic chemicals in digestive fluids might be compound-309 specific.

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#### 311 Coingestion experiments

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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 non-normally distributed data: \*, \*\*, \*\*\* = p<sub>value</sub> < 0.05, 0.01 and 0.001, respectively.

319 The *in vitro* digestion of CMTT along with surrogate fish prey (G. pulex) affected the solubilization of 320 the tire-associated compounds. When comparing the digestion of CMTT alone to the coingestion 321 scenario, only the solubilization of BT was not impacted by the addition of food (figure 3). The 322 solubilization of ANI, HBT, MBT, 6PPD-Q and DPG was reduced by a factor of 1.8 to 5.6 in the 323 coingestion scenario. In contrast, the solubilization of 6PPD, FLT, PYR and BPY was enhanced by a 324 factor of 2.3 to 2.7 with the addition of food. The DOC of both fluids reached 12700 mg L<sup>-1</sup> in the coingestion scenario but only 1250 mg L<sup>-1</sup> without coingestion of food organic matter. Reduced 325 solubilization of the most polar compounds in the presence of food could be due to the increased 326 327 hydrophobic properties of the gut fluids due to the solubilization of organic matter from the food 328 particles, preventing solubilization of the hydrophilic compounds. Oppositely, the enhanced

329 solubilization of the PAHs and 6PPD were related to the more hydrophobic nature of the fluids in the 330 coingestion scenario and to the presence of higher DOC concentration. The effect of DOC or dissolved 331 organic matter (DOM) on the solubilization of PAHs from microplastic particles has been studied and 332 the dissolution of Phenanthrene was enhanced by 3.7 fold when DOM increased from 0 to 1000 mg L<sup>-</sup> <sup>162</sup>. From a broader standpoint, the presence of increasing levels of DOC in aqueous solution favours 333 the transfer of hydrophobic organic compounds from polymers<sup>63,64</sup> and nonaqueous phase liquids<sup>65</sup> to 334 335 water. Finally, lower solubility of polar drugs and enhanced solubility of nonpolar drugs was observed 336 in the fed state compared to the fasted state in simulated human intestinal fluids<sup>66</sup>. This corroborates 337 our findings and highlights that solubilization of hydrophobic compounds in simulated gut fluids is not 338 only controlled by the intra-particle diffusion but can also be impacted by external mass transfer as a 339 function of the fluid's characteristics. As a consequence, a strong exponential relationship between 340 the bioaccessible fraction and the octanol-water partition coefficient ( $K_{ow}$ ) of the compounds was 341 found for both scenarios (with or without coingestion of G. pulex) (figure 4). The decreased 342 solubilization of the more polar compounds and the increased solubilization of the apolar compounds 343 in the coingestion scenario is emphasized by the lower slope of the regression line compared to CMTT digestion only (slope = -0.34 with Cl<sub>95%</sub>: [-0.39; -0.30] and slope = -0.52 with Cl<sub>95%</sub>: [-0.59; -0.45], 344 respectively). This highlights that both the compound properties (log Kow) and the fluid's composition 345 drive the solubilization of the tire-associated compounds in our model fish digestive fluids. 346

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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<sub>ow</sub>) without (blue dots) or with coingestion of G.pulex (red dots). The lines represent the linear regression model and the shaded area represent the 95% confidence interval.

352It should be noted that 6PPD-Q was highly solubilized with regards to its hydrophobic properties (log353 $K_{ow} = 3.96^{67}$ ) (figure 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

355 compounds that are distributed homogenously in the CMTT particles and for which bioaccessibility

356 was lower.

#### 357 Limitations and environmental implications

In our in vitro digestion experiments, only a small to moderate percentage of tire-associated 358 359 compounds (between 0.4 % and 11.3 %) was found to be solubilized and bioaccessible in the simulated 360 gastrointestinal fluids within a representative gut transit time for fish. These values are low compared 361 to studies 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<sup>68</sup>. 362 363 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 364 365 of the polymer matrix, are homogeneously distributed within the particles and are more strongly 366 bound to the matrix. Nonetheless, the *in vitro* digestion of 10 g of CMTT L<sup>-1</sup> of digestive fluids without 367 coingestion of G. pulex resulted in marked concentrations of tire-associated compounds, such that they can be compared with LC<sub>50</sub> available in the literature. Concentrations of 6PPD, DPG, 6PPD-Q and 368 369 MBT approached or were above  $LC_{50}$  values determined for salmonid species (table 2). It should be 370 noted that LC<sub>50</sub> were determined in vivo and account for all exposure routes whereas our study 371 focused only on the exposure via the digestive track. The LC<sub>50</sub> for 6PPD-Q was determined for rainbow 372 trout and that recent studies showed contrasting toxicity of this compound for other fish species and crustaceans (LC50 from 0.095 – 309  $\mu$ g L<sup>-1 25–28,30</sup>) suggesting species-specific mode of actions for this 373 compound<sup>29</sup>. 374

Table 2: Concentrations of 4 main tire-associated chemicals after an *in vitro* digestion of 10 g L<sup>-1</sup> of CMTT compared to the corresponding LC<sub>50</sub> determined for salmonid species (Coho salmon (6PPD), Rainbow trout (MBT, DPG and 6PPD-Q)).

	МВТ	DPG	6PPD	6PPD-Q
<i>In vitro</i> digestate (μg L <sup>-1</sup> )	3111	2184	1262	15
LC <sub>50</sub> (μg L <sup>-1</sup> ) (ref)	1300 – 6200 (69)	11000 (70)	250 (25)	1.96 (27)

## 377

378 One limitation of our study is the use of a high concentration of CMTT in the in vitro digestion 379 experiments (10 g L<sup>-1</sup>) 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<sup>31</sup>, the level of 380 381 exposure of fish to TRWP remains poorly documented and the concentration of CMTT and the 382 food/CMTT ratio used in our study are likely overestimated compared to an environmentally realistic 383 scenario. Moreover, we used a static in vitro digestion setup mimicking a finite bath digestion scenario. 384 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 385 concentration gradient for further solubilization of contaminants from CMTT<sup>71-73</sup>. Another limitation 386 387 of this study is that it relies on the use of CMTT as a surrogate for environmental TRWP. It has been 388 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<sup>5,8</sup>. The different 389 390 surface areas of CMTT and TRWP could impact the solubilization kinetics of the associated compounds 391 as well as their overall bioaccessibility. Furthermore, TRWP will undergo various types of weathering 392 (thermooxidation, photodegradation, mechanical shear stress, biodegradation) once released in the environment that may affect its chemical and physical properties<sup>7</sup>. The effects of aging on tire particles 393 394 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

401 compounds from such aged tire particles and TRWP.

402 Overall, our study shows that the ingestion of CMTT by fish, as a surrogate for environmental TRWP, 403 could lead to exposure of a cocktail of identified and probably other still unknown chemicals. The 404 coingestion of food organic matters impacted the bioaccessible fraction of the chemicals in the fish 405 digestive fluids. Thus, the bioaccessibility and further uptake of tire-associated compounds by the 406 epithelial cells and related toxicity to fish based on refined environmental concentrations should be 407 investigated. Finally, the bioaccumulation potential of the more hydrophobic tire-associated 408 chemicals (6PPD-Q, DPG) needs to be determined.

# 409 **Supporting information:**

410 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 (SF<sub>INTESTINAL</sub>), solubilization kinetics of tire-associated

412 surface tension of simulated intestinal find (SFINESTINAL), solubilization knetics of the associated 413 compounds from unaltered and artificially aged CMTT in water, SF<sub>GASTRIC</sub> and SF<sub>INTESTINAL</sub>, the profile of

the 16 measured PAHs in unaltered CMTT and aged CMTT can be found in the supplementary information.

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# 427 **Graphical Abstract:**



## 428

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