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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 ¹H 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/cm³ (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 TSPd₄ (sodium tetra deuterated trimethylsilyl
155 propionate) and methanol-d₄, acetone-d₆, 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 μm) 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 μm , removing large debris, to obtain a fine black powder. Indeed, previous work showed
178 that the fraction [0-250 μm] accounted for *ca.* 90 w% of the sample mass in the [0-850 μm]
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 ± 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⁻¹ or 10^6 spore.mL⁻¹,
210 depending on the strain, in 1 mL mineral salt medium (MSM, Table S2) containing or not
211 (biotic control) 10 ± 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 (CO₂) and the biomass production (ATP). The CO₂ 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⁻¹. Detection of CO₂ was performed using selected ion monitoring
222 (SIM) mode (m/z 44 (¹²C CO₂) and m/z 45 (¹³C CO₂)) with 70 eV of electronic impact ionization
223 energy. Quantification of CO₂ was calculated using a calibration curve carried out with CO₂

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 μL 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 ^1H NMR and GC/MS.

232

233 2.5. NMR analysis

234 2.5.1. ^1H NMR

235 The supernatants from extraction and biodegradation experiments (600 μL) were dried
236 under a nitrogen flux and resuspended in 600 μL of a deuterated solvent supplemented with a
237 reference (TSPd₄ (or TMS). Deuterated solvents were used for locking (field stability) and
238 shimming (field homogeneity) while TSPd₄ 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⁻¹) of a pure and uncrosslinked SBR, totally soluble in CDCl₃. ¹H 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 (¹H-¹³C 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. ¹³C decoupling (GARP4) was performed during ¹H
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 μL) were dried
270 under a nitrogen flux. Samples were 2-fold concentrated by dissolving them in 100 μL 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 μ m-Phenomenex) operating at 30°C and an injection volume of 5 μ 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; Llompart 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 μL of the RP extract were dried under a nitrogen flux and were 6-fold concentrated
298 in 100 μL 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 μm) 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 μL ; 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 10⁶,
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 (He/O_2) to quantify C(CO_2), H(H_2O), N(N_2) and S(SO_2). For O analysis, RP were pyrolyzed
314 in Ag capsids at 1065°C to form 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

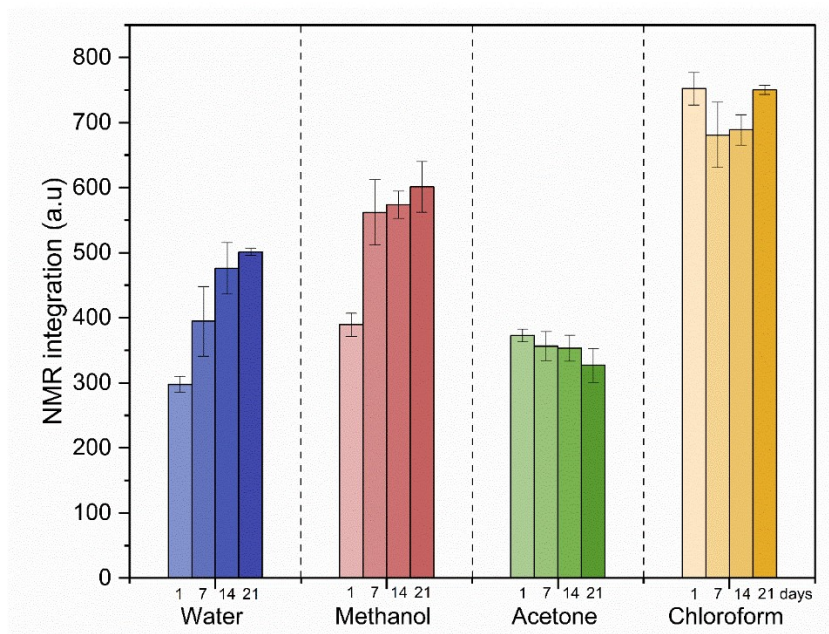
320

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 ^1H 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.



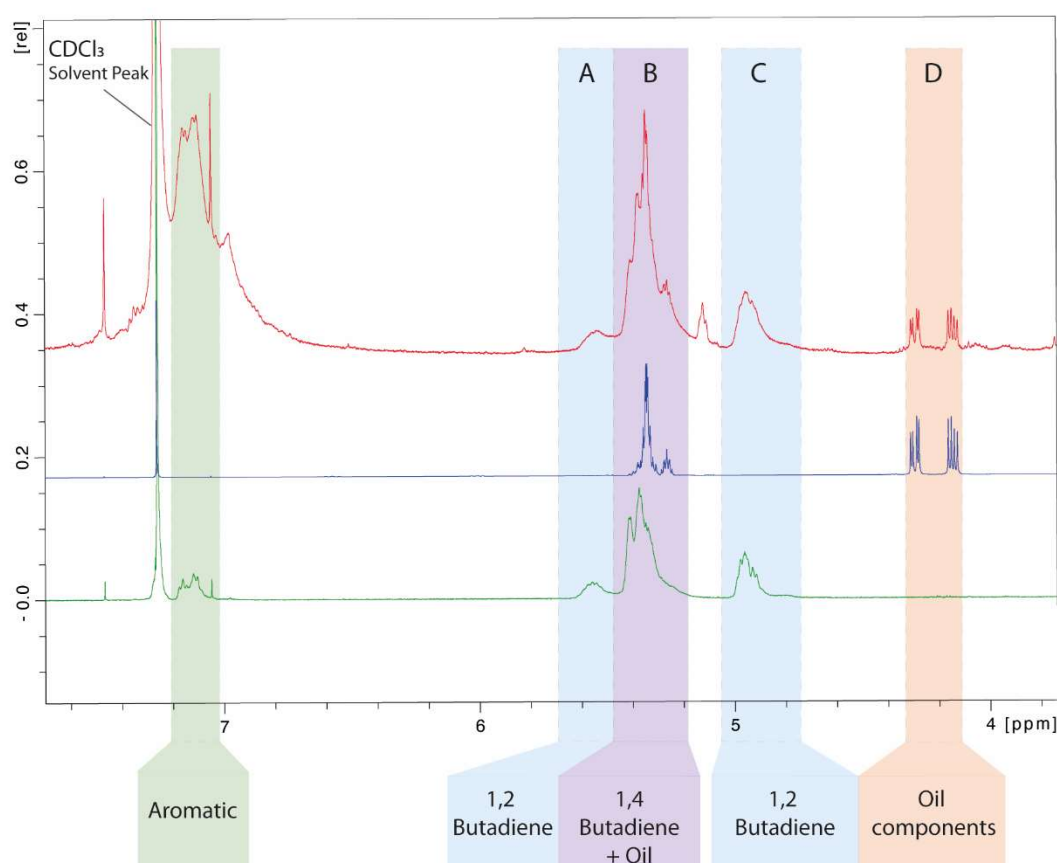
337

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

363 estimated around 40 wt% by elemental analysis of the particle (carbon: 28.55 ± 1.05 wt%,
364 hydrogen: 3.23 ± 0.15 wt%, nitrogen: 0.36 ± 0.18 wt%, sulfur: 0.74 ± 0.04 wt% and oxygen
365 6.75 ± 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.

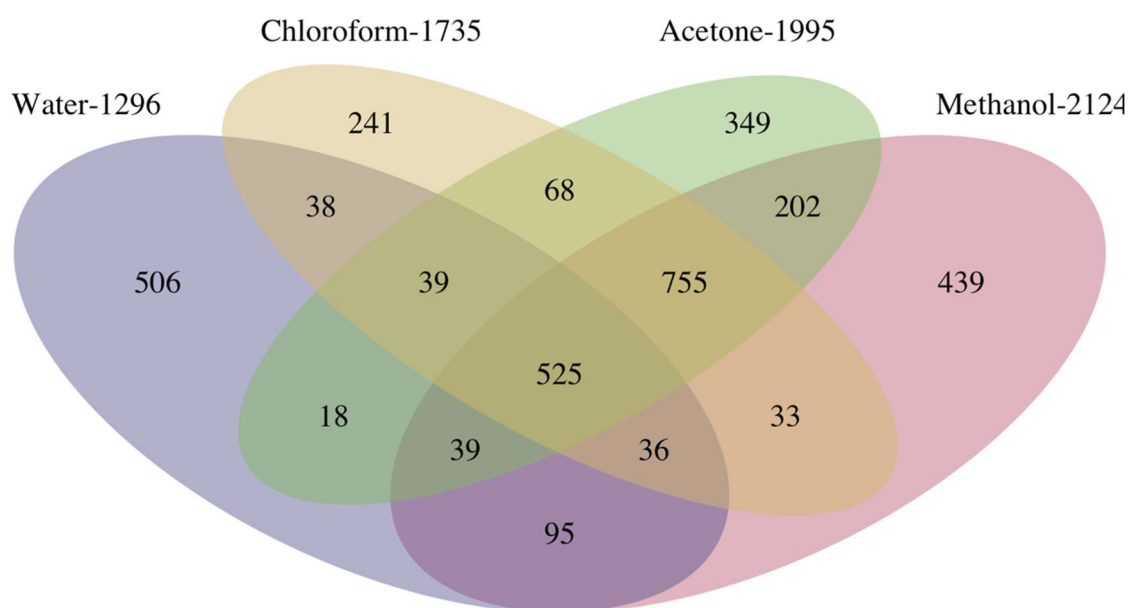


368
369 **Fig. 2.** Liquid state ^1H NMR spectra of a SBR standard ($0.2 \text{ mg}\cdot\text{mL}^{-1}$) in CDCl_3 (green), oil
370 (blue) and a 21-day chloroform extract of $10 \text{ mg}\cdot\text{mL}^{-1}$ of RP (red)

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 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.



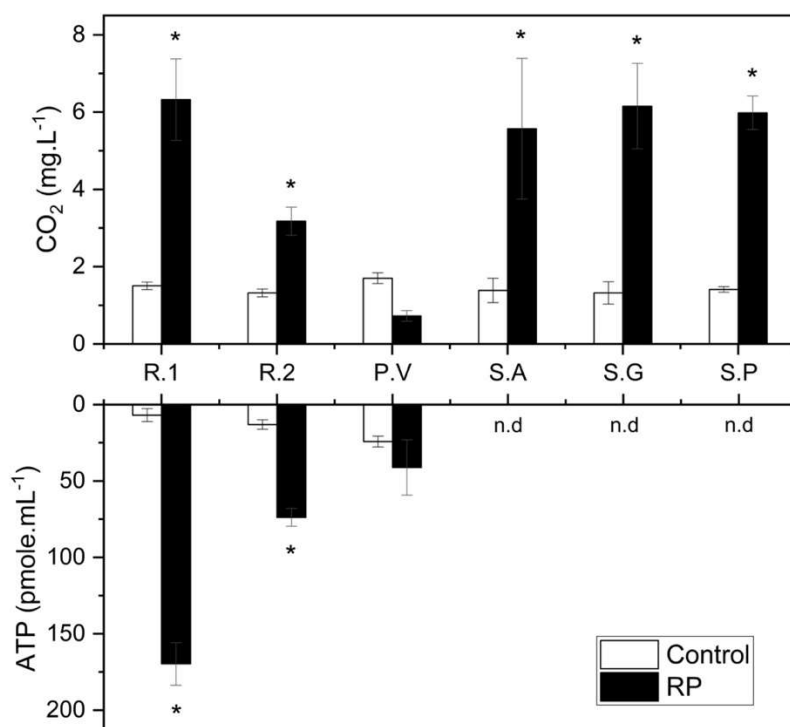
386
 387 **Fig. 3.** Venn diagram representing the number of peaks detected by LC/ESI⁺-HRMS mode in
 388 the 21-day extracts for the 4 solvents tested: water (blue), chloroform (yellow), acetone (green)
 389 and methanol (red)

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 CO₂
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 CO₂ production and
427 only a slight one for ATP content.



428

429 **Fig. 4.** CO₂ 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).

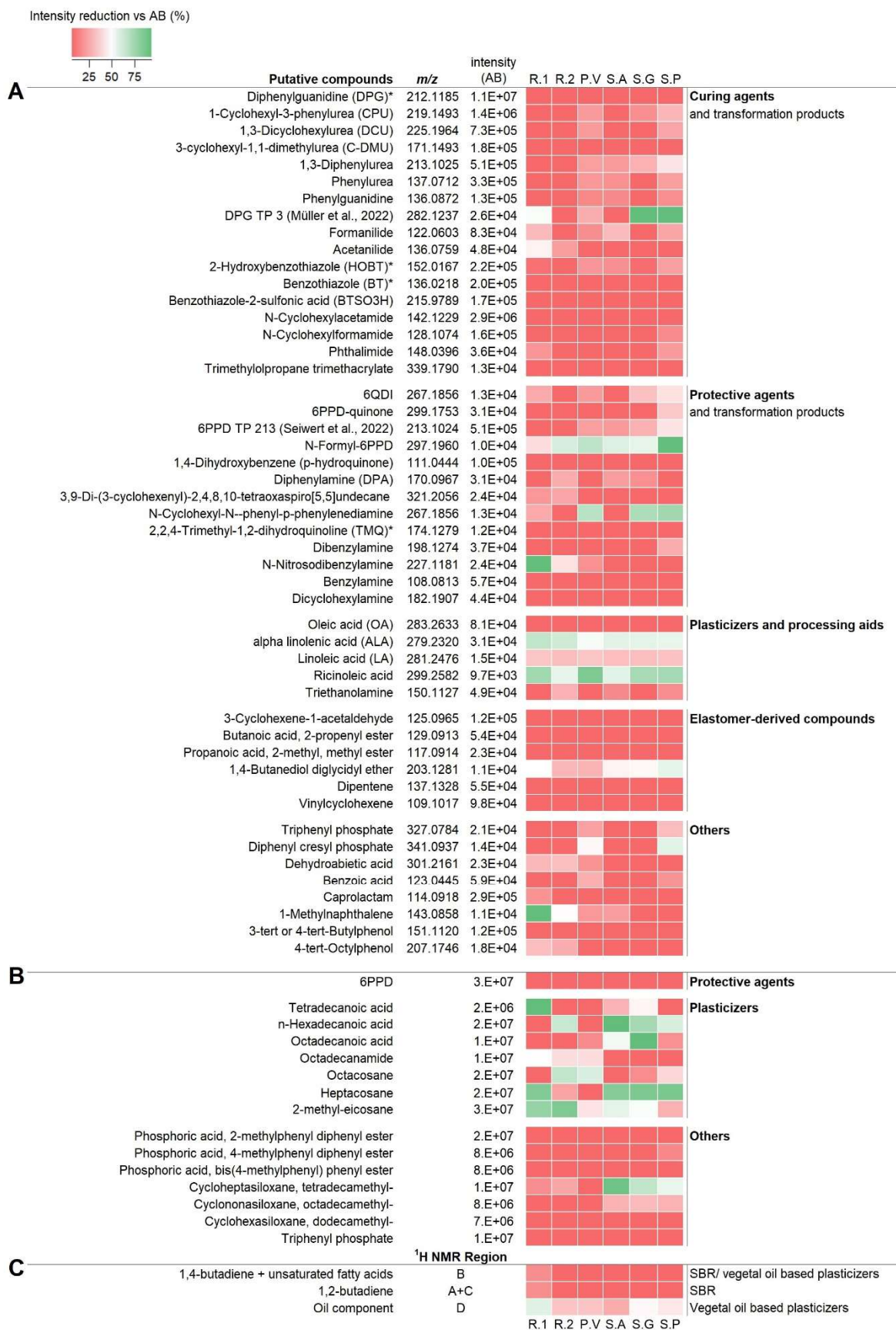
434 Several sources of carbon can be used to support the bacterial growth and may be found
435 either in the culture medium through the leaching of RP water soluble compounds or directly
436 on solid particles. Therefore after 14 days of incubation, the culture supernatants and the
437 chloroform extracts 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 ¹H-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 ¹H 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)** ¹H-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, CO₂ 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, ^1H 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 ^1H 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* BTSO₃) 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

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693

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