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### ► To cite this version:

Laurie Calarnou, Mounir Traïkia, Martin Leremboure, Lucie Malosse, Séverin Dronet, et al.. Assessing biodegradation of roadway particles via complementary mass spectrometry and NMR analyses. *Science of the Total Environment*, 2023, 900, pp.165698. 10.1016/j.scitotenv.2023.165698 . hal-04245621

**HAL Id: hal-04245621**

**<https://hal.science/hal-04245621>**

Submitted on 17 Oct 2023

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1 **Assessing biodegradation of roadway particles**

2 **via complementary mass spectrometry and NMR analyses**

3  
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10  
11 **Abstract**

12 Roadway particles (RP) that can be collected with on-vehicle system, consist of a mixture of  
13 Tire and road wear particles (TRWP) with other traffic-derived particles (exhaust or non-  
14 exhaust) and/or biogenic compounds and represent a significant source of xenobiotics,  
15 susceptible to reach the different environmental compartments. The study of the RP fate is thus  
16 a major challenge to tackle in order to understand their degradation and impact. They offer a  
17 variety of carbon sources potentially usable by microorganisms, ranging from the tire-derived  
18 plasticizers, vulcanizing agents, protective agents and their transformation products, to other  
19 traffic, road and environmental-derived contaminants. A multi-analytical approach was  
20 implemented to characterize RP and study their biodegradation. Kinetics of RP extractions were  
21 monitored during 21 days in water, methanol, acetone and chloroform to identify leaching and  
22 extractable compounds and monitor the particle composition. The results confirmed that  
23 hundreds of readily leachable chemicals can be extracted from RP directly into water according

24 to a dynamic process with time while additional poorly soluble compounds remain in the  
25 particles. Mass spectrometry (LC-HRMS and GC-MS) allowed us to propose 296 putative  
26 compounds using an extensive rubber database. The capacity of 6 bacterial strains, belonging  
27 to *Rhodococcus*, *Pseudomonas* and *Streptomyces* genera, to biodegrade RP was then evaluated  
28 over 14 days of incubation. The selected strains were able to grow on RP using various  
29 substrates. Elastomer monitoring by <sup>1</sup>H NMR revealed a significant 12% decrease of the  
30 extractable SBR fraction when the particles were incubated with *Rhodococcus ruber*. After  
31 incubation, the biodegradation of 171 compounds among leachable and extractable compounds  
32 was evaluated. Fatty acids and alkanes from rubber plasticizers and paraffin waxes were the  
33 most degraded putative compounds by the six strains tested, reaching 75% of biodegradation  
34 for some of them.

35

## 36 **Keywords**

37 Tire and road wear particles (TRWP), Styrene butadiene rubber (SBR), Additives,  
38 *Rhodococcus*, *Streptomyces*

39

## 40 **1. Introduction**

41 Tires are made of complex polymer-based composite materials containing a wide variety  
42 of compounds designed to provide specific properties and performances. The tread composition  
43 typically includes natural and/or synthetic elastomers (30-70 wt%), such as styrene butadiene  
44 rubber (SBR), butadiene rubber (BR) and natural rubber (NR), fillers (25-35 wt%), plasticizers  
45 (0-25 wt%), vulcanizing agents (2-5 wt%) and other additives (2-10 wt%) such as antioxidants,  
46 coupling agents, processing aids (Babbit, 2010; Baumann, W., Ismeier, 1998). During the tire  
47 life, the tread material undergoes different solicitations induced by friction with road surface  
48 and climatic conditions, that impact its structure and chemical composition. Tire aging and wear

49 lead to the formation of heterogeneous agglomerates made of an intimate mixture of damaged  
50 tire particles, road pavement particles and exogenous organic and inorganic materials in  
51 variable proportions and compositions to form the Tire and road wear particles: TRWP  
52 (Baensch-Baltruschat et al., 2020; Smith and Veith, 1982; Sommer et al., 2018; Wagner et al.,  
53 2022). There is some inconsistency in the literature regarding the terminology used to refer to  
54 wear particles depending on their generation and collection processes, as already stated by  
55 Baensch-Baltruschat et al. (2020). Accordingly, following the definition used by Kreider et al.  
56 (2010), the term Roadway particles (RP) will be used in the present paper to refer to actual  
57 outdoor-collected samples using an on-vehicle particle collection system, as such samples  
58 might contain both TRWP and other traffic-derived particles (exhaust or non-exhaust) and/or  
59 biogenic compounds.

60 The emission of TRWP is the main mechanism of tire mass loss. It was estimated between  
61 0.2 and 5.5 kg per year per inhabitant (Baensch-Baltruschat et al., 2020) but accurate  
62 quantification of TRWP emissions remains challenging. This is currently assessed via tire  
63 emission factors or material flow analyses due to a lack of direct measurements (Mennekes and  
64 Nowack, 2022) TRWP are mostly elongated or round particles (Kovochich et al., 2021) with a  
65 size ranging from a few nanometers to several hundred micrometers (Klöckner et al., 2020;  
66 Rosso et al., 2023) and densities ranging from 1.3 to above 2.2 g/cm<sup>3</sup> (Klöckner et al., 2021b;  
67 Kovochich et al., 2021) that can be transported by air and deposited or washed off the road and  
68 transferred to aquatic or soil ecosystems (Klöckner et al., 2020; Baensch-Baltruschat et al.,  
69 2020). TRWP were monitored and quantified in various environmental compartments (air, soil,  
70 fresh water, marine environment roadside, runoff, estuaries, river and sea sediments) (Baensch-  
71 Baltruschat et al., 2021; Goßmann et al., 2021; Klöckner et al., 2021a; Leads and Weinstein,  
72 2019; Panko et al., 2013; Parker-Jurd et al., 2021; Rauert et al., 2022a; Seiwert et al., 2020;  
73 Thomas et al., 2022a; Unice et al., 2012) evidencing them as a significant source of dispersion

74 of contaminants (Baensch-Baltruschat et al., 2020; Kole et al., 2017; Unice et al., 2019; Wagner  
75 et al., 2018) and emphasizing the urgent need to assess their impact and fate in the environment.  
76 These studies were based on various analytical methods but did not account for the aging or  
77 degradation of the particles in the environment, which could bias their identification and  
78 quantification.

79 Indeed, once in the environment, TRWP can be submitted to several abiotic and biotic  
80 degradation processes (photo-oxidation, thermo-oxidation, ozonation, shear stress,  
81 biodegradation) leading to the modification of their composition and to the formation of various  
82 transformation products (TP) that can be released as smaller particles or soluble compounds  
83 (Fohet et al., 2023; Wagner et al., 2022). Such processes increase the complexity of the  
84 chemical composition of TRWP and make their global environmental study challenging.  
85 Moreover, most of the few studies on TRWP environmental fate focus on one of its individual  
86 component or family: for example, abiotic oxidative transformation of some additives (N-(1,3-  
87 dimethylbutyl)-N'-phenyl-*p*-phenylenediamine (6PPD), diphenylguanidine (DPG), *n*-  
88 cyclohexyl-2-benzothiazole-sulfenamide (CBS) (Hu et al., 2022; Seiwert et al., 2022a; Tian et  
89 al., 2021; Unice et al., 2015) or tire elastomers (Thomas et al., 2022b).

90 Concerning biodegradation, while the first investigations on tires emerged 40 years ago  
91 (Cadle and Williams, 1980), no assay has been directly published on TRWP or RP yet. In fact,  
92 tire biodegradation was mainly considered as an alternative to mechanical or chemical recycling  
93 approaches. Indeed, it was addressed either to study the potential of microbial treatments for  
94 devulcanization of end-of-life tires and cryoground tire rubber (GTR) (Anderl, 2020; Holst et  
95 al., 1998; Li et al., 2022; Saputra et al., 2021; Stevenson et al., 2008) or to study the detailed  
96 biotic transformations of tire components individually (pure elastomers, additives or  
97 plasticizers) by bacterial or fungal isolates. Several rubber-degrading bacteria (*e.g.*  
98 *Streptomyces coelicolor* 1A, *Gordonia polyisoprenivorans* VH2, *Xanthomonas* sp. 35Y) were

99 isolated for their activity towards poly(1,4-*cis*-isoprene) (*i.e.* natural rubber NR or synthetic  
100 isoprene rubber IR) and metabolic pathways were proposed (Bode et al., 2000; Hiessl et al.,  
101 2012; Sharma et al., 2018). Only a few papers report studies on SBR biodegradation, which is  
102 the most common elastomer entering into car tire tread composition. Recently, Aboelkheir et  
103 al. (2019) compared the ability of three bacterial strains (*Bacillus subtilis*, *Pseudomonas*  
104 *aeruginosa* and *Streptomyces* sp.) to degrade vulcanized SBR. They evidenced chemical  
105 surface modifications of the rubber with all of them, suggesting cleavages of sulfur and carbon  
106 bonds. Literature on biodegradation of tire additives is also scarce. Few studies reported the  
107 biotic degradation of vulcanizing agents such as benzothiazole derivatives by *Rhodococcus*  
108 isolates (Besse et al., 2001; De Wever et al., 2001, 1998; Haroune et al., 2004) or more recently,  
109 acetanilide and hexamethoxymethylmelamine (HMMM), by the fungus *Trametes versicolor*  
110 ATCC 42530 (Wiener and Lefevre, 2022). Likewise, biodegradability of aliphatic  
111 hydrocarbons, used as plasticizers or processing aids in tires, was reported with bacteria  
112 (Abbasian et al., 2015; Sharma and Pant, 2000; Zampolli et al., 2014) and fungi (Daccò et al.,  
113 2020; Hidayat and Tachibana, 2012).

114 However, biodegradability of all these compounds was not assessed within TRWP. The  
115 biodegradation of the organic fraction of TRWP remains unknown and the characterization of  
116 the diverse potential carbon sources usable by microorganisms is one of the key points to  
117 understand TRWP environmental fate. To date, the characterization of the elastomer in tire  
118 particles themselves is poorly documented and only addressed in a few studies dealing with the  
119 ecotoxicological impact of TRWP (Halle et al., 2020; Khan et al., 2019). The chemical  
120 composition of TRWP was mainly elucidated through the analysis of their leachates by LC-  
121 ESI-HRMS (Halsband et al., 2020; Thomas et al., 2023; Unice et al., 2015). Recently, Müller  
122 et al. (2022) combined non-target and suspect screening strategies to analyze extracts of  
123 different pieces of tire treads. They detected 214 organic substances of which more than one

124 hundred leachates as potential water pollutants. Recent research works mainly focused on the  
125 detection and transformation of tire additives such as protective and vulcanizing agents or  
126 plasticizers (Halsband et al., 2020; Müller et al., 2022; Selbes et al., 2015; Unice et al., 2015;  
127 Zahn et al., 2019). Although SBR and IR are widely used markers for the detection and  
128 quantification of TRWP in the environment by pyrolysis-gas chromatography-mass  
129 spectrometry (Py-GC/MS) (Goßmann et al., 2021; Miller et al., 2022a, 2022b; Rauert et al.,  
130 2022a, 2022b; Unice et al., 2013, 2012), the elastomer part of tire, is relatively poorly  
131 characterized in TRWP transformation studies.

132 To compensate this lack of knowledge, we studied the biodegradability and ultimate fate  
133 of a RP sample. It has the advantage to contain perfectly representative TRWP, at least from  
134 the target vehicle and from other vehicles due to particle resuspension (Charbouillot et al., 2023;  
135 Järllskog et al., 2022; Kreider et al., 2010). This study implemented complementary analytical  
136 tools to characterize the complex RP chemical composition and to investigate the  
137 biodegradation of both potential leachable compounds and solid particles by 6 bacterial strains,  
138 belonging to 3 ubiquitous genera, *Rhodococcus*, *Pseudomonas* and *Streptomyces*. These genera  
139 are well-known for their ability to biodegrade environmental contaminants (Kim et al., 2018;  
140 Martinkova et al., 2009; Nazari et al., 2022; Wasi et al., 2013) such as aliphatic and polycyclic  
141 aromatic hydrocarbons (PAH)(Abbasian et al., 2015; Dean-Ross et al., 2001; Ghosal et al.,  
142 2016), pesticides (Briceño et al., 2018; Gilani et al., 2016) but also various polymers such as  
143 polyethylene (Matjašič et al., 2021), polystyrene (Mohan et al., 2016; Mor and Sivan, 2008),  
144 polyester (Ghosh et al., 2013; Mohanan et al., 2020) or natural rubber (Ali Shah et al., 2013;  
145 Andler et al., 2022; Chengalroyen and Dabbs, 2013; Joseph et al., 2022). The biodegradation  
146 of SBR was evaluated by Nuclear Magnetic Resonance spectroscopy (NMR). The leaching of  
147 organic compounds and their transformation products was monitored by chromatography

148 methods coupled to mass spectrometry (LC/HRMS and GC/MS). The biodegradation of RP  
149 was also investigated by monitoring the growth and respiration of microorganisms.

150

## 151 **2. Material and Methods**

### 152 *2.1. Chemicals*

153 All the solvents used for extraction were HPLC analytical grade (Sigma Aldrich).  
154 Deuterated water supplemented with a 0.2 mM TSPd<sub>4</sub> (sodium tetra deuterated trimethylsilyl  
155 propionate) and methanol-d<sub>4</sub>, acetone-d<sub>6</sub>, chloroform-d supplemented with 0.03 v/v% TMS  
156 (tetramethylsilane) were purchased from Eurisotop. Analytical NMR standards for styrene  
157 butadiene rubber (SBR; 15 w% styrene, 20 w% 1,2-butadiene, 65 w% 1,4-butadiene) and  
158 sunflower oil (8001-21-6) were provided by Michelin. Analytical LC/HRMS standards for N-  
159 (1,3-dimethylbutyl)-N'-phenyl-*p*-phenylenediamine (6PPD-793-24-8), N,N'-  
160 diphenylguanidine (DPG-102-06-7), 2,2,4-Trimethyl-1,2-dihydroquinoline (TMQ-26780-96-  
161 1), 2,2-Dithiobisbenzothiazole (MBTS-120-78-5), N-Cyclohexyl-2-benzothiazolsulfenamide  
162 (CBS-95-33-0), Mercaptobenzothiazole (MBT-149-30-4), Benzothiazole (BT-95-16-9), 2-  
163 Hydroxybenzothiazole (HOBT-934-34-9) were purchased from Sigma Aldrich.

164

### 165 *2.2. Environmental TRWP (RP) collection*

166 RP were collected in 2019 with an on-road driving collection device Charbouillot et al.  
167 (2023), from a summer tire (Michelin Primacy 4, 225/45 R17 91W), on the high- speed track on  
168 the Michelin test center of Ladoux, near Clermont-Ferrand (France) as described in Fohet et al.  
169 (2023). Briefly, the device was positioned close to the rear left wheel to limit external pollution  
170 (*i.e.* vegetation, minerals) from the right side of roads. The device (Fig. S1) was composed of  
171 three nozzles connected to three vacuums located within a trailer attached to the car and  
172 powered by a power generating unit (GYW-9 M5, Genelec, 230 V, 6000 W). Three filters



173 (FESTO, MS9-LFM-B, pore size: 1  $\mu\text{m}$ ) located between the nozzles and the vacuums allowed  
174 to collect the particles resulting from the contact between the tire and the road, including TRWP  
175 of the target tire as well as other non-tire particles and possibly TRWP that were previously  
176 emitted by other vehicles and resuspended by our testing vehicle. The raw sample was sieved  
177 to 250  $\mu\text{m}$ , removing large debris, to obtain a fine black powder. Indeed, previous work showed  
178 that the fraction [0-250  $\mu\text{m}$ ] accounted for *ca.* 90 w% of the sample mass in the [0-850  $\mu\text{m}$ ]  
179 range (Charbouillot et al., 2023). Moreover, sharper granulometry is preferable for  
180 biodegradation experiments to avoid kinetic effects due to specific surface area limitations  
181 (Chinaglia et al., 2018) and reduce dispersion between replicates. These particles were stored  
182 for about a year in the dark at room temperature prior our study.

183  
184

### 185 2.3. RP extractions

186 RP were autoclaved (120  $^{\circ}\text{C}$ , 1 bar) to avoid any parasitic biological action on the  
187 materials.  $10 \pm 0.3$  mg of RP were extracted with 1 mL of sterile distilled water, methanol,  
188 acetone or chloroform in sterile glass vials and incubated on a horizontal shaker at 200 rpm and  
189 25 $^{\circ}\text{C}$  in an incubator (Bioconcept, Firlabo). The aqueous samples were previously sonicated  
190 for 20 min in an ultrasonic bath to avoid aggregate formation. All extractions were performed  
191 in triplicate and samples were collected after 1, 7, 14 and 21 days of incubation. After  
192 decantation, the supernatants were aliquoted and stored at -20 $^{\circ}\text{C}$  for one month at most before  
193 analyses.

194

### 195 2.4. RP biodegradation

196 *Culture and inoculum preparation:* The bacterial strains were selected as follows: *R. ruber*  
197 (ATCC 29672) and *P. vancoverensis* (DSM 8368) for their capacity to degrade polyolefins  
198 (polyethylene and polypropylene) (Bonhomme et al., 2003; Eyheraguibel et al., 2017;

199 Fontanella et al., 2013) and PAH (Barnsley, 1975), respectively ; *R. rhodochrous* (ATCC  
200 15906), *S. anulatus* (Lab 2112), *S. griseolus* (Lab 912), previously isolated in our laboratory  
201 from an old tire and test track soil (Michelin, Clermont-Ferrand , France) and *S. phaeofaciens*  
202 (NRRL 8092) for their capacity to grow on natural rubber (Borel, 1980). The *Rhodococcus*  
203 and *Pseudomonas* strains were grown in 10 mL of Tryptic soy broth in 100 mL flasks at 25°C  
204 for 1 day. The *Streptomyces* strains were cultivated on agar Emerson medium (Table S1) in  
205 Petri dishes for 1 week at 25°C. Spore concentrations were determined using Malassez counting  
206 chamber and microscope (Olympus) and spore suspensions were conserved in 15% glycerol  
207 (w/v) at -80°C before inoculation.

208 *Biodegradation experiment.* Biodegradation assays were performed in 4 mL hermetic sterile  
209 glass vials in triplicate for each strain. Strains were inoculated at  $10^6$  cell.mL<sup>-1</sup> or  $10^6$  spore.mL<sup>-1</sup>,  
210 depending on the strain, in 1 mL mineral salt medium (MSM, Table S2) containing or not  
211 (biotic control)  $10 \pm 0.3$  mg of autoclaved RP as the sole carbon source. Incubations of RP in  
212 MSM without bacteria were also prepared as the abiotic controls. Incubations were performed  
213 for 14 days in the same experimental conditions as those used for extractions.

214 *Bacterial growth.* The bacterial growth was assessed by measuring the cellular respiration  
215 (CO<sub>2</sub>) and the biomass production (ATP). The CO<sub>2</sub> production was monitored by GC/MS using  
216 Thermo Trace ultra-GC coupled to a Thermo Trace DSQ mass spectrometer. Chromatographic  
217 separation was obtained on a Agilent J&W GS Gaspro 60 m x 0.32 mm column. At the end of  
218 incubation, 50 µL of sample was withdrawn from the headspace using a gas-tight syringe and  
219 injected into column at an initial temperature of 100 °C. Analyses were performed under  
220 isothermal conditions (60 °C) for a total run of 6 min. Helium was used as the carrier gas at a  
221 constant flow of 1 mL.min<sup>-1</sup>. Detection of CO<sub>2</sub> was performed using selected ion monitoring  
222 (SIM) mode ( $m/z$  44 (<sup>12</sup>C CO<sub>2</sub>) and  $m/z$  45 (<sup>13</sup>C CO<sub>2</sub>)) with 70 eV of electronic impact ionization  
223 energy. Quantification of CO<sub>2</sub> was calculated using a calibration curve carried out with CO<sub>2</sub>

224 standard samples at different known concentrations. ATP measurements were carried out on  
225 the final total cell extract according to the Biothema ATP kit extraction procedure.

226 *Preparation of samples for analysis.* After 14 days of incubation, 200  $\mu\text{L}$  of the culture were  
227 centrifuged at 9.7 g during 5 min and the supernatant triplicates were analyzed by LC/HRMS.  
228 Simultaneously, for one replicate of each condition, the residual RP were lyophilized overnight  
229 in a RP2V SERAIL freeze dryer. RP were then extracted in 1 mL of chloroform during 1 day  
230 in the same experimental conditions as those previously described. Chloroform extracts were  
231 analyzed by  $^1\text{H}$  NMR and GC/MS.

232

## 233 2.5. NMR analysis

### 234 2.5.1. $^1\text{H}$ NMR

235 The supernatants from extraction and biodegradation experiments (600  $\mu\text{L}$ ) were dried  
236 under a nitrogen flux and resuspended in 600  $\mu\text{L}$  of a deuterated solvent supplemented with a  
237 reference (TSPd<sub>4</sub> (or TMS). Deuterated solvents were used for locking (field stability) and  
238 shimming (field homogeneity) while TSPd<sub>4</sub> and TMS constituted a reference for chemical  
239 shifts (0 ppm) and quantification (Reference value = 100). All spectra were recorded on a  
240 Bruker 500 MHz Avance III spectrometer, equipped with a 5 mm Prodigy cryoprobe inverse-  
241 triple tuned (TCI)  $^1\text{H}/^{13}\text{C}/^{15}\text{N}$  with z-gradient coil probe (Bruker Biospin) with 5 mm-diameter  
242 tubes. 128 scans were collected (3.24 s acquisition time, 4.0 s relaxation delay, a spectral  
243 window of 20 ppm, FID size of 65536 data points zero-filled to 131072 point). Water signal  
244 was eliminated by pre-saturation (noesygprr1d sequence). An exponential filter (line  
245 broadening LB=0.3 Hz) was applied before Fourier transformation and an automatic baseline  
246 correction was performed on spectra before integration. Under these conditions, the limit of  
247 quantification is 0.01 mM. Relative integrations were expressed in arbitrary unit (a.u).

248 *SBR calibration curve.* A SBR calibration curve was prepared using five concentrations (from  
249 0.10 to 0.80 mg.mL<sup>-1</sup>) of a pure and uncrosslinked SBR, totally soluble in CDCl<sub>3</sub>. <sup>1</sup>H NMR  
250 spectra were recorded for each solution with the same acquisition parameters as those described  
251 previously. The specific region of 1,2-butadiene protons ( $\delta_{\text{H}} \sim 4.80\text{--}5.05$  ppm) was integrated  
252 relative to the internal TMS standard. A calibration curve was established between the relative  
253 integration and the concentration of SBR. For semi-quantification of SBR in RP chloroform  
254 extracts, the same specific region was integrated and correlated to the corresponding SBR  
255 concentration. The chloroform extraction of SBR from RP was determined **in triplicate** after **1,**  
256 **7, 14, 21 days, and showed the same** efficiency and recovery rate regardless of the extraction  
257 time.

258

#### 259 2.5.2. HSQC NMR

260 2D heteronuclear (<sup>1</sup>H-<sup>13</sup>C HSQC) experiments were performed with quadrature phase  
261 detection in dimensions, using Echo-Antiecho detection mode in the indirect one. For each 512  
262 increments in the indirect dimension, 2K data points were collected and 64 transients were  
263 accumulated in the direct dimension. <sup>13</sup>C decoupling (GARP4) was performed during <sup>1</sup>H  
264 acquisition time. Indirect data was zero-filled to 2k and a  $\pi/2$  shifted square sine-bell function  
265 was applied in both dimensions before Fourier transformation. All spectra were recorded at 298  
266 K and treated with TopSpin version 4.0.7 software.

267

#### 268 2.6. LC/HRMS analyses

269 The supernatants from extraction and biodegradation experiments (200  $\mu$ L) were dried  
270 under a nitrogen flux. Samples were 2-fold concentrated by dissolving them in 100  $\mu$ L of a  
271 water / acetonitrile mixture (50:50 v:v). LC/HRMS analyses were performed with an Ultimate  
272 3000 RSLC chromatographic system (ThermoScientific) coupled to an Exactive Orbitrap mass

273 spectrometer (Thermo Fisher Scientific) equipped with an electrospray (ESI) source. The  
274 chromatographic separations were performed using a Kinetex EVO C18 column (2.1 x 100  
275 mm; 1.7  $\mu$ m-Phenomenex) operating at 30°C and an injection volume of 5 $\mu$ L. The flow rate  
276 was fixed at 0.45 mL/min with 0.1% of formic acid in water (A) and in acetonitrile (B) for  
277 mobile phases at the following gradient: initial, 95% A; 0–7.5 min linear, 1% A linear; 7.5–8.5  
278 min, 1% A; 8.5–9 min, 95% A linear, 9–11 min 95% A following by washing and reconditioning  
279 of the column. The mass spectrometer was operated in positive ion mode with capillary voltage  
280 at 3,2 kV and –3 kV, respectively and a capillary temperature set at 320 °C. The detection was  
281 performed with full scan from m/z 50 to 750 using a resolution set at 70 000 at m/z 200. MS  
282 raw data were processed using Xcalibur Software (version 4.2) and python scripts. High  
283 resolution mass spectrometry was used to determine monoisotopic mass of ionized compounds.  
284 The m/z values whose peak intensity was above  $10^4$  and over 3 times that of the biotic control,  
285 were annotated by putative matches with a database containing 1219 compounds related to tire  
286 or technical rubber components and their transformation products. The database combines the  
287 lists of suspected compounds published by Müller et al. (2022) (792 compounds), EPA  
288 CompTox Chemical database “TYRES” (182 out of 274 compounds) and an inhouse database  
289 (245 out of 355 compounds) compiled from the literature data (Fohet et al., 2023; Halsband  
290 et al., 2020; He et al., 2011; Hu et al., 2022; Joseph et al., 2015; Klöckner et al., 2020; Kreider  
291 et al., 2010; Llompарт et al., 2013; Rauert et al., 2022a; Seiwert et al., 2022a; Thomas et al.,  
292 2022b; Warhurst and Fewson, 1994). Database was curated to avoid duplicate entries and the  
293 monoisotopic mass of the [M+H] ion of all entries was compared to the experimental m/z value  
294 with a mass tolerance of 5 ppm for putative assignment.

295

## 296 2.7. GC/MS analyses

297 600  $\mu\text{L}$  of the RP extract were dried under a nitrogen flux and were 6-fold concentrated  
298 in 100  $\mu\text{L}$  of a suitable solvent (hexane or chloroform for chloroform extracts and acetonitrile  
299 for water, methanol and acetone samples). Analyses were performed on an Agilent 6890A GC,  
300 equipped with a J&W Scientific HP-5MS (Methyl siloxane) column (30 m x 0.25 mm; 0.25  
301  $\mu\text{m}$ ) and coupled to an Agilent 5973N MSD (single quadrupole). Oven temperature: linear  
302 gradient 100°C-325°C for 22.5 min; 325°C for 10 min; sample volume: 2  $\mu\text{L}$ ; Carrier gas:  
303 helium at a constant flow of 1  $\text{mL}\cdot\text{min}^{-1}$ . Mass spectrometer acquisitions were made in scan  
304 mode, from 50 to 600  $m/z$  with 70 eV of electronic impact ionization energy. Chemical  
305 identifications were performed on the chromatographic peaks with area strictly greater than 10<sup>6</sup>,  
306 using the NIST17 standard reference database with a score greater than 80%.

307

## 308 2.8. Elemental analyses CHNS-O

309 The elemental composition of RP was analyzed in triplicate by the CHNS/O elemental  
310 analyzer FlashSmart from ThermoFischer based on fast catalytic pyrolysis. CHNS analysis was  
311 performed under combined reductive (tin capsids) and oxidative ( $\text{V}_2\text{O}_5/\text{O}_2$ ) conditions at 960°C  
312 and with a 110  $\text{mL}\cdot\text{min}^{-1}$  carrier gas (He) flow and a 250  $\text{mL}\cdot\text{min}^{-1}$  combustion gas flow  
313 ( $\text{He}/\text{O}_2$ ) to quantify C( $\text{CO}_2$ ), H( $\text{H}_2\text{O}$ ), N( $\text{N}_2$ ) and S( $\text{SO}_2$ ). For O analysis, RP were pyrolyzed  
314 in Ag capsids at 1065°C to form  $\text{O}_2$ . Gas were separated on GC columns under He flow and  
315 analyzed by a thermal conductivity detector (TCD). The calibration was performed using 2,5-  
316 bis(5-*tert*-butyl-benzoxazol-2-yl)thiophene (BBOT) standards. Masses of samples were lower  
317 than 2 mg.

318

## 319 3. Results

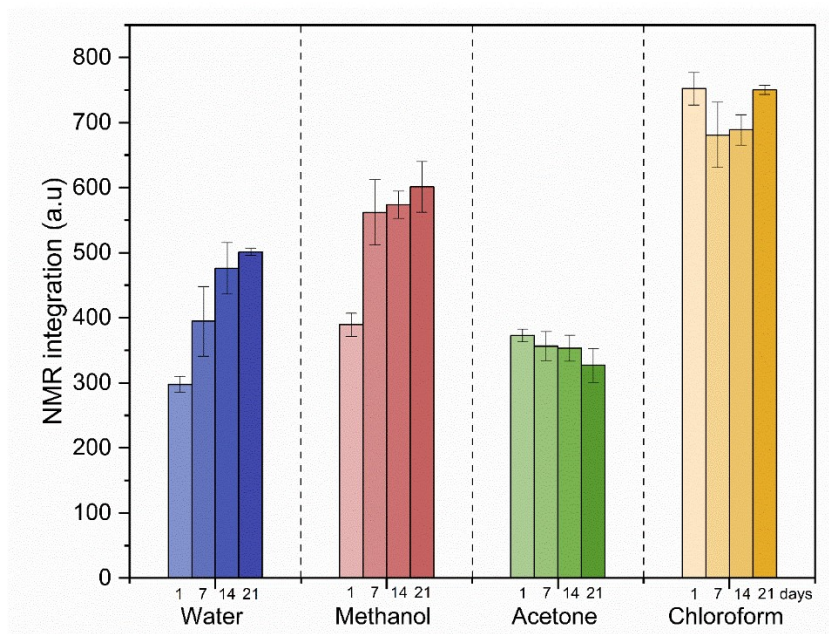
320

321 3.1. Characterization of the RP carbon sources

322

323 3.1.1. Analysis of the leachate profiles and SBR by NMR spectroscopy

324 <sup>1</sup>H NMR liquid state analysis was used to gather semi-quantitative and qualitative  
325 information on the compounds extracted with different solvents. A specific profile of the RP  
326 extracts can be observed for each solvent in terms of composition and concentration (Fig. S2).  
327 Integration of all the NMR signals versus that of the reference was used to study the effect of  
328 extraction time on its efficiency (Fig. 1). It is noteworthy that the integration value obtained  
329 cannot be compared between solvents as different compounds can be extracted according to  
330 the solvent used. For the water and methanol extracts, an increase in the entire NMR signal was  
331 measured with time. The extraction yield was increased by 33%, 60% and 68% in water and  
332 by 44%, 48% and 55% in methanol when the extraction time was extended from 1 to 7, 14 and  
333 21 days, respectively, indicating a gradual release of RP leachable compounds. This trend was  
334 not observed for acetone and chloroform which are less polar solvents and provide a better  
335 extraction of RP hydrophobic compounds. The maximum extraction was reached within 1 day  
336 as these solvents induce a better swelling of the particles and a quicker extraction.



337

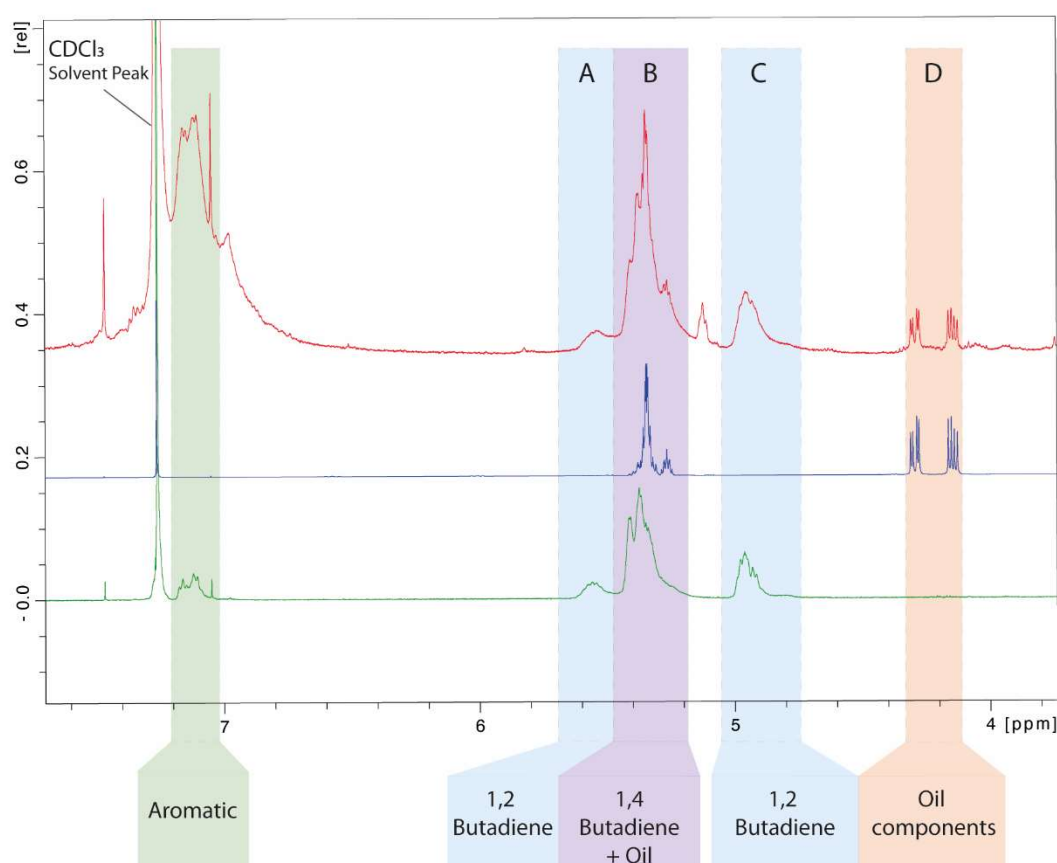
338 **Fig. 1.** Relative integration of the entire  $^1\text{H}$  NMR signal compared to that of an internal standard  
339 in function of the extraction time and solvent. The integration values cannot be compared  
340 between solvents as different compounds can be extracted. The mean values ( $n=3$ )  $\pm$  standard  
341 deviation are presented.

342

343 All the RP extracts (Fig. S2A-S2D) presented an intense  $^1\text{H}$  NMR signal in aliphatic ( $\delta_{\text{H}} \sim 0.0$ – $1.9$  ppm) and functionalized aliphatic ( $\delta_{\text{H}} \sim 1.9$ – $3.1$  ppm) structure regions. The water  
344  $\sim 0.0$ – $1.9$  ppm) and functionalized aliphatic ( $\delta_{\text{H}} \sim 1.9$ – $3.1$  ppm) structure regions. The water  
345 extract exhibited high signals in the region of oxygenated groups ( $\delta_{\text{H}} \sim 3.1$ – $4.3$  ppm). On the  
346 contrary, no signal related to unsaturated ( $\delta_{\text{H}} \sim 5.3$ – $7.0$  ppm) or aromatic ( $\delta_{\text{H}} \sim 7$ – $10$  ppm)  
347 functional groups was detected in water extract, while these signals were clearly present in  
348 chloroform extracts (as well as in acetone and methanol to a lesser extent). The signal ( $\delta_{\text{H}} \sim$   
349  $7.0$ – $7.2$  ppm) was assigned to the styrene aromatic protons and that at ( $\delta_{\text{H}} \sim 4.8$ – $5.7$  ppm) to the  
350 butadiene protons after comparison with the  $^1\text{H}$  NMR spectrum of a SBR standard (Fig. 2).  
351 Nevertheless, NMR analysis of complex samples often involves signal and dynamic range  
352 overlaps according to the relative concentrations of each compound. Complementary analysis  
353 (2D-NMR) showed that the regions assigned to 1,2-butadiene ethylenic protons ( $\delta_{\text{H}} \sim 5.5$ – $5.7$   
354 ppm, region A and  $\delta_{\text{H}} \sim 4.8$ – $5.05$  ppm, region C ; Fig. 2, S3 and S4) can be used as a marker  
355 to evidence the solubilization of SBR chains in chloroform extract (named CHL fraction). The  
356 integration value of these specific regions was thus used to build a SBR calibration curve using  
357 a pure SBR standard dissolved in chloroform at different concentrations. The concentration of  
358 the SBR fraction extracted in chloroform was then estimated, assuming that the extracted chains  
359 have the same microstructure as that of the SBR standard used for calibration. Regardless of  
360 the extraction time, the amount of CHL fraction was  $0.12 \text{ mg}\cdot\text{mL}^{-1}$  which represented  
361 approximately 1.2 wt% of the RP mass present. This value could be underestimated as the  
362 extraction recovery rate may be incomplete. As the content of RP organic fraction was



363 estimated around 40 wt% by elemental analysis of the particle (carbon:  $28.55 \pm 1.05$  wt%,  
364 hydrogen:  $3.23 \pm 0.15$  wt%, nitrogen:  $0.36 \pm 0.18$  wt%, sulfur:  $0.74 \pm 0.04$  wt% and oxygen  
365  $6.75 \pm 0.56$  wt%), the CHL fraction would represent approximately 3% of the organic fraction  
366 of RP. NMR analyses therefore enable the monitoring of a non-negligible part of the elastomer  
367 and the determination of whether it is impacted by degradation processes.



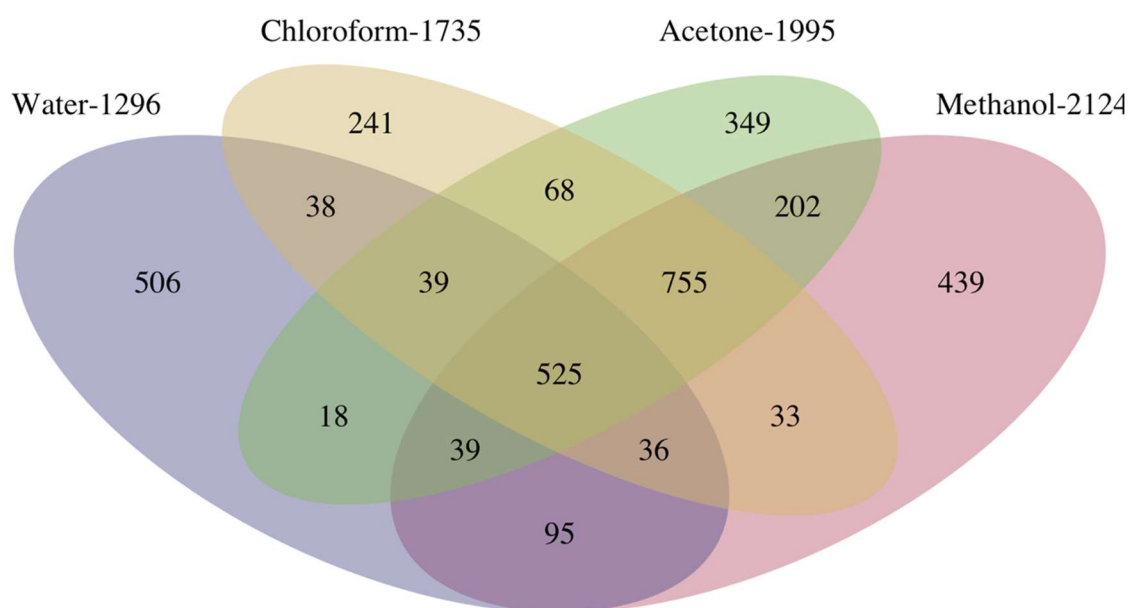
368  
369 **Fig. 2.** Liquid state <sup>1</sup>H NMR spectra of a SBR standard ( $0.2 \text{ mg}\cdot\text{mL}^{-1}$ ) in CDCl<sub>3</sub> (green), oil  
370 (blue) and a 21-day chloroform extract of  $10 \text{ mg}\cdot\text{mL}^{-1}$  of RP (red)

371

### 372 3.1.2. Analysis of potential leachable compounds by mass spectrometry

373 Full scan analysis of the different RP extracts generated after 21 days of incubation  
374 revealed the presence of numerous peaks whatever the solvent used. Nevertheless, as expected,  
375 the number of peaks was greater in organic solvents (respectively methanol  $2124 \text{ m/z}$ , acetone

376 1995 m/z, chloroform 1735 m/z) than in water (1296 m/z) (Fig. 3). 525 peaks were detected in  
 377 all extracts regardless of the solvent. These peaks represented 41% of the total peaks detected  
 378 in water and 25 to 30% of the total peaks detected in other solvents. A higher number of peaks  
 379 (755) were detected in all organic solvent extracts, corresponding to 36 to 44% of the total  
 380 peaks detected in these solvents. Some peaks were specifically detected in a single solvent and  
 381 represented 39% (506) of total peaks in water, 21% (439) in methanol, 17% (349) in acetone  
 382 and 14% (241) in chloroform. This higher rate (39%) of water-specific peaks might be ascribed  
 383 either to the preferential extraction of the most polar compounds and/or to a poor stability in  
 384 water of some of the parent compounds extracted leading to the formation of new  
 385 transformation products.



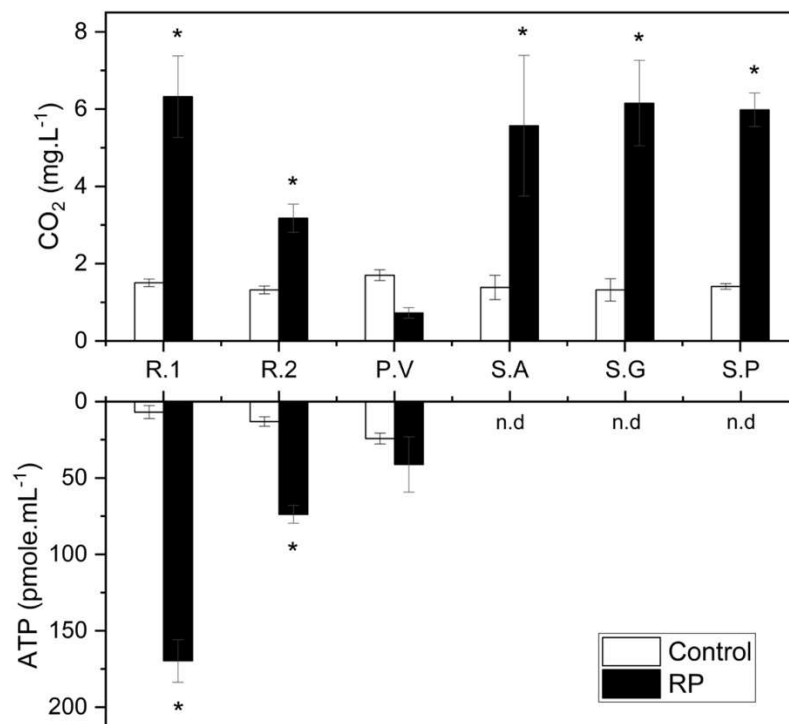
386  
 387 **Fig. 3.** Venn diagram representing the number of peaks detected by LC/ESI<sup>+</sup>-HRMS mode in  
 388 the 21-day extracts for the 4 solvents tested: water (blue), chloroform (yellow), acetone (green)  
 389 and methanol (red)

390 The molecular composition of RP extracts was characterized using a database containing  
391 1219 compounds related to rubber and additives for tire and/or technical rubber goods  
392 applications, and some of their transformation products. The exact mass of 259 ions detected in  
393 the extracts matched with that of the monoisotopic [M+H] ions of compounds from this  
394 database, which corresponds to the identification confidence level 5 used in mass spectrometry  
395 (Schymanski et al., 2014) (Table S3). Among them, 8 compounds were identified with a  
396 reference standard and are indicated in Table S3 (confidence level 1). The number of identified  
397 compounds were found to be slightly higher in organic solvents compared to water extraction.  
398 181 putative compounds were detected in water, compared to 214 in methanol, 201 in acetone  
399 and 208 in chloroform (Table S3). The list of compounds comprised several additives entering  
400 the tire formulation, including the curing agents diphenylguanidine (DPG), 2,2-  
401 Dithiobisbenzothiazole (MBTS), N-Cyclohexyl-2-benzothiazolsulfenamide (CBS) and the  
402 antioxidants N-(1,3-Dimethylbutyl)-N-phenyl-*p*-phenylenediamine (6PPD) and 2,2,4-  
403 Trimethyl-1,2-dihydroquinoline (TMQ). Some compounds reported as possible transformation  
404 products of DPG, MBTS/CBS and 6PPD were also detected in RP extracts, such as 8 urea  
405 residues (e.g., 1-Cyclohexyl-3-phenylurea (CPU), 1,3-diphenylurea, phenylurea), 6  
406 benzothiazole derivatives (e.g. benzothiazole, 2-hydroxybenzothiazole, 2-  
407 mercaptobenzothiazole) and more than 40 transformation products of 6PPD (e.g. 6PPD-  
408 quinone, 6QDI, N-Formyl-6PPD). To complete the characterization of molecular composition  
409 of RP extracts, GC/MS analyses were performed and led to 43 matches (including 6 compounds  
410 already detected in LC/MS) with the NIST17 standard reference database (Table S4). GC/MS  
411 analysis of chloroform extracts allowed a better detection of low-polarity compounds, such as  
412 fatty acids and alkanes, composing vegetal oil-based plasticizers and paraffin waxes,  
413 respectively. Overall, the utilization of both LC/HRMS and GC/MS led to the identification of  
414 296 putative compounds in RP extracts.

415

### 416 3.2. Biodegradation of RP

417 The complex organic composition of RP represents many sources of carbon that can be  
418 potential substrates for biodegradation. To assess the RP biodegradation, 6 bacterial pure strains  
419 were incubated in minimal salt medium (MSM) in the presence or absence of RP as the sole  
420 carbon source. After 14 days of culture, a bacterial growth was clearly observed for all the  
421 strains cultivated in the presence of RP, except for *P. vancoverensis*. A net increase of CO<sub>2</sub>  
422 production was recorded for *Rhodococcus* and *Streptomyces* strains, indicating up to 4 times  
423 higher cellular respiration than that of the biotic controls (Fig. 4). The concomitant biomass  
424 production was confirmed by the ATP increase for *Rhodococcus* strains. No ATP was detected  
425 for *Streptomyces* strains. The low extraction yield could be explained by the difficulty in  
426 breaking their cell wall. *P. vancoverensis* did not show any increase in CO<sub>2</sub> production and  
427 only a slight one for ATP content.



428

429 **Fig. 4.** CO<sub>2</sub> production and ATP concentration after 14 days of RP incubation with R.1 =  
430 *Rhodococcus ruber* ATCC 29672; R.2 = *Rhodococcus rhodochrous* ATCC 15906; P.V =  
431 *Pseudomonas vancouverensis* DSM 8368; S.A = *Streptomyces anulatus* Lab 2112; S.G =  
432 *Streptomyces griseolus* Lab 912; S.P = *Streptomyces phaeofaciens* NRRL 8092. The star  
433 represents statistically significant differences (Student test, p value < 0.05).

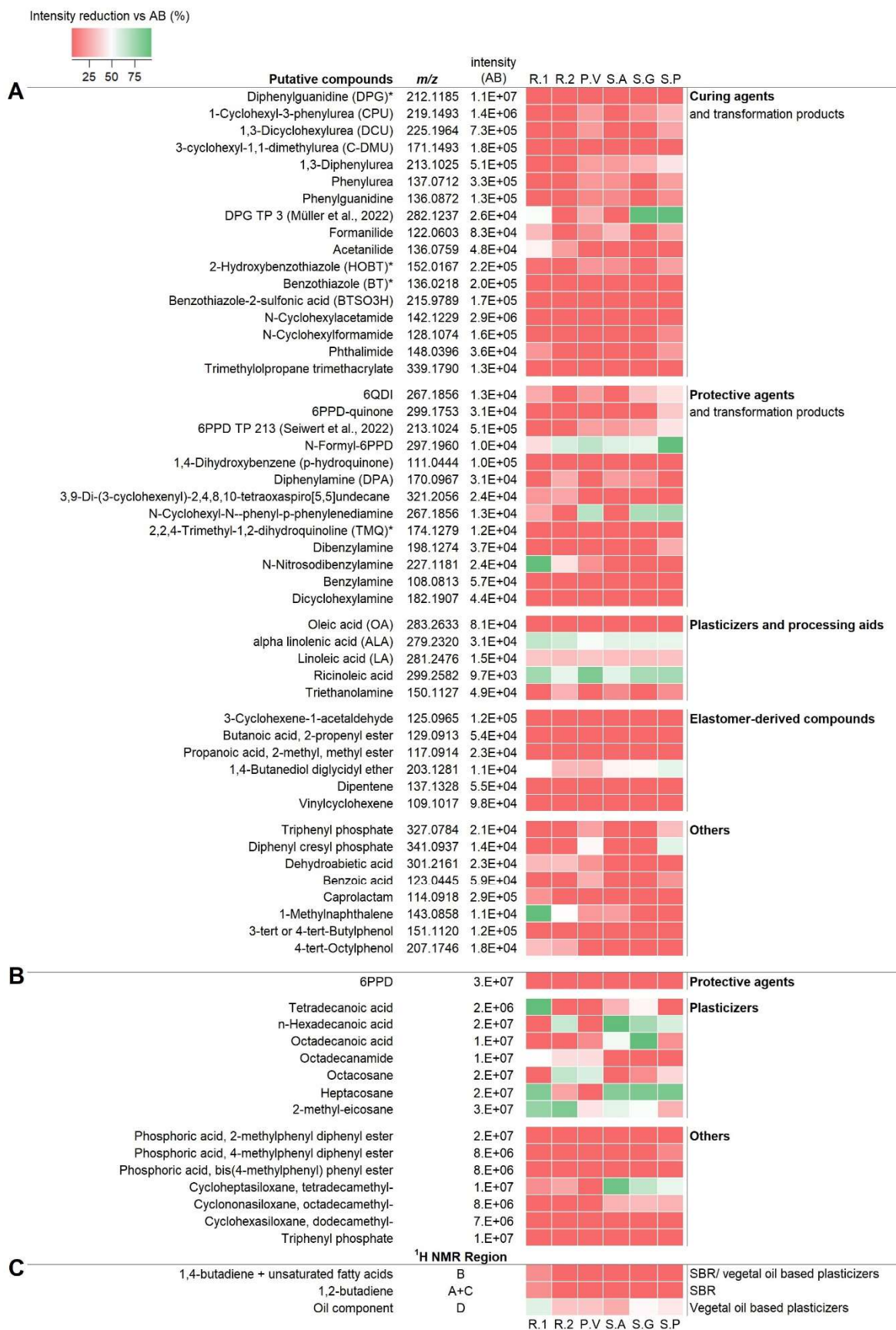
434           Several sources of carbon can be used to support the bacterial growth and may be found  
435 either in the culture medium through the leaching of RP water soluble compounds or directly  
436 on solid particles. Therefore after 14 days of incubation, the culture supernatants and the  
437 chloroform extracts of residual particles were analyzed in both abiotic and biotic conditions by  
438 the different analytical techniques presented previously. 996 peaks were detected by LC/HRMS  
439 in the supernatant and 61 by GC/MS in the CHL fraction of abiotic sample. Among them, 146  
440 peaks were annotated in the supernatant by putative matches with our compiled database and  
441 25 peaks were identified in the CHL fraction with the NIST database. Based on the current state  
442 of knowledge, some of these compounds were associated to tire-derived components and were  
443 thus sorted into several categories among curing agents, protective agents, plasticizers,  
444 elastomers or transformation products thereof. The possible source of some other putative  
445 compounds could not be as clearly ascribed (e.g. putative phthalates, phosphates, PAH) and  
446 were thus simply affiliated under the “others” category as RP may contain various sources of  
447 carbon beside tire components. In total, the degradation of these 171 markers was monitored in  
448 the biotic samples. The comparative results of 64 selected putative compounds monitored by  
449 LC/HRMS (49) and GC/MS (15) are presented for the 6 bacterial strains tested in Fig. 5 as well  
450 as those for SBR and oil by <sup>1</sup>H-NMR. The complete list of compounds identified by LC/HRMS  
451 and GC/MS and the corresponding heatmap are available in Tables S5 and S6.

452           Among the monitored compounds, plasticizers would preferentially be attacked by the  
453 investigated bacteria whether they are released into the supernatant (LC/HRMS) or still  
454 embedded within the particle (GC/MS). The putative fatty acids, namely linoleic (LA), alpha  
455 linolenic (ALA) and ricinoleic acids (in LC/HRMS), were partly degraded by the six strains (>  
456 50% for ALA and > 25% for LA). The degradation of ricinoleic acid would be the most  
457 significant (> 75%) for *R. ruber*, *P. vancouverensis*, *S. griseolus* and *S. phaeofaciens* strains.  
458 The degradation of the putative tetradecanoic, n-hexadecanoic or octadecanoic acids by *R.*  
459 *ruber*, *S. anulatus*, *S. griseolus* was also observed by GC/MS. Similarly, the putative alkane  
460 compounds (heptacosane, octacosane, 2-methyl-eicosane) were degraded by the different  
461 strains. Among the curing agents, diphenylguanidine (DPG), benzothiazole (BT) and 2-  
462 hydroxybenzothiazole (HOBT) were unequivocally identified. No degradation was observed  
463 with any of the strains for DPG and BT, whereas HOBT was slightly decreased after 14 days  
464 with *S. phaeofaciens*, *S. anulatus* and *P. vancouverensis* (18%, 13% and 16% degradation,  
465 respectively). Several peaks were assigned to different possible transformation products of  
466 DPG or CBS previously reported in the literature. Among them, benzothiazole-2-sulfonic acid  
467 and N-cyclohexylacetamide did not show any degradation whereas incubation with *S.*  
468 *phaeofaciens* led to a decline of most of the compounds possibly derived from DPG (1-  
469 cyclohexyl-3-phenylurea (CPU), 1,3-dicyclohexylurea (DCU), phenylurea, phenylguanidine,  
470 formanilide). Similarly, the *m/z* 282.1237 ion matching with a potential DPG reaction product  
471 (DPG TP3) reported by Müller et al. (2022) decreased by more than 75% with both *S. griseolus*  
472 and *S. phaeofaciens*. Among the protective agents, several *m/z* ions were tentatively assigned  
473 to transformation products of 6PPD (antioxidant) such as N-Formyl-N-(1,3-dimethylbutyl)-N-  
474 phenyl-*p*-phenylenediamine (N-formyl-6PPD), 6QDI, 6PPD-quinone, *p*-hydroquinone or the  
475 TP213 reported by Seiwert et al. (2022). N-formyl-6PPD was degraded by all strains (> 25%  
476 to more than 75% with *S. phaeofaciens*), while 6PPD-quinone decreased significantly (30%) in

477 the presence of *S. phaeofaciens* only. Various putative phenolic compounds, possibly deriving  
478 from phenols used as rubber antioxidants, were also detected (Table S5). They showed no or  
479 very limited degradation except for the  $m/z$  257.1547 ion potentially assigned to 4,4'-  
480 methylenebis(2,6-dimethylphenol) or 2,2'-methylenebis(4,6-dimethylphenol) which decreased  
481 with *R. rhodochrous* (46%) and *P. vancouverensis* (59%).

482 Chloroform extractions were also carried out on RP recovered after bacterial incubation .  
483 Assuming an identical extraction efficiency between the particles incubated with the bacterial  
484 strains and the negative control, the  $^1\text{H}$  NMR spectra of the extracts revealed that the  
485 degradation of elastomer only occurred with *R. ruber*. A decrease (12%) of the 1,2-butadiene  
486 ethylenic proton signals (Fig. 2 Regions A and C:  $\delta_{\text{H}} \sim 5.5\text{--}5.7$  ppm and 4.80–5.05 ppm) relative  
487 to those of the abiotic control was recorded within only 14 days of culture, confirming the  
488 alteration of the SBR chains (Fig. S5). A concomitant 13% reduction of the region B signals  
489 ( $\delta_{\text{H}} \sim 5.2\text{--}5.5$  ppm; ethylenic protons of both oil unsaturated fatty acids and 1,4-butadiene  
490 moieties of SBR) supports this trend. An important reduction of the specific oil signal (Region  
491 D:  $\delta_{\text{H}} \sim 4.1\text{--}4.3$  ppm) was also measured for all the strains tested (from 22% up to 60% with *R.*  
492 *ruber*), showing that both SBR and oil were degraded.

493





495 **Fig. 5.** Comparative heatmap of signal intensity reduction for 64 selected putative compounds,  
496 belonging to different categories of Roadway particles (RP) detected by **A)** LC/HRMS (49)  
497 (aqueous leachates), **B)** GC/MS (15) (chloroform extracts); **C)** <sup>1</sup>H-NMR (chloroform extracts)  
498 after 14-day biodegradation by 6 bacterial strains with an abiotic control (AB). Strains are  
499 abbreviated as follows R.1 = *Rhodococcus ruber* ATCC 29672; R.2 = *Rhodococcus*  
500 *rhodochrous* ATCC 15906; P.V = *Pseudomonas vancouverensis* DSM 8368; S.A =  
501 *Streptomyces anulatus* Lab 2112; S.G = *Streptomyces griseolus* Lab 912; S.P = *Streptomyces*  
502 *phaeofaciens* NRRL 8092. Intensities are not comparable between LC/HRMS and GC/MS. (\*)  
503 Compounds identified with a reference standard.

504

#### 505 **4. Discussion**

506

507 To date, only a few studies have addressed the fate of TRWP in the environment. Cadle  
508 and Williams (1980) investigated the soil degradation of tire wear particles generated with an  
509 indoor test facility by driving a tire on a steel pulley drum without any road-derived mineral  
510 encrustations. No biodegradation studies have been reported due to the difficulty of  
511 representative TRWP collection and the challenge to characterize them exhaustively. Indeed,  
512 TRWP have a complex and heterogeneous composition, corresponding to a mixture of hundreds  
513 of organic and inorganic compounds present in new tires but also coming from the tire aging  
514 by different processes (photo-, thermo oxidation, ozonation), combined with road surface and  
515 environmental components (Wagner et al., 2022). This composition evolves over time as  
516 TRWP are continuously submitted to various abiotic and biotic mechanisms once emitted in  
517 the environment (Fohet et al., 2023). Described as solid hetero-aggregates, they can also release  
518 leaching compounds according to the conditions. In the environment, they can also be mixed  
519 with other traffic-derived particles (such as brake dust, exhaust emission, other wear particles

520 re-suspended, ...) as well as other sources of particles (e.g. biogenic, residential, industrial...),  
521 forming Roadway particles (Kreider et al., 2010). Therefore, RP offer a variety of carbon  
522 sources for microorganisms, ranging from elastomer to numerous organic molecules from  
523 various chemical families, that can be potentially degraded. It is essential to consider all these  
524 carbon sources present in the particles to determine which type of compounds is degraded or  
525 not and to have a global overview of their environmental fate. Such a complex RP sample,  
526 collected in real driving conditions and combining at least representative TRWP from the target  
527 tire, but also TRWP from other vehicles and other traffic-derived particles (Charbouillot et al.,  
528 2023) was used as the test material for our study. A multi-analytical approach was implemented  
529 to identify and monitor leaching compounds and particle composition, including elastomers,  
530 plasticizers, vulcanizing agents, protective agents and their transformation products, before and  
531 after biodegradation.

532 We evaluated the RP-degrading capacity of 6 bacterial strains, belonging to 3 ubiquitous  
533 genera, *Rhodococcus*, *Pseudomonas* and *Streptomyces*. In this study, CO<sub>2</sub> and ATP  
534 measurements showed that all these strains, except *P. vancouverensis*, were able to grow on  
535 RP. To date, microbial growth on tire rubber, oxidized or not, was reported for several bacterial  
536 isolates and consortia by respirometric measurements (Alejo et al., 2022; Marchut-Mikołajczyk  
537 et al., 2019; Tsuchii and Tokiwa, 2006) but never demonstrated on TRWP. Our results  
538 confirmed that these strains, isolated from the environment, can also biodegrade organic  
539 compounds contained in RP and suggest that the biodegradation could occur in natural  
540 conditions. The next step was to study the biodegradation of the various potential carbon  
541 sources by bacteria.

542 Starting with the elastomer phase, it has previously been reported that worn tire tread  
543 surface exhibits a liquid-like layer composed of an intimate blend of mechanochemically-  
544 degraded rubber and road minerals, eventually leading to TRWP, from which decrosslinked

545 polymer chains can be extracted (Smith and Veith, 1982; Togashi and Mouri, 1999). For the  
546 first time,  $^1\text{H}$  NMR analysis was used to monitor the potentially extractable elastomer chains  
547 from TRWP, before and after bacterial incubation in liquid minimal medium with RP as the  
548 sole source of carbon. As expected, the chloroform extraction of RP (CHL fraction) induced  
549 the swelling of the rubber phase and the release of the soluble fraction from the rubber network,  
550 among which SBR chains were identified *via* NMR analysis. This tool allowed us to estimate  
551 that they would account for *ca.* 1.2 wt% of RP at least, which would represent *ca.* 3 wt% of RP  
552 organic fraction based on elemental analyses. Under the *proviso* that 1°) the contribution of  
553 organic sources deriving from a potentially non-tire origin within RP was negligible (*i.e.* RP  $\approx$   
554 multi-TRWP only), 2°) the elastomer can account for *ca.* 50 wt% of the organic fraction of  
555 TRWP, as determined by thermogravimetric analysis reported in Kreider et al. (2010), then one  
556 could estimate that these extractable chains of SBR would account for *ca.* 6% of the elastomer,  
557 which could represent a significant carbon source. After only 14 days of biodegradation, a  
558 significant decrease of the RP 1,2-butadiene ethylenic proton signal was observed with  
559 *Rhodococcus ruber*, suggesting the attack of this soluble fraction representing a degradation of  
560 0.8% of the total elastomer. Such quantitative NMR analyses have already been applied to  
561 monitor oligomers and assess biodegradation of other polymers such as polyethylene (PE)  
562 (Bonhomme et al., 2003; Eyheraguibel et al., 2017), polypropylene (Fontanella et al., 2013) and  
563 polyester (Cheng et al., 2022). Our results confirm the relevance of  $^1\text{H}$  NMR to address the fate  
564 of elastomer in TRWP providing specific qualitative and semi-quantitative information  
565 particularly necessary in such complex particles. This approach also brings complementary  
566 results to the soluble/gel fraction determination technics which have been preferentially used to  
567 assess microbial devulcanization of rubbers (Allan et al., 2021; Kaewpetch et al., 2019; Yao et  
568 al., 2013). Generally, a mass ratio of both fractions after swelling in an organic solvent is used  
569 to evidence the decrease of the polymer crosslinking and the increase of the soluble fraction

570 following the enzymatic breakages of sulphur bonds. Our observations rather suggested a  
571 degradation of SBR soluble oligomers by *R. ruber*. Only a few bacterial strains were described  
572 as SBR degraders. The biodegradation activity of *Bacillus subtilis*, *Streptomyces* sp. and  
573 *Pseudomonas aeruginosa* (Aboelkheir et al., 2019) or *Lactobacillus plantarum* (Olejnik et al.,  
574 2022) was shown on vulcanized SBR. The authors highlighted mass loss and surface  
575 modifications of rubber after 4 weeks, suggesting cleavages of sulfur and carbon bonds. Such  
576 cleavages can also occur during the use phase of tires by abiotic aging (photo-, thermo  
577 oxidation) and mechano-chemical reaction induced by the rubber-ground friction, resulting in  
578 a decrease of the molecular weights (Togashi and Mouri, 1999) and in an enhancement of  
579 microbial and/or enzymatic degradations. Furthermore, rubber oligomers with a lower  
580 molecular weight might be more rapidly biodegraded, as it was suggested by Tsuchii et al.  
581 (1984,1985) in the case of natural rubber vulcanizates and 1,4-polybutadiene degraded by  
582 *Nocardia* sp. 835A and *Moraxella* sp. 912, respectively. These results also highlight that the  
583 TRWP quantification in environmental matrices by Py-GC-MS using elastomer markers might  
584 be biased as a part of the elastomer may have been previously consumed by rubber-degrading  
585 microorganisms at the time of the analysis.

586         Beyond elastomers, a large part of RP organic carbon comes from formulation additives.  
587 The bioavailability of these compounds depends on their physico-chemical properties. They  
588 can be either leached out over time or remain in the TRWP and their biodegradation can thus  
589 occur in the environment when they are released or directly into the particles. First, we  
590 characterized by NMR, the extraction kinetics of the readily leachable and extractable  
591 compounds using various polarity solvents. A gradual release of leachable compounds was  
592 observed over 21 days of extraction in polar solvents (water and methanol), confirming the  
593 dynamic process of leaching in the environment. This corroborated previous studies about the  
594 effect of tire exposure time on organic leachate concentrations, such as an increase during 30

595 days of exposure in groundwater (Selbes et al., 2015) or during 14 days in seawater (Halsband  
596 et al., 2020). Müller et al. (2022) showed that 45.8% of chemicals were leached to less than  
597 10% of their theoretical potential after 28 days of constant contact to water indicating that the  
598 length of exposure is a key parameter in the release of chemicals from TRWP. All these results  
599 also emphasized the fact that the poorly leachable constituents may be slowly released into the  
600 environment. Non-polar solvent extraction indicated that some compounds were only extracted  
601 in acetone and/or chloroform. This observation confirmed that numerous compounds remain in  
602 the TRWP during the release of particles in the environment and emphasized that organic  
603 solvent extraction is necessary to assess the degradation of these chemicals. Among the putative  
604 compounds detected in this study, curing agents, protective agents, plasticizers and elastomers  
605 were particularly interesting to consider.

606         At the end of the biodegradation experiment, 996 peaks were detected by LC/HRMS in  
607 the supernatant of the abiotic control. Some of them (15%, 146 putative compounds) seem to  
608 match with compounds from an in-house database compiling rubber additives for tire and  
609 technical rubber good applications, and some of their transformation products. Nevertheless, a  
610 large number of ions remains unknown as it is very difficult to predict all the tire transformation  
611 products that can be formed and to list all the chemicals coming from the road and environment.  
612 As it was shown with tire tread pieces (Müller et al., 2022), crumb rubber granulates (Halsband  
613 et al., 2020), chips of shredded scrap tires (Selbes et al., 2015) and TRWP generated using an  
614 interior drum system with asphalt pavement (Marwood et al., 2011; Unice et al., 2015), our  
615 results confirmed that hundreds of chemicals can be extracted from RP directly into water and  
616 can thus diffuse in the environment (Seiwert et al., 2020). Numerous compounds, putatively or  
617 unequivocally identified in our RP extracts, have already been reported in tire particle leachates  
618 such as the widely used tire antioxidant 6PPD and its transformation products (*e.g.* 6PPD-  
619 quinone) (Hu et al., 2022; Seiwert et al., 2022a; Tian et al., 2021; Wagner et al., 2022), as well

620 as benzothiazoles, DPG, phenol derivatives, PAH and phthalates (Halsband et al., 2020; He et  
621 al., 2011; Kreider et al., 2010; Unice et al., 2015). The comparison of the above-mentioned  
622 leaching studies is limited due to the use of diverse substrates: leachates from crumb rubber  
623 granulates or scrap tires might contain compounds from internal parts of tire that are not in  
624 contact with the road and should not be detected in representative TRWP. On the other hand,  
625 the use of very representative TRWP, such in our RP sample, is impaired with the occurrence  
626 of compounds from non-tire sources. For instance, Kreider et al. (2010) previously reported  
627 significantly higher concentration of PAH in RP compared to TRWP generated on road  
628 simulator or to tread particles, suggesting a non-tire contribution in RP. Some putative  
629 compounds detected in this study might thus originate from other vehicle components or from  
630 the road bitumen, such as 1-methylnaphthalene (PAH), triphenyl phosphate (flame retardant  
631 used in lubricating oils or hydraulic fluids), phthalates, etc.

632         Mass spectrometry analyses allow to better monitor these various carbon sources and to  
633 compare their intensity before and after biodegradation, provided that no matrix effect impairs  
634 the comparison. Therefore, our non-target screening gave some new insights about the potential  
635 biodegradability of 171 chemicals. Among them, the putative compounds ascribed to fatty  
636 acids, particularly alpha-linolenic acid, linoleic acid and ricinoleic acid composing tire vegetal  
637 oils were degraded by the 6 strains tested within 14 days. Likewise, the putative alkanes  
638 extracted from RP (e.g. heptacosane, 2-methyl-eicosane), which might originate from mineral  
639 oils or paraffin waxes entering tire composition, seemed to be also potential substrates for  
640 bacteria. *Rhodococcus*, *Streptomyces* and *Pseudomonas* were largely reported to degrade long  
641 aliphatic chain hydrocarbons (Baoune et al., 2019; Larkin et al., 2005; Rojo, 2009; Sharma and  
642 Pant, 2000; Zampolli et al., 2014). While these compounds were attacked by all the 6 strains  
643 tested, the degradation of most of the other compounds was strain-dependent. Among the  
644 vulcanizing agents, the diphenylguanidine (DPG) and benzothiazole (BT) always identified in

645 the TRWP studies, were not degraded by our strains. To date, only a few bacterial strains  
646 belonging to the *Rhodococcus* genus (*R. pyridinovorans* PA, *R. rhodochrous* OBT18, *R.*  
647 *erythropolis* BTSO<sub>3</sub>) and isolated from activated sludges or industrial effluents, were reported  
648 to degrade BT and some of its derivatives or transformation products so far (De Wever et al.,  
649 1997, 1998; Gaja and Knapp, 1997; Haroune et al., 2002). Concerning DPG and phenylurea  
650 residues, Dvořák et al. (2014) reported their total mineralization by an adapted sludge for  
651 industrial wastewater treatment. There is also a growing interest for transformation products,  
652 particularly since the recent identification of 6PPD-quinone as an ozonation product deriving  
653 from the antioxidant and antiaging rubber agent 6PPD, this transformation product having a  
654 lethal toxicity in coho salmon above a given threshold (Tian et al., 2021). In our study, a 30%  
655 decrease of 6PPD-quinone was observed after 14 days with *S. phaeofaciens*, while present  
656 literature focused on abiotic fate of 6PPD and its transformation products (Fohet et al., 2023;  
657 Hu et al., 2022; Seiwert et al., 2022b). Recently, the occurrence and predominance of  
658 biodegradation of 6PPD-quinone in soils was demonstrated (Xu et al., 2023), highlighting the  
659 importance of considering biodegradation when assessing the environmental fate of additives.

660

## 661 **5. Conclusion**

662

663 Since a few years, TRWP, and more generally RP, have emerged as environmental  
664 contaminants of interest and the understanding of their fate is urgently required to assess their  
665 global impact and potential toxicity. Nevertheless, the complexity of their chemical  
666 composition is a limiting factor for achieving comprehensive studies, in particular to assess  
667 their biodegradability. The use of complementary analytical tools (NMR, LC/HRMS, GC/MS)  
668 in order to identify the different potential carbon sources of RP for microorganisms and then to  
669 monitor their biodegradation by 6 selected bacterial strains belonging to *Rhodococcus*,

670 *Pseudomonas* and *Streptomyces* genera constituted a powerful strategy to obtain a first  
671 overview of RP fate. The tested strains were able to grow on RP (except for *P. vancouverensis*)  
672 by degrading various carbon sources as demonstrated with the monitoring of SBR by NMR and  
673 hundreds of organic components by MS before and after incubation. Significant degradation of  
674 fatty acids and alkanes from oil and paraffin waxes was observed for the 6 strains. *Rhodococcus*  
675 *ruber* was particularly efficient towards SBR oligomers while *Streptomyces phaeofaciens*  
676 partially degraded several markers tentatively assigned to 6PPD and DPG transformation  
677 products (e.g. 6PPD-quinone, 6QDI, N-formyl-6PPD, CPU, DCU, phenylurea,  
678 phenylguanidine). Further work would be required to (1) complete the characterization of RP  
679 components as many remained unidentified and (2) unequivocally identify some markers of  
680 interest detected by LC/HRMS or GC/MS and that can be assigned to a tire origin, in order to  
681 further investigate their biodegradation mechanism and pathway. Longer incubation  
682 experiments and a wider screening of microbial strains, in *consortium* or not, would help to  
683 identify the definitively recalcitrant compounds and to estimate the degradation timescale of  
684 the degrading ones in more realistic environmental conditions. These tasks are key points to  
685 consider for assessing the environmental fate and biodegradation of RP in future studies.

686

#### 687 **Declaration of competing interest**

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

690

#### 691 **Acknowledgement**

692 This study was funded by the Manufacture Française des Pneumatiques Michelin.

693



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