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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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**Assessing biodegradation of roadway particles
via complementary mass spectrometry and NMR analyses**

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Abstract

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

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

Keywords

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

1. Introduction

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

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

The emission of TRWP is the main mechanism of tire mass loss. It was estimated between 0.2 and 5.5 kg per year per inhabitant (Baensch-Baltruschat et al., 2020) but accurate quantification of TRWP emissions remains challenging. This is currently assessed via tire emission factors or material flow analyses due to a lack of direct measurements (Mennekes and Nowack, 2022) TRWP are mostly elongated or round particles (Kovochich et al., 2021) with a size ranging from a few nanometers to several hundred micrometers (Klöckner et al., 2020; Rosso et al., 2023) and densities ranging from 1.3 to above 2.2 g/cm³ (Klöckner et al., 2021b; Kovochich et al., 2021) that can be transported by air and deposited or washed off the road and transferred to aquatic or soil ecosystems (Klöckner et al., 2020; Baensch-Baltruschat et al., 2020). TRWP were monitored and quantified in various environmental compartments (air, soil, fresh water, marine environment roadside, runoff, estuaries, river and sea sediments) (Baensch-Baltruschat et al., 2021; Goßmann et al., 2021; Klöckner et al., 2021a; Leads and Weinstein, 2019; Panko et al., 2013; Parker-Jurd et al., 2021; Rauert et al., 2022a; Seiwert et al., 2020; Thomas et al., 2022a; Unice et al., 2012) evidencing them as a significant source of dispersion

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

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

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

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

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

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

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

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

2. Material and Methods

2.1. Chemicals

All the solvents used for extraction were HPLC analytical grade (Sigma Aldrich). Deuterated water supplemented with a 0.2 mM TSPd₄ (sodium tetra deuterated trimethylsilyl propionate) and methanol-d₄, acetone-d₆, chloroform-d supplemented with 0.03 v/v% TMS (tetramethylsilane) were purchased from Eurisotop. Analytical NMR standards for styrene butadiene rubber (SBR; 15 w% styrene, 20 w% 1,2-butadiene, 65 w% 1,4-butadiene) and sunflower oil (8001-21-6) were provided by Michelin. Analytical LC/HRMS standards for N-(1,3-dimethylbutyl)-N'-phenyl-*p*-phenylenediamine (6PPD-793-24-8), N,N'-diphenylguanidine (DPG-102-06-7), 2,2,4-Trimethyl-1,2-dihydroquinoline (TMQ-26780-96-1), 2,2-Dithiobisbenzothiazole (MBTS-120-78-5), N-Cyclohexyl-2-benzothiazolsulfenamide (CBS-95-33-0), Mercaptobenzothiazole (MBT-149-30-4), Benzothiazole (BT-95-16-9), 2-Hydroxybenzothiazole (HOBT-934-34-9) were purchased from Sigma Aldrich.

2.2. Environmental TRWP (RP) collection

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

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

2.3. RP extractions

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

2.4. RP biodegradation

Culture and inoculum preparation: The bacterial strains were selected as follows: *R. ruber* (ATCC 29672) and *P. vancoverensis* (DSM 8368) for their capacity to degrade polyolefins (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

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

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

2.5. NMR analysis

2.5.1. ^1H NMR

The supernatants from extraction and biodegradation experiments (600 μ L) were dried under a nitrogen flux and resuspended in 600 μ L of a deuterated solvent supplemented with a reference (TSPd₄ (or TMS). Deuterated solvents were used for locking (field stability) and shimming (field homogeneity) while TSPd₄ and TMS constituted a reference for chemical shifts (0 ppm) and quantification (Reference value = 100). All spectra were recorded on a Bruker 500 MHz Avance III spectrometer, equipped with a 5 mm Prodigy cryoprobe inverse-triple tuned (TCI) $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ with z-gradient coil probe (Bruker Biospin) with 5 mm-diameter tubes. 128 scans were collected (3.24 s acquisition time, 4.0 s relaxation delay, a spectral window of 20 ppm, FID size of 65536 data points zero-filled to 131072 point). Water signal was eliminated by pre-saturation (noesygprr1d sequence). An exponential filter (line broadening LB=0.3 Hz) was applied before Fourier transformation and an automatic baseline correction was performed on spectra before integration. Under these conditions, the limit of quantification is 0.01 mM. Relative integrations were expressed in arbitrary unit (a.u).

SBR calibration curve. A SBR calibration curve was prepared using five concentrations (from 0.10 to 0.80 mg.mL⁻¹) of a pure and uncrosslinked SBR, totally soluble in CDCl₃. ¹H NMR spectra were recorded for each solution with the same acquisition parameters as those described previously. The specific region of 1,2-butadiene protons ($\delta_H \sim 4.80\text{--}5.05$ ppm) was integrated relative to the internal TMS standard. A calibration curve was established between the relative integration and the concentration of SBR. For semi-quantification of SBR in RP chloroform extracts, the same specific region was integrated and correlated to the corresponding SBR concentration. The chloroform extraction of SBR from RP was determined in triplicate after 1, 7, 14, 21 days, and showed the same efficiency and recovery rate regardless of the extraction time.

2.5.2. HSQC NMR

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

2.6. LC/HRMS analyses

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

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

2.7. GC/MS analyses

600 μ L of the RP extract were dried under a nitrogen flux and were 6-fold concentrated in 100 μ L of a suitable solvent (hexane or chloroform for chloroform extracts and acetonitrile for water, methanol and acetone samples). Analyses were performed on an Agilent 6890A GC, equipped with a J&W Scientific HP-5MS (Methyl siloxane) column (30 m x 0.25 mm; 0.25 μ m) and coupled to an Agilent 5973N MSD (single quadrupole). Oven temperature: linear gradient 100°C-325°C for 22.5 min; 325°C for 10 min; sample volume: 2 μ L; Carrier gas: helium at a constant flow of 1 mL.min⁻¹. Mass spectrometer acquisitions were made in scan mode, from 50 to 600 m/z with 70 eV of electronic impact ionization energy. Chemical identifications were performed on the chromatographic peaks with area strictly greater than 10⁶, using the NIST17 standard reference database with a score greater than 80%.

2.8. Elemental analyses CHNS-O

The elemental composition of RP was analyzed in triplicate by the CHNS/O elemental analyzer FlashSmart from ThermoFischer based on fast catalytic pyrolysis. CHNS analysis was performed under combined reductive (tin capsids) and oxidative (V₂O₅/O₂) conditions at 960°C and with a 110 mL.min⁻¹ carrier gas (He) flow and a 250 mL.min⁻¹ combustion gas flow (He/O₂) to quantify C(CO₂), H(H₂O), N(N₂) and S(SO₂). For O analysis, RP were pyrolyzed in Ag capsids at 1065°C to form O₂. Gas were separated on GC columns under He flow and analyzed by a thermal conductivity detector (TCD). The calibration was performed using 2,5-bis(5-*tert*-butyl-benzoxazol-2-yl)thiophene (BBOT) standards. Masses of samples were lower than 2 mg.

3. Results

3.1. Characterization of the RP carbon sources

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

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

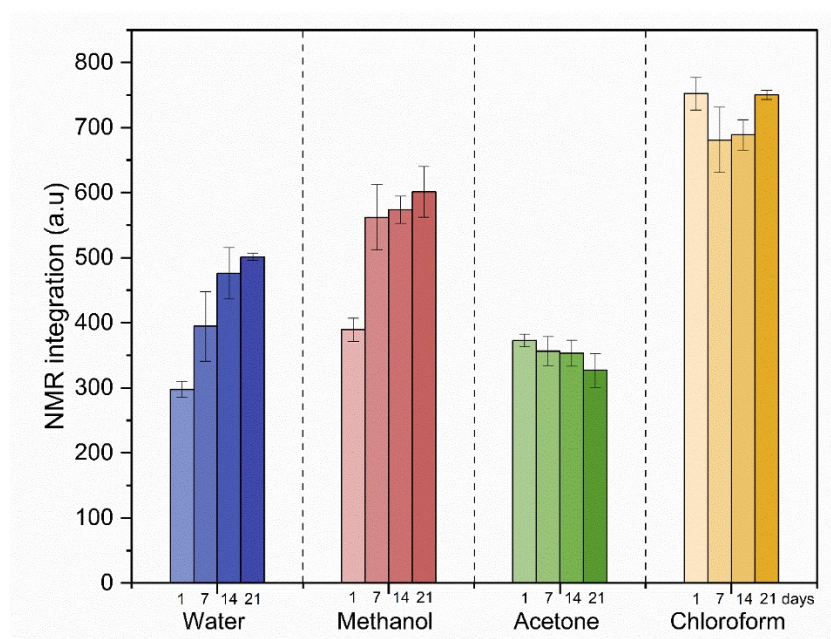


Fig. 1. Relative integration of the entire ^1H NMR signal compared to that of an internal standard in function of the extraction time and solvent. The integration values cannot be compared between solvents as different compounds can be extracted. The mean values ($n=3$) \pm standard deviation are presented.

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 extract exhibited high signals in the region of oxygenated groups ($\delta_{\text{H}} \sim 3.1\text{--}4.3$ ppm). On the 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) functional groups was detected in water extract, while these signals were clearly present in chloroform extracts (as well as in acetone and methanol to a lesser extent). The signal ($\delta_{\text{H}} \sim 7.0\text{--}7.2$ ppm) was assigned to the styrene aromatic protons and that at ($\delta_{\text{H}} \sim 4.8\text{--}5.7$ ppm) to the butadiene protons after comparison with the ^1H NMR spectrum of a SBR standard (Fig. 2). Nevertheless, NMR analysis of complex samples often involves signal and dynamic range overlaps according to the relative concentrations of each compound. Complementary analysis (2D-NMR) showed that the regions assigned to 1,2-butadiene ethylenic protons ($\delta_{\text{H}} \sim 5.5\text{--}5.7$ 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 to evidence the solubilization of SBR chains in chloroform extract (named CHL fraction). The integration value of these specific regions was thus used to build a SBR calibration curve using a pure SBR standard dissolved in chloroform at different concentrations. The concentration of the SBR fraction extracted in chloroform was then estimated, assuming that the extracted chains have the same microstructure as that of the SBR standard used for calibration. Regardless of the extraction time, the amount of CHL fraction was 0.12 mg.mL^{-1} which represented approximately 1.2 wt% of the RP mass present. This value could be underestimated as the extraction recovery rate may be incomplete. As the content of RP organic fraction was

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

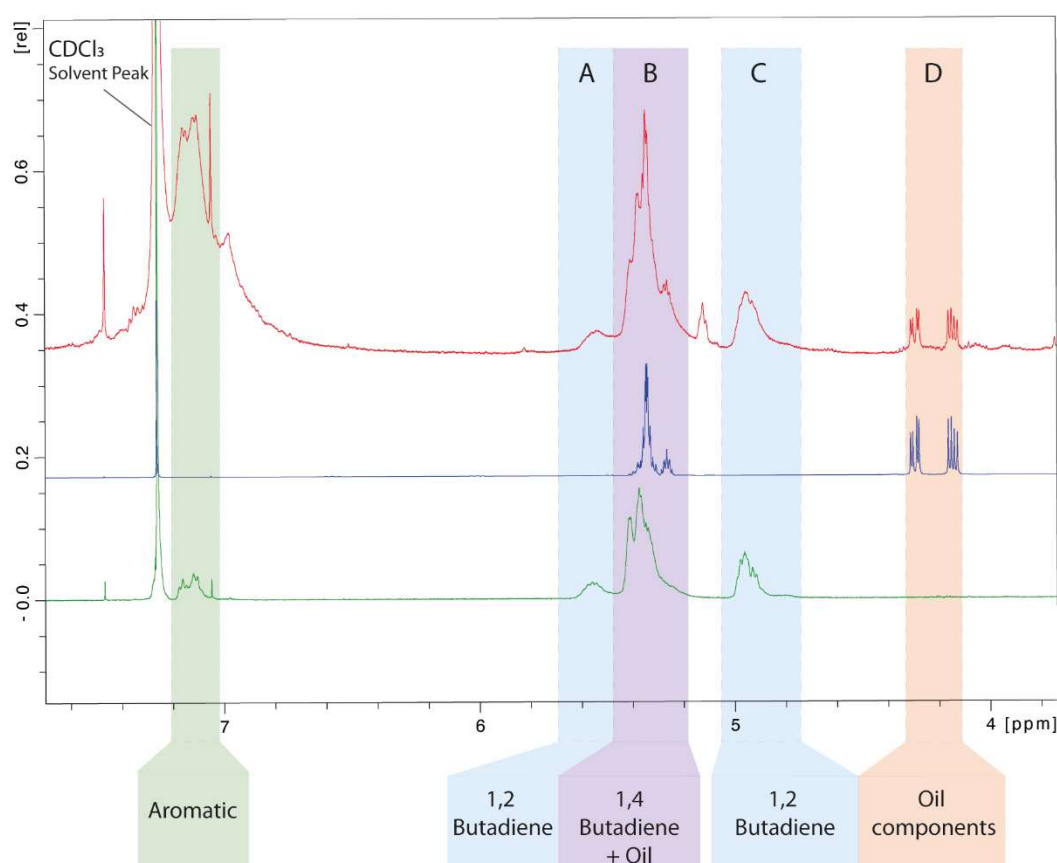


Fig. 2. Liquid state ^1H NMR spectra of a SBR standard (0.2 mg.mL^{-1}) in CDCl_3 (green), oil (blue) and a 21-day chloroform extract of 10 mg.mL^{-1} of RP (red)

3.1.2. Analysis of potential leachable compounds by mass spectrometry

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

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

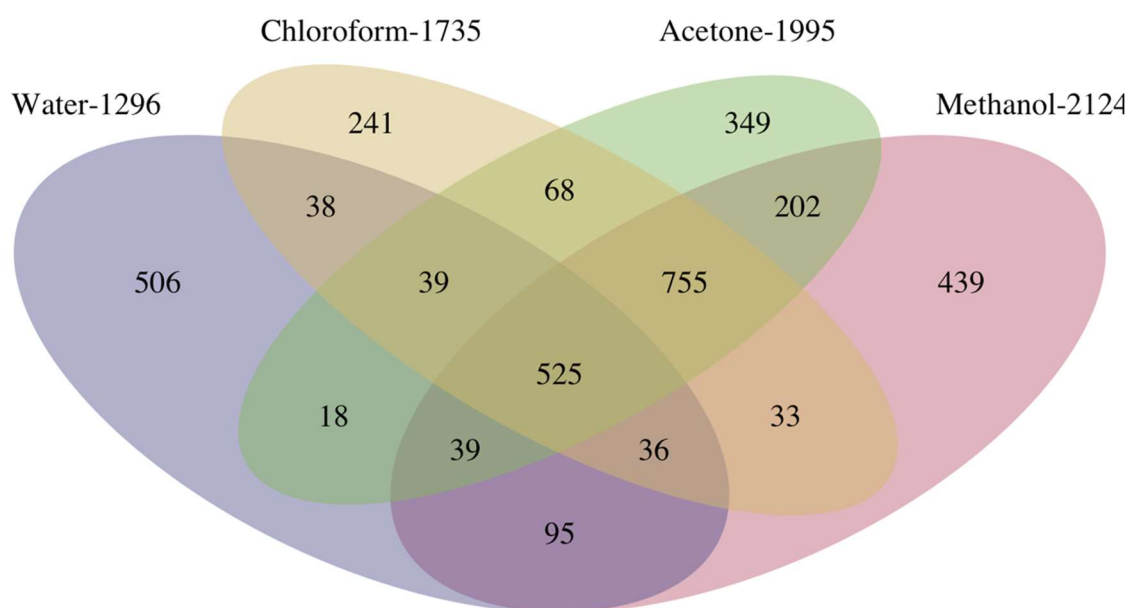


Fig. 3. Venn diagram representing the number of peaks detected by LC/ESI⁺-HRMS mode in the 21-day extracts for the 4 solvents tested: water (blue), chloroform (yellow), acetone (green) and methanol (red)

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

3.2. Biodegradation of RP

The complex organic composition of RP represents many sources of carbon that can be potential substrates for biodegradation. To assess the RP biodegradation, 6 bacterial pure strains were incubated in minimal salt medium (MSM) in the presence or absence of RP as the sole carbon source. After 14 days of culture, a bacterial growth was clearly observed for all the strains cultivated in the presence of RP, except for *P. vancouverensis*. A net increase of CO₂ production was recorded for *Rhodococcus* and *Streptomyces* strains, indicating up to 4 times higher cellular respiration than that of the biotic controls (Fig. 4). The concomitant biomass production was confirmed by the ATP increase for *Rhodococcus* strains. No ATP was detected for *Streptomyces* strains. The low extraction yield could be explained by the difficulty in breaking their cell wall. *P. vancouverensis* did not show any increase in CO₂ production and only a slight one for ATP content.

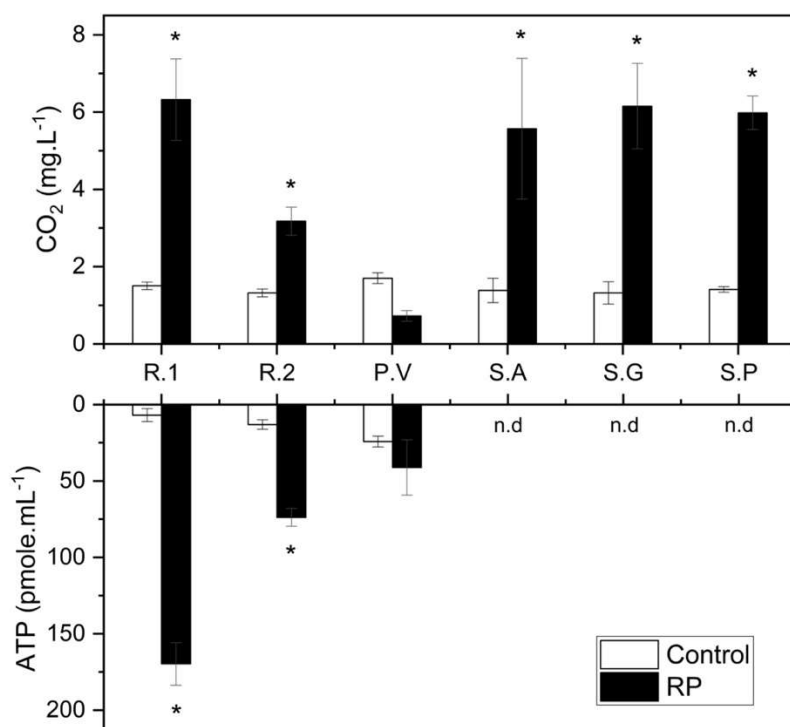


Fig. 4. CO₂ production and ATP concentration after 14 days of RP incubation with R.1 = *Rhodococcus ruber* ATCC 29672; R.2 = *Rhodococcus rhodochrous* ATCC 15906; P.V = *Pseudomonas vancouverensis* DSM 8368; S.A = *Streptomyces anulatus* Lab 2112; S.G = *Streptomyces griseolus* Lab 912; S.P = *Streptomyces phaeofaciens* NRRL 8092. The star represents statistically significant differences (Student test, p value < 0.05).

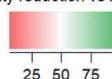
Several sources of carbon can be used to support the bacterial growth and may be found either in the culture medium through the leaching of RP water soluble compounds or directly on solid particles. Therefore after 14 days of incubation, the culture supernatants and the chloroform extracts of residual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

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

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

Intensity reduction vs AB (%)



A

Putative compounds	m/z	intensity (AB)	R.1	R.2	P.V	S.A	S.G	S.P	
Diphenylguanidine (DPG)*	212.1185	1.1E+07							Curing agents and transformation products
1-Cyclohexyl-3-phenylurea (CPU)	219.1493	1.4E+06							
1,3-Dicyclohexylurea (DCU)	225.1964	7.3E+05							
3-cyclohexyl-1,1-dimethylurea (C-DMU)	171.1493	1.8E+05							
1,3-Diphenylurea	213.1025	5.1E+05							
Phenylurea	137.0712	3.3E+05							
Phenylguanidine	136.0872	1.3E+05							
DPG TP 3 (Müller et al., 2022)	282.1237	2.6E+04							
Formanilide	122.0603	8.3E+04							
Acetanilide	136.0759	4.8E+04							
2-Hydroxybenzothiazole (HOBT)*	152.0167	2.2E+05							
Benzothiazole (BT)*	136.0218	2.0E+05							
Benzothiazole-2-sulfonic acid (BTSO3H)	215.9789	1.7E+05							
N-Cyclohexylacetamide	142.1229	2.9E+06							
N-Cyclohexylformamide	128.1074	1.6E+05							
Phthalimide	148.0396	3.6E+04							
Trimethylolpropane trimethacrylate	339.1790	1.3E+04							Protective agents and transformation products
6QDI	267.1856	1.3E+04							
6PPD-quinone	299.1753	3.1E+04							
6PPD TP 213 (Seiwert et al., 2022)	213.1024	5.1E+05							
N-Formyl-6PPD	297.1960	1.0E+04							
1,4-Dihydroxybenzene (p-hydroquinone)	111.0444	1.0E+05							
Diphenylamine (DPA)	170.0967	3.1E+04							
3,9-Di-(3-cyclohexenyl)-2,4,8,10-tetraoxaspiro[5,5]undecane	321.2056	2.4E+04							
N-Cyclohexyl-N-phenyl-p-phenylenediamine	267.1856	1.3E+04							
2,2,4-Trimethyl-1,2-dihydroquinoline (TMQ)*	174.1279	1.2E+04							
Dibenzylamine	198.1274	3.7E+04							
N-Nitrosodibenzylamine	227.1181	2.4E+04							
Benzylamine	108.0813	5.7E+04							
Dicyclohexylamine	182.1907	4.4E+04							
Oleic acid (OA)	283.2633	8.1E+04							Plasticizers and processing aids
alpha linolenic acid (ALA)	279.2320	3.1E+04							
Linoleic acid (LA)	281.2476	1.5E+04							
Ricinoleic acid	299.2582	9.7E+03							
Triethanolamine	150.1127	4.9E+04							
3-Cyclohexene-1-acetaldehyde	125.0965	1.2E+05							Elastomer-derived compounds
Butanoic acid, 2-propenyl ester	129.0913	5.4E+04							
Propanoic acid, 2-methyl, methyl ester	117.0914	2.3E+04							
1,4-Butanediol diglycidyl ether	203.1281	1.1E+04							
Dipentene	137.1328	5.5E+04							
Vinylcyclohexene	109.1017	9.8E+04							Others
Triphenyl phosphate	327.0784	2.1E+04							
Diphenyl cresyl phosphate	341.0937	1.4E+04							
Dehydroabietic acid	301.2161	2.3E+04							
Benzoic acid	123.0445	5.9E+04							
Caprolactam	114.0918	2.9E+05							
1-Methylnaphthalene	143.0858	1.1E+04							
3-tert or 4-tert-Butylphenol	151.1120	1.2E+05							
4-tert-Octylphenol	207.1746	1.8E+04							

B

6PPD	3.E+07								Protective agents
Tetradecanoic acid	2.E+06								Plasticizers
n-Hexadecanoic acid	2.E+07								
Octadecanoic acid	1.E+07								
Octadecanamide	1.E+07								
Octacosane	2.E+07								
Heptacosane	2.E+07								
2-methyl-eicosane	3.E+07								
Phosphoric acid, 2-methylphenyl diphenyl ester	2.E+07								Others
Phosphoric acid, 4-methylphenyl diphenyl ester	8.E+06								
Phosphoric acid, bis(4-methylphenyl) phenyl ester	8.E+06								
Cycloheptasiloxane, tetradecamethyl-	1.E+07								
Cyclononasiloxane, octadecamethyl-	8.E+06								
Cyclohexasiloxane, dodecamethyl-	7.E+06								
Triphenyl phosphate	1.E+07								

C

	¹ H NMR Region	R.1	R.2	P.V	S.A	S.G	S.P	
1,4-butadiene + unsaturated fatty acids	B							SBR/ vegetal oil based plasticizers
1,2-butadiene	A+C							
Oil component	D							

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

4. Discussion

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

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

We evaluated the RP-degrading capacity of 6 bacterial strains, belonging to 3 ubiquitous genera, *Rhodococcus*, *Pseudomonas* and *Streptomyces*. In this study, CO₂ and ATP measurements showed that all these strains, except *P. vancouverensis*, were able to grow on RP. To date, microbial growth on tire rubber, oxidized or not, was reported for several bacterial isolates and consortia by respirometric measurements (Alejo et al., 2022; Marchut-Mikołajczyk et al., 2019; Tsuchii and Tokiwa, 2006) but never demonstrated on TRWP. Our results confirmed that these strains, isolated from the environment, can also biodegrade organic compounds contained in RP and suggest that the biodegradation could occur in natural conditions. The next step was to study the biodegradation of the various potential carbon sources by bacteria.

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

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

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

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

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

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

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

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

the TRWP studies, were not degraded by our strains. To date, only a few bacterial strains belonging to the *Rhodococcus* genus (*R. pyridinovorans* PA, *R. rhodochrous* OBT18, *R. erythropolis* BTSO₃) and isolated from activated sludges or industrial effluents, were reported to degrade BT and some of its derivatives or transformation products so far (De Wever et al., 1997, 1998; Gaja and Knapp, 1997; Haroune et al., 2002). Concerning DPG and phenylurea residues, Dvořák et al. (2014) reported their total mineralization by an adapted sludge for industrial wastewater treatment. There is also a growing interest for transformation products, particularly since the recent identification of 6PPD-quinone as an ozonation product deriving from the antioxidant and antiaging rubber agent 6PPD, this transformation product having a lethal toxicity in coho salmon above a given threshold (Tian et al., 2021). In our study, a 30% decrease of 6PPD-quinone was observed after 14 days with *S. phaeofaciens*, while present literature focused on abiotic fate of 6PPD and its transformation products (Fohet et al., 2023; Hu et al., 2022; Seiwert et al., 2022b). Recently, the occurrence and predominance of biodegradation of 6PPD-quinone in soils was demonstrated (Xu et al., 2023), highlighting the importance of considering biodegradation when assessing the environmental fate of additives.

5. Conclusion

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

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

Declaration of competing interest

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

Acknowledgement

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

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