



## Uptake of chemicals from tire wear particles into aquatic organisms - search for biomarkers of exposure in blue mussels (*Mytilus edulis*)

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### ABSTRACT

Little is known about the exposure of aquatic biota to tire and road wear particles (TRWP) washed away from roads. Mussels were exposed for 7 days to model TRWP (m-TRWP), produced by milling tire tread particles with pure sand, and analyzed for 21 tire-related compounds by liquid chromatography-high resolution-mass spectrometry (LC-HRMS). Upon exposure to 0.5 g/L of m-TRWP, 15 compounds were determined from 944 µg/kg wet weight (diphenylguanidine, DPG) over 18 µg/kg for an oxidation product of N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine (6-PPDQ) to 0.6 µg/kg (4-hydroxydiphenyl amine). Transfer into mussels was highest for PTPD, DTPD and 6-PPDQ and orders of magnitude lower for 6-PPD. During 7 days depuration the concentration of all determined chemicals decreased to remaining concentrations between ~50 % (PTPD, DTPD) and 6 % (6-PPD). Suspect and non-target screening found 37 additional transformation products (TPs) of tire additives, many of which did not decrease in concentration during depuration, among them ten likely TPs of DPG, two of 6-PPD and PTPD and two of 1,2-dihydro-2,2,4-trimethylquinoline. A wide variety of chemicals is taken up by mussels upon exposure to m-TRWP and a wide range of TPs is formed, enabling the differentiation of biomarkers of exposure to TRWP and biomarkers of exposure to tire-associated chemicals.

### 1. Introduction

Tire and road wear particles (TRWP), generated by the abrasion of tires tread on roads, are a significant source of anthropogenic polymers in the environment (Wagner et al., 2022; Wagner et al., 2018; Wik and Dave, 2009). Consequently, there are significant concerns about their impact on environmental health, particularly due to the chemicals they release into aquatic environments (Mayer et al., 2024; Müller et al., 2022). Well-known tire additives are vulcanization accelerators (e.g. diphenylguanidine (DPG) and benzothiazoles) and antioxidants (e.g. N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine (6-PPD)), as well as their transformation products (TPs) (Seiwert et al., 2020). These chemicals can reach considerable concentrations in rivers affected by

road runoff: concentrations of 0.5 µg/L for DPG and 2.3 µg/L for 6-PPD quinone (6-PPDQ), the oxidation product of 6-PPD formed during tire ageing (Seiwert et al., 2022; Weyrauch et al., 2023), have been observed in Canadian river waters following storm events (Johannessen et al., 2022). Several TPs can be formed during tire manufacturing, use or abrasion, or after leaching from TRWP due to abiotic and biotic processes (Foscari et al., 2024; Foscari et al., 2023; Müller et al., 2022; Seiwert et al., 2022; Seiwert et al., 2020; Weyrauch et al., 2023). One TP of 6-PPD, 6-PPDQ, is highly toxic to Coho salmon (*Oncorhynchus kisutch*) (Tian et al., 2022). Beyond that, tire leachates have been shown to affect other aquatic organisms, such as algae, mussels and crustaceans (Capolupo et al., 2020; Day et al., 1993; Halle et al., 2021).

Studies with mussels have also been performed: blue mussels (*Mytilus*

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*edulis*) exposed to both tire particles and their leachates showed a 46 % reduction in filtration rates after 3 weeks of exposure, with a median effect concentration ( $EC_{50}$ ) of 3 g/L of tire wear particles (TWP) (Thomsen et al. (2024)). The lysosomal membrane stability, gamete fertilization, embryonic development ( $EC_{50}$  0.5 % of leachate solution), larvae motility ( $EC_{50}$  36 % of leachates solution) and survival have been shown to be affected by tire leachates (Capolupo et al., 2020).

While such lab experiments have proven that chemicals originating from TWP exhibit detrimental effects on mussels, the extent to which marine mussels are exposed to TRWP and chemicals released from it remains largely unknown.

In a recent study, concentrations of 15 tire-related chemicals in 21 estuarine species collected in the Pearl River estuary were reported, with 6-PPDQ, DPPD (*N, N'*-diphenyl-*p*-phenylenediamine) and DTPD (*N, N'*-bis(2-methylphenyl)-1,4-benzenediamine) being most prominent (Wei et al., 2024). However, mussels were not included in that study.

A better understanding is needed on the bioavailability of TWP-related chemicals by mussels upon exposure to TWP to assess potential impact and to evaluate if any of these chemicals can be suggested as biomarkers. For this purpose, laboratory exposure experiments were performed with blue mussels (*Mytilus edulis*) exposed to model tire road wear particles (m-TRWP) and the concentration of 21 tire-related chemicals was determined. In addition, suspect and non-target screening was performed to account for TPs that may have formed in mussels upon exposure to TRWP.

## 2. Materials and methods

### 2.1. Production of model tire road wear particles

Model tire road wear particles (m-TRWP) were produced to obtain a more realistic material allowing repeatable experiments. Cryo-milled tire tread (CMTT<sub>mix</sub>; Foscari et al., 2024) was ball-milled with pure sand (SiO<sub>2</sub>) in a ratio of 1:4 (w/w) with a Mixer Mill MM400 (Retsch, Germany). This m-TRWP had a mean particle size of  $94 \pm 41 \mu\text{m}$  in volume distribution, compared to  $129 \pm 54 \mu\text{m}$  for the CMTT<sub>mix</sub>. The tire content of the m-TRWP (20 %) is lower than typically reported for TRWP (45 %) (Weyrauch et al., 2023). This discrepancy needs to be considered if the quantitative data of this lab experiment with m-TRWP are to be transferred to real TRWP. The calculated density of the m-TRWP is  $1.5 \text{ g/cm}^3$ , which is well in the range of real TRWP (Klockner et al., 2019).

The smaller particle size and higher density were advantageous for the exposure experiment and the uptake into the mussels. Moreover, it is assumed that the heteroaggregate m-TRWP should be a better model for real TRWP than CMTT. The size and shape characteristics of the material are reported in the supplementary information (Fig. S1). The obtained heteroaggregate enhances the uptake of CMTT by mussels and reduces both the hydrophobicity and particle agglomeration. Extractables were analyzed from the CMTT before the final milling with the quartz sand. For this, CMTT was dispersed in methanol (0.1 g/L) and subjected to ultrasonic bath for 1 h. The resulting extracts were filtered and analyzed following the procedure described in Section 2.4. The concentration of extractables in m-TRWP was then calculated from the concentrations determined for the CMTT and the CMTT percentage in the m-TRWP (Table S8).

### 2.2. Exposure experiment

Blue mussels (*Mytilus edulis*) were collected from the Belgian part of the North Sea near the city of De Panne on February 23, 2022. Before the start of the exposure experiment, the mussels underwent a depuration phase lasting 15 days to eliminate any ingested materials potentially present in their gastrointestinal tracts.

Mussels were allocated to three separate aquaria, each containing 25 L of seawater, with a total of 60 individuals per aquarium. The high-

concentration treatment aquaria contained a total of 12.5 g of the m-TRWP (consisting of 2.5 g CMTT and 10 g sand) corresponding to 0.5 g<sub>m-TRWP/L</sub>, while the low-concentration aquaria contained 1.25 g of m-TRWP (0.25 g CMTT and 1 g sand) corresponding to 0.05 g<sub>m-TRWP/L</sub>. The exposure concentrations utilized in this study were selected based on preliminary exposure studies conducted on the same species (Thomsen et al., 2024), as well as on other organisms (Zhang et al., 2024). A third aquarium, without m-TRWP exposure, served as the control (Fig. 1). The experiment was conducted over a 14-day period and m-TRWP introduced at the start of the experiment. After a 7-day exposure period, the mussels were transferred to another aquarium with fresh seawater to undergo a depuration phase lasting an additional 7 days.

### 2.3. Sampling and sample preparation

Mussel and tank water samples were collected at four time points: day 1 (24 h), day 3, day 7 (end of the exposure period), and day 14 (end of the depuration period). For each sampling, 3 organisms were collected and mussel shell length was measured, and wet soft tissue was weighed and kept at  $-20 \text{ }^\circ\text{C}$  until analysis. The wet weight of the mussels was subsequently used to calculate the chemical content associated with their soft tissue. The soft tissue of each individual mussel was freeze-dried (Christ Alpha 1-4 LSC plus), ball-milled with Mixer Mill MM400 (Retsch, Germany) for homogenization and later on analyzed separately.

#### Sample preparation for organic analysis:

The homogenized mussel powder was extracted using 5 mL of methanol (MeOH), which has been shown to provide higher recovery for the compounds of interest (> 50 %) compared to acetonitrile (ACN) (Fig. S2). Furthermore, the use of MeOH streamlined the sample preparation process by eliminating the need for evaporation and reconstitution steps, which significantly reduced the amount of compounds of interest in the quality test (Fig. S3). Tank water samples were filtrated by regenerated cellulose membrane syringe filters (0.45  $\mu\text{m}$ ).

### 2.4. QA/QC

To minimize contamination, the use of plastic and rubber materials was avoided as much as possible throughout the exposure experiment. Potential contamination was assessed by including a control aquarium, in which no m-TRWP exposure occurred, and where sampling and analysis was conducted in parallel with the exposed systems. Additionally, sample processing blanks were included and prepared using the same glass vials employed for sampling, storage, and shipment. All samples were stored at  $-20 \text{ }^\circ\text{C}$  and thawed immediately prior to analysis. During sample preparation, only non-plastic materials were used, and all handling was performed using cotton laboratory coats and nitrile gloves. Control samples from the unexposed aquaria were processed and interpreted using the same procedures as for exposed mussels, and results are reported in Table S3. Instrumental and water media blank controls analyzed alongside the samples showed concentrations below LOD/LOQ levels, otherwise subtracted from sample values.

### 2.5. Analysis

A number of 21 tire-derived compounds (additives and their transformation products) were analyzed in the test material, in mussels and tank water (analyte details in Table S2). Extracts (diluted 1:1 with ultrapure water) and tank water were directly injected into the UPLC-HRMS. The chemicals used, UPLC-HRMS analytical method and the identification of TPs were the same as described in Foscari et al. (2024). Analytical-grade chemicals, including methanol, acetonitrile, formic acid (for LC-MS analysis), and various salts, were used without further purification. Ultrapure water was supplied by a Merck MilliQ system. Aqueous samples and particle extracts were analyzed via UPLC-TOF-MS,

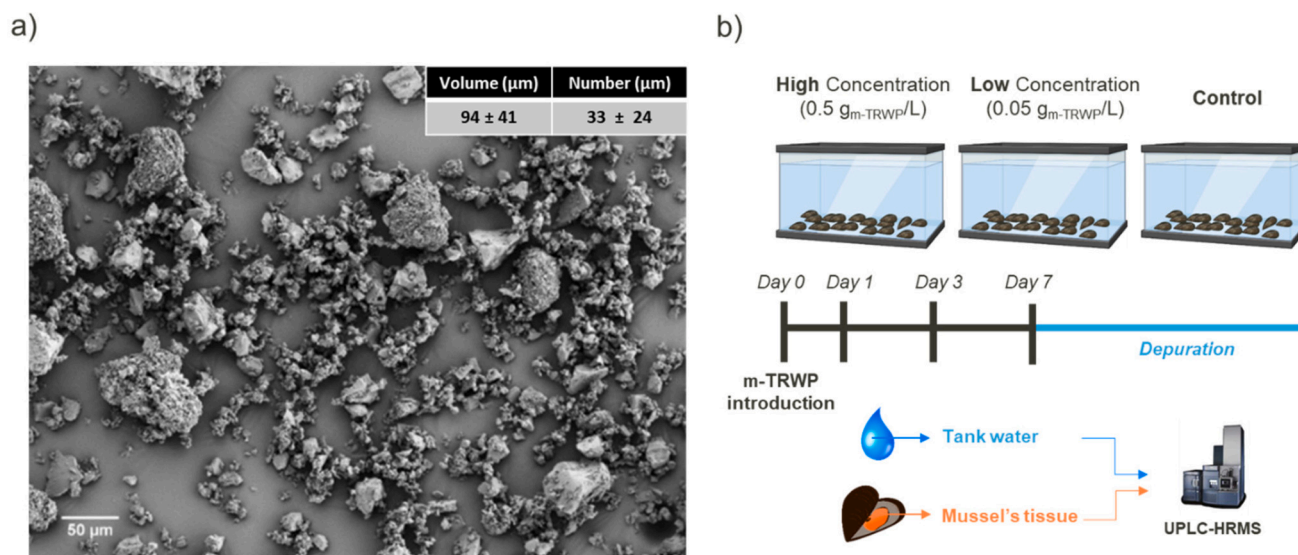


Fig. 1. a) Scanning electron microscopy image showing m-TRWP used for mussel exposure, its mean particle size based on volume and number; b) experimental setup of the mussel exposure experiments.

employing an ACQUITY UPLC system coupled with a XEVO XS Q-TOF-MS, as described by Klockner et al. (2021). Transformation products (TPs) were identified using reversed phase liquid chromatography – high resolution-mass spectrometry (RPLC-HRMS) data within a retention time range of 1–12 min and a mass range of  $m/z$  50–1200 both positive and negative ionization. MarkerLynx software facilitated peak picking, and subsequent data evaluation was conducted in Excel. Peak areas were normalized to the total marker intensities of the chromatograms (done by MarkerLynx) and after removing all peaks which have been present in the blank and the non-exposed controls, relative intensities above 1 were selected. The peak intensity of these peaks were checked to be  $>3$  S/N. Formulas were assigned with a 5 ppm mass tolerance. Fragment ions were used to propose potential structures and confirm the elemental composition of the molecular ion. More details, including the extracted ion chromatograms of the quantified target analytes, are provided in the *supplementary information*. Quantitative data of the mussels are given related to the initial wet weight of the mussels.

## 2.6. Calculation of concentration ratios

**Internal concentration vs tank water concentration ratio:** The internal concentration-to-water concentration ratio was calculated by dividing the concentration of each target chemical in mussel soft tissue ( $\mu\text{g}/\text{kg}$  wet weight) by its corresponding concentration in tank water (expressed as  $\mu\text{g}/\text{L}$ ) at the end of the exposure period (Day 7) (Table S3). This ratio provides an indication of the degree of chemical accumulation in mussels relative to its presence in the aqueous phase (Table 1). Considering that the water content of mussel tissue exceeded 85 % (Table S7), 1 kg of mussel tissue was assumed to correspond to 1 L in volume.

**Tissue-to m-TRWP concentration ratio:** This ratio was calculated from the internal concentration in mussel ( $\mu\text{g}/\text{kg}$  wet weight) and the concentrations in m-TRWP ( $\mu\text{g}/\text{g}$ ) (Table S8), resulting in a dimension of  $\text{g}/\text{kg}$  (Table 1, Table S8).

**Residual internal concentration after depuration:** The residual concentration of each chemical in mussels at the end of the 7-day depuration period was expressed as the percentage of the concentration determined at the end of the 7-day exposure phase (Table S3).

## 3. Results and discussion

For a TRWP exposure experiment with blue mussels (*Mytilus edulis*), it is important that the particle size simulates the diet of mussels as closely as possible to ensure efficient uptake. Blue mussels are capable of ingesting prey up to 1.2 mm in length (Jonsson et al., 2009), but its primary diet consists of phytoplankton smaller than  $100 \mu\text{m}$  (Møhlenberg and Riisgård, 1978; Newell et al., 1989). It has been shown that particle size influences the extent of uptake into mussel, with smaller particles being more effectively assimilated. This, in turn, affects both retention efficiency and clearance rate (Dral, 1967; Strohmeier et al., 2012). Therefore m-TRWP were prepared, which exhibit a higher density and a smaller mean particle size than the CMTT mix from which it was produced (Fig. 1a, Fig. S7). Both of these properties make m-TRWP more comparable to real TRWP (Weyrauch et al., 2023).

### 3.1. Tire additives and their derivatives in mussel

A total of 21 tire-related chemicals were quantified by LC-HRMS (Table S2). These consist of vulcanization accelerators and antioxidants used in tire production together with some derivatives formed already during the manufacturing process and some transformation products (TPs) formed after their release from the particles. The polarity of the analytes spanned a  $\log D$  range from 4.87 (DTPD) to  $-1.47$  (PG) (Table 1).

All quantifiable compounds were found at significantly higher concentrations in mussel's tissue than in tank water, at both high and low exposure concentrations (0.5 and 0.05  $\text{g}/\text{L}$  m-TRWP, respectively) (Fig. 2, Table 1). In these exposure experiments with m-TRWP, tire-related chemicals can be taken up by mussels in two ways: (i) from the tank water after the chemicals were leached from m-TRWP, or (ii) directly from m-TRWP after ingestion of the particles by the mussel. As the m-TRWP act as a continuous source of tire-related chemicals and as many of these chemicals are not stable in water (Han et al., sub.; Xu et al., 2025), a stable concentration of the chemicals in the tank water over the 7 days of exposure cannot be assumed. Therefore, bioaccumulation factors, i.e. the concentration ratios of chemicals between the organism and the tank water at equilibrium, cannot be derived.

A comparative analysis of the relative contribution of single compounds to the sum concentration of all quantified compounds in m-TRWP extracts, mussel's tissue and tank water (both after a 7-day exposure in the high-concentration aquarium) revealed that

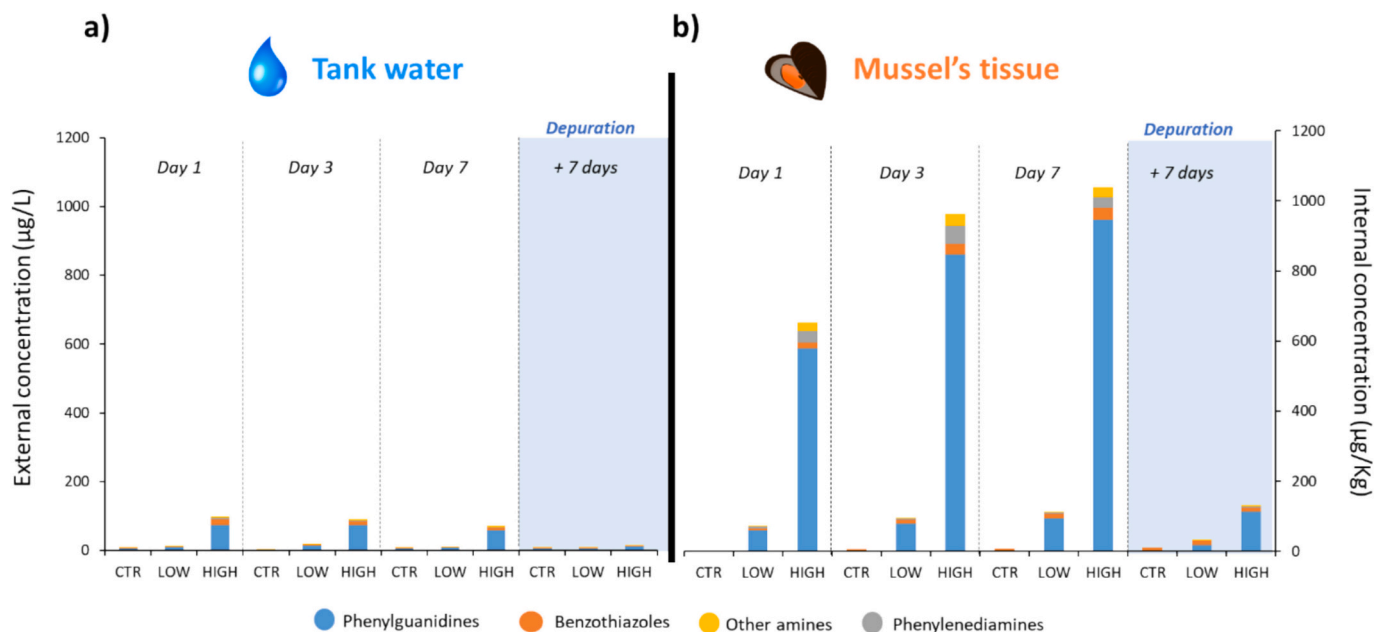
**Table 1**  
Concentrations determined in mussels tissue upon 7-day exposure to m-TRWP, concentration ratio of the mussels after 7 days and the m-TRWP, concentration ratio of the mussels (internal) and water (external), and relative concentration in mussel tissue after a 7-day depuration.  $N = 3$  individuals.

Compound name	abbreviation	Class	LogD <sub>1)</sub>	Maximum internal concentration <sup>2)</sup>		Internal concentration vs tank water concentration (Day 7)		Ratio of internal to TRWP concentration		Residual internal concentration after depuration <sup>3)</sup> (+ 7 days)
				0.5 g/L	0.05 g/L	0.5 g/L	0.05 g/L	0.5 g/L	0.05 g/L	0.5 g/L
				$\mu\text{g}/\text{Kg}_{\text{mussel}}(\text{ww})$		$L_{\text{water}}/\text{Kg}_{\text{mussel}}(\text{ww})$		$\text{g}_{\text{m-TRWP}}/\text{Kg}_{\text{mussel}}(\text{ww})$		%
Diphenylguanidine	DPG	Vulcanization accelerator	1.54	943.5 ± 132.2 (7)	94.3 ± 16.1 (7)	16 ± 2	13 ± 2	7.28 ± 1.12	0.73 ± 0.13	12 ± 2
Phenylguanidine	PG	TP of vulc. Acc.	-1.47	0.8 ± 0.6 (7)	< LOQ	5 ± 4	-	7.46 ± 5.76	< LOQ	68 ± 21
Triphenylguanidine	TPG	Vulcanization accelerator	4.21	4.5 ± 1.3 (3)	0.3 ± 0.1 (3)	31 ± 3	4 ± 1	7.48 ± 1.29	0.60 ± 0.23	37 ± 5
2-Methylthiobenzothiazole	2-MTBT	TP of vulc. Acc.	2.19	28.1 ± 8.4 (7)	12.3 ± 5.1 (7)	7 ± 2	7 ± 3	452 ± 139	198 ± 83	31 ± 1
2-hydroxybenzothiazole	2-OHBT	TP of vulc. Acc.	1.74	1.8 ± 0.7 (3)	< LOQ	2 ± 1	-	0.49 ± 0.20	< LOQ	7 ± 4
Dicyclohexylamine	DCH	TP of vulc. Acc.	0.62	32.8 ± 7.9 (3)	2.0 ± 0.7 (7)	8 ± 1	5 ± 2	7.40 ± 0.96	0.57 ± 0.21	10 ± 1
Methyldicyclohexyl amine	Me-DCH	TP of vulc. Acc.	0.96	2.5 ± 0.7 (3)	0.2 ± 0.0 (3)	8 ± 2	2 ± 1	6.01 ± 1.75	0.48 ± 0.37	19 ± 2
Tributylamine	TBA	Impurity of vulc. Acc.	1.69	5.9 ± 1.8 (3)	0.4 ± 0.1 (3)	7 ± 2	2 ± 1	-	-	8 ± 3
<i>N, N'</i> -diphenyl-1,4-phenylenediamine	DPPD	Antioxidant	3.95	18.4 ± 2.3 (3)	1.4 ± 0.5 (1)	54 ± 17	-	1.89 ± 0.62	0.02 ± 0.03	2 ± 2
<i>N, N'</i> -di-( <i>p</i> -tolyl)- <i>p</i> -phenylenediamine	DTPD	Antioxidant	4.87	10.1 ± 1.0 (7)	1.0 ± 0.2 (7)	56 ± 7	-	8.57 ± 0.88	0.87 ± 0.20	55 ± 7
<i>N</i> -phenyl- <i>N'</i> -( <i>o</i> -tolyl)- <i>p</i> -phenylenediamine	PTPD	Antioxidant	4.33	21.2 ± 2.3 (7)	2.1 ± 1.0 (3)	41 ± 1	-	9.20 ± 1.00	0.73 ± 0.22	46 ± 6
<i>N</i> -(1,3-dimethylbutyl)- <i>N'</i> -phenyl- <i>p</i> -phenylenediamine	6-PPD	Antioxidant	4.21	12.4 ± 12.4 (3)	0.2 ± 0.1 (1)	4 ± 2	-	0.03 ± 0.02	< LOQ	8 ± 8
6-PPD quinone	6-PPDQ	TP of antiox.	3.14	17.7 ± 4.6 (3)	4.4 ± 1.4 (1)	54 ± 17	-	9.84 ± 2.30	1.36 ± 0.70	18 ± 7
4-Hydroxydiphenyl amine	4-HDPA	TP of antiox.	2.8	0.6 ± 0.4 (7)	0.1 ± 0.1 (3)	56 ± 7	-	0.37 ± 0.26	< LOQ	26 ± 12
4-Aminodiphenyl amine	4-ADPA	TP of antiox.	1.82	0.6 ± 0.1 (7)	< LOQ	41 ± 1	-	1.80 ± 0.47	< LOQ	26 ± 4

<sup>1)</sup> Chemicalize was used for prediction of logD values, 12.2024, <https://chemicalize.com/>, developed by ChemAxon.

<sup>2)</sup> Day of maximum internal concentration given in brackets.

<sup>3)</sup> As percent of the concentration at the end of the exposure phase (day 7).



**Fig. 2.** Concentrations of phenylguanidines, benzothiazoles, other amines and phenylenediamines in a) tank water (left) and b) mussels tissue (right). CTR: control experiment with no m-TRWP; LOW: low concentration of m-TRWP (0.05 g<sub>-TRWP</sub>); HIGH: high concentration of m-TRWP (0.5 g<sub>-TRWP</sub>).

vulcanization accelerators were predominant across all three matrices (Fig. 3). Among the vulcanization accelerators, DPG comprises the highest relative concentrations (50 %) to the m-TRWP extract and even exceeds 80 % in both, mussel tissue and tank water.

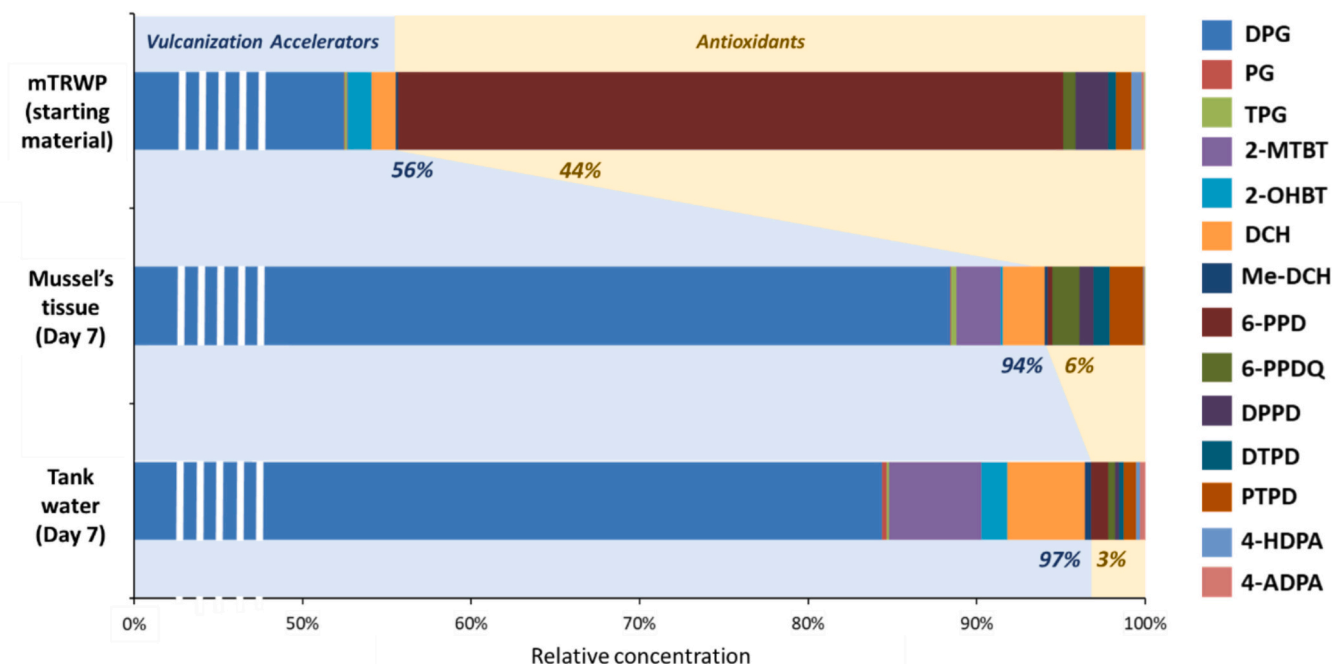
Although benzothiazoles are generally considered dominant among vulcanization accelerators (Weyrauch et al., 2023), their quantification in this study was hampered due to their high concentrations detected in mussel samples from the control aquarium without m-TRWP, suggesting unidentified sources of benzothiazoles present during the exposure period (Table S3).

In contrast, the contribution of antioxidants was considerably lower

than those of vulcanization accelerators. In the m-TRWP extracts, antioxidants constituted approximately 45 % of the total quantified compounds, with 6-PPD being most prevalent (40 %). In comparison, the relative concentrations of antioxidants were markedly reduced in mussel's tissue (6 %) and tank water (3 %) (Fig. 3). Most of this relative decrease is, indeed, due to 6-PPD (Fig. 3).

3.1.1. Vulcanization accelerators

Phenylguanidines were the predominant class of compounds in mussel tissue (Fig. 2), primarily due to the high DPG concentration. After 7 days of exposure, the concentration of DPG reached 942 (±132) µg/kg (wet



**Fig. 3.** Relative mean concentrations of the quantifiable compounds in extracts of m-TRWP (starting material), of mussel's tissue (N = 3 individuals) and in tank water at the end of the exposure period (day 7). These latter two were obtained from the high-concentration aquarium (0.5 g<sub>-TRWP</sub>/L).

weight), with a concentration  $\sim 16$  times higher in the mussel than in the tank water (Fig. 4a, Table S3). DPG is the predominant compound leaching from tires in both, laboratory (Foscarini et al., 2024; Müller et al., 2022; Weyrauch et al., 2023) and environmental settings (Challis et al., 2021). Its hydrolysis product phenylguanidine (PG) was detected as well, but at much lower concentrations ( $0.8 \pm 0.6$   $\mu\text{g}/\text{kg}$ ). The DPG concentrations in mussels of the low-concentration aquarium ( $0.05$   $\text{g}_{\text{TRWP}}/\text{L}$ ) were approximately 10-fold lower than those in the high-concentration aquarium ( $0.5$   $\text{g}_{\text{TRWP}}/\text{L}$ ) for all sampling days (Table 1, Table S3), reflecting the linear relationship between m-TRWP exposure and DPG accumulation.

*Benzothiazoles* are another abundant class of vulcanization accelerators commonly identified in tire-leachates (Foscarini et al., 2023; Jeong et al., 2022; Müller et al., 2022). However, most *benzothiazoles* analyzed in this study, such as 2-mercaptobenzothiazole (2-MBT), 2-methylthio-benzothiazole (2-MTBT), 2-hydroxybenzothiazole (2-OHBT), amino-benzothiazole ( $\text{NH}_2\text{-BT}$ ), benzothiazole (BT) and benzothiazole-2-sulfonic acid (BTSA), were present in the mussel extract of the non-exposed control experiment in concentrations comparable to those of the exposed ones (Table S3). This may be attributed to unidentified sources of benzothiazoles present during the exposure period.

Despite this, 2-MTBT, a biotransformation product of 2-MBT (Reemtsma et al., 1995), was found at substantially higher concentrations in mussels exposed to high m-TRWP concentration than in controls. After 7 days of exposure, 2-MTBT reached a concentration of  $28 (\pm 8)$   $\mu\text{g}/\text{kg}$  which is 7 times higher than the concentration found in the tank water (Fig. 4b, Table S3).

Among the *other amines* considered, dicyclohexylamine (DCH), a hydrolysis product of the vulcanization accelerator *N,N'*-dicyclohexyl-2-benzothiazole sulfenamide (DCBS; Seiwert et al., 2020), was one of the compounds detected at the highest concentration in mussel tissue (Fig. 3). DCH reached a maximum concentration of  $33 (\pm 8)$   $\mu\text{g}/\text{kg}$  at day 3, with a concentration ratio to the tank water of  $8 (\pm 1)$  L/kg observed at the end of the exposure period (day 7) (Table 1). Methyl-dicyclohexylamine (Me-DCH), exhibited the highest concentration at day 3 in both high and low concentration aquaria, reflecting the 10-fold difference in concentration between the two conditions ( $2.5$  and  $0.2$   $\mu\text{g}/\text{kg}$ , respectively) (Table 1).

### 3.1.2. Antioxidants

*Para-phenylenediamines* (PPDs) are widely used as antioxidants in tire rubber (Huntink et al., 2004). *N*-(1,3-dimethylbutyl)-*N'*-phenyl-*p*-

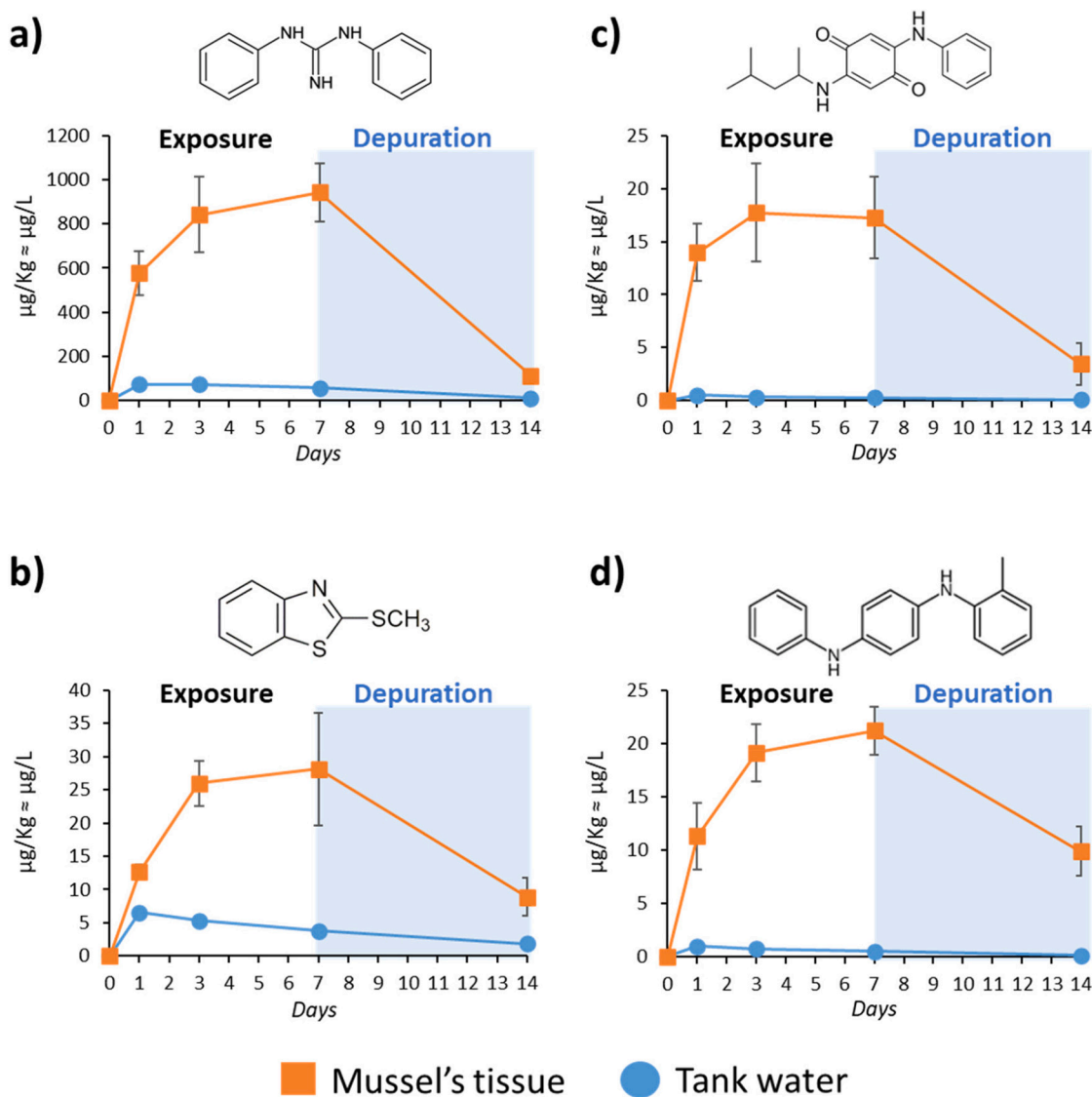


Fig. 4. Concentrations of a) diphenylguanidine, b) 2-methylthio-benzothiazole, c) 6-PPD quinone and d) *N*-(1,3-dimethylbutyl)-*N'*-phenyl-*p*-phenylenediamine during the whole experiment in the tank water (blue line) and mussel's soft tissue (orange line).  $N = 3$  individuals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phenylenediamine (6-PPD), its oxidation product 6-PPD quinone (6-PPDQ) (Seiwert et al., 2022; Tian et al., 2021), and its hydrolysis and oxidation products 4-hydroxydiphenylamine (4-HDPA) and 4-aminodiphenylamine (4-ADPA) were determined (Seiwert et al., 2022). Additionally, three *N,N'*-mixed phenyl and tolyl derivatives (BENPAT) components, *N,N'*-diphenyl-1,4-phenylenediamine (DPPD), *N,N'*-di-(*p*-tolyl)-*p*-phenylenediamine (DTPD) and *N*-phenyl-*N'*-(*o*-tolyl)-*p*-phenylenediamine (PTPD) (Zhang et al., 2020, 2020), were found in both tank water and mussels tissue (Table 1).

Among the detected PPDs, PTPD was predominant, with a concentration of 21 ( $\pm 2$ )  $\mu\text{g}/\text{kg}$  in mussel tissue - approximately 40-fold higher than the external concentration measured on day 7 (Fig. 4d, Table S3). Notably, PTPD has not been previously reported in exposure studies on tire-associated chemicals. The related antioxidants DPPD and DTPD were the third and fourth most abundant compounds detected, with mussel concentrations of 18 ( $\pm 2$ )  $\mu\text{g}/\text{kg}$  and 10 ( $\pm 1$ )  $\mu\text{g}/\text{kg}$ , respectively (Table S3). This finding aligns with a recent study by Wei et al. (2024) which reported high bioaccumulation of DPPD and DTPD ( $\log \text{BAF} > 3.7$  in 21 aquatic species from the Pearl River estuary).

The second most abundant compound in the mussels was 6-PPDQ, with its highest concentration reached between day 3 and 7 (17  $\pm$  4  $\mu\text{g}/\text{kg}$ ) (Fig. 4c, Table 1). The more polar TPs of 6-PPD, 4-HDPA and 4-ADPA ( $\log D$  2.8 and 1.82, respectively), both reached relatively low concentration of 0.6  $\mu\text{g}/\text{kg}$  at day 7 at 0.5 g/L m-TRWP exposure and close to LOQ values at 0.05 g/L (Table 1).

6-PPD exhibited its highest values at day 3 and day 1 in the high and low concentration aquaria, respectively. Subsequently, the internal concentrations declined markedly, consistent with the rapid decrease observed in the surrounding water. This trend can be attributed to the short half-life of 6-PPD in water (8 h at pH 7; ECHA, 2022); this may also apply for the mussel tissue.

### 3.2. Mussels' internal exposure to m-TRWP

For the estimation of the internal exposure it is interesting to learn, how the concentration of a chemical in m-TRWP translates into its concentration in the mussels exposed to these particles in suspension. Therefore, the concentration ratio between the mussel and the m-TRWP was calculated for each chemical, for the high and low exposure concentration (Table 1, Table S8). For the high exposure experiment (0.5 g/L m-TRWP) many of the analyzed compounds show a similar concentration ratio of 10–6 g/kg between mussel and m-TRWP (PTPD  $\approx$  DTPD  $>$  6-PPDQ  $>$  DPG  $\approx$  PG  $\approx$  TPG  $\approx$  DCH  $>$  Me-DCH). For the more hydrophobic compounds (PTPD, DTPD, 6-PPDQ, TPG ( $\log D$  4.9–3.1, Table 1)) one may assume that the internal concentration in the mussel tissue originates from ingested m-TRWP. In contrast, the more polar compounds DPG, PG, and DCH ( $\log D$  values  $<$  1.5; Table 1) may have been taken up from the tank water, where they were determined at substantial concentrations (Table S3).

Other compounds show lower ratios between 2 and 0.1 g/kg (DPPD  $\approx$  4-ADPA  $>$  2-OHBT  $>$  4-HDPA). The latter three are more polar TPs of tire-additives ( $\log D$  1.7–2.8; Table 1); their lower ratios may either reflect a more effective excretion from the mussel or their comparatively fast further transformation in the mussel. Remarkably, the lowest ratio of 0.03 g/kg only was found for 6-PPD (Table 1). As discussed above this corresponds to its short hydrolysis half-life. The PPDs with only aromatic amine substituents (DPPD, DTPD, PTPD) all show significantly longer hydrolysis half-lives than 6-PPD (Han et al., subm.; Xu et al., 2025), translating here into a higher relative concentration in the mussel tissue compared to the m-TRWP, with a ratio of 2–9 g/kg (Table 1).

The compound 2-MTBT exhibits an exceptionally high concentration ratio of around 500 g/kg (Table 1). As mentioned above. 2-MTBT is known as biomethylation product of 2-MBT, formed by *S*-methyltransferases (Reemtsma et al., 1995). This transformation obviously occurred in the mussel, either by the mussel itself or its microbiome.

For most of the compounds that could be determined in the mussels

exposed to the 10-fold lower m-TRWP concentration (0.05 g/L), the concentration ratio between the mussel and the m-TRWP was roughly also 10-fold lower than for the high exposure (Table 1). This indicates a linear relationship between m-TRWP exposure concentration and the corresponding release and uptake of tire-related chemicals. This does not apply to 2-MTBT; the methylation leading to 2-MTBT may be relatively more effective at the lower internal exposure to 2-MBT.

### 3.3. Depuration phase

A depuration phase was added by transferring the exposed mussels into fresh seawater for an additional 7 days. In this phase, m-TRWP with their remaining chemicals load are expected to be excreted from the mussels. Of the chemicals taken up into the mussel tissue more polar organic compounds are expected to be released more rapidly than less polar ones, as demonstrated previously for aromatic hydrocarbons (Farrington et al., 1982; Hansen et al., 1979). Compounds with a rapid concentration decrease in the depuration phase may, thus, be proposed as “*biomarkers of exposure to TRWP*”, because their presence in the mussel would require the ongoing exposure to TRWP.

Conversely, less polar compounds are expected to decrease more slowly during depuration. This would also allow for their ongoing biotransformation during this phase, so that the concentration of biotransformation products, if not too polar, may continue to increase during the depuration phase. Compounds with such a behavior may be considered “*biomarkers of exposure to tire-related chemicals*”, because they can still be found after the exposure to TRWP has ended.

The concentration of all quantified compounds drastically decreased in the mussels during the depuration phase (Fig. 2, Table S3). In the mussels previously exposed to the high m-TRWP concentration, however, all compounds remained detectable (Table 1). On the contrary, in the case of the low exposure concentration (0.05 g/L), only DPG, TPG, 6-PPDQ, DCH, DTPT and PTPD could be determined, with concentration levels close to their LOQ (Table S2, S3). At both exposure concentrations, DTPD and PTPD exhibited the weakest concentrations decrease of the tire-chemicals ( $>$  40 % remaining), followed by TPG, 2-MTBT, 4-HDPA, 4-ADPA, 6-PPDQ, Me-DCH, DPG and DCH with retained concentrations of above 10 % and 6-PPD, TBA, 2-OHBT and DPPD with  $<$  10 %, only (Table 1, S3). Nevertheless, all these compounds are expected to reach concentration levels below the LOQ upon extended depuration.

### 3.4. Transformation products

For a more comprehensive evaluation of the exposure of mussels to tire-related chemicals, the LC-HRMS data were screened in two ways: i) by a suspect screening for compounds that have been found in previous studies (Alhelou et al., 2019; Foscari et al., 2024; Grasse et al., 2023; Müller et al., 2022) and ii) by a non-target screening for intense peaks exclusively found in the tissue of the exposed mussel but not in the non-exposed control.

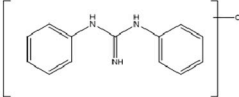
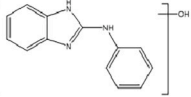
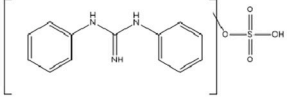
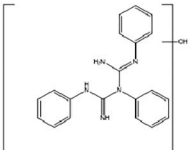
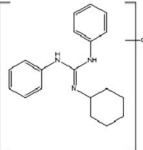
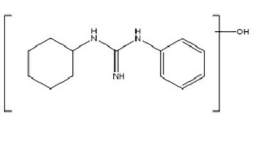
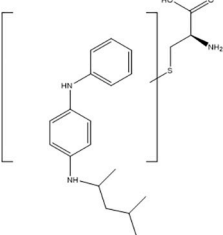
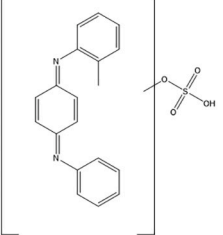
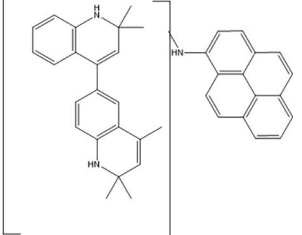
As a result, 37 additional TPs in mussel tissue were detected and identified with different confidence (Table S4 - S6), including 13 compounds not previously reported (Table S4, Table S5). Of these, 18 TPs persisted in the mussels even after the depuration phase, maintaining concentrations comparable to those observed during the exposure period (Table 2, Table S4, S5). In addition to the 37 identified TPs, 12 compounds were detected in the tissue of the exposed mussels for which no parent chemical could be attributed. The majority of these compounds persisted through the depuration phase in both high- and low-concentration aquaria, with some exhibiting increasing trends. Interestingly, most of these 12 compounds were not detectable in the surrounding tank water (Table S6).

#### 3.4.1. DPG related TPs

High DPG concentrations are consistently accompanied by various derivative compounds, formed during the vulcanization process

**Table 2**

Overview of selected transformation products of tire additives detected in mussel tissue, their concentration trend during depuration and relative concentration after depuration. N = 3 individuals. More detailed information in Table S4, Table S5.

ID <sup>1)</sup>	Molecular ion formula	Concentration trend during depuration <sup>2)</sup>		Residual concentration in mussel after depuration <sup>3)</sup>	Peak area after depuration
		Tank Water	Mussel	%	$\times 10^3$ AU
<i>DPG related</i>					
DPG_228_1	C <sub>13</sub> H <sub>14</sub> N <sub>3</sub> O	↘	≈	90 ± 32	5
DPG_228_2	C <sub>13</sub> H <sub>14</sub> N <sub>3</sub> O	↘	≈	118 ± 14	186
DPG_226	C <sub>13</sub> H <sub>12</sub> N <sub>3</sub> O	≈	↗	147 ± 29	1
DPG_306	C <sub>13</sub> H <sub>12</sub> N <sub>3</sub> O <sub>4</sub> S	↘	≈	128 ± 69	2
DPG_234	C <sub>13</sub> H <sub>20</sub> N <sub>3</sub> O	<i>n.d.</i>	≈	108 ± 58	0,5
DPG_310_2	C <sub>19</sub> H <sub>24</sub> N <sub>3</sub> O	↘	↗	191 ± 22	20
DPG_310_3	C <sub>19</sub> H <sub>24</sub> N <sub>3</sub> O	↘	↗	125 ± 22	3
DPG_310_4	C <sub>19</sub> H <sub>24</sub> N <sub>3</sub> O	↘	↗	135 ± 4	4
DPG_346_1	C <sub>20</sub> H <sub>20</sub> N <sub>3</sub> O	<i>n.d.</i>	≈	96 ± 13	3
DPG_346_2	C <sub>20</sub> H <sub>20</sub> N <sub>3</sub> O	<i>n.d.</i>	≈	98 ± 8	2
<i>Proposed structures:</i>					
					
DPG_228_1,2	DPG_226	DPG_306			
					
DPG_346_1,2	DPG_310_2,3,4	DPG_234			
<i>PPDs related</i>					
6PPD_388	C <sub>21</sub> H <sub>30</sub> N <sub>3</sub> O <sub>2</sub> S	<i>n.d.</i>	≈	89 ± 25	9
PTPD_367	C <sub>19</sub> H <sub>15</sub> N <sub>2</sub> O <sub>4</sub> S	↘	≈	78 ± 6	3
<i>TMQ related</i>					
TMQ_546_1	C <sub>39</sub> H <sub>36</sub> N <sub>3</sub>	<i>n.d.</i>	≈	90 ± 10	4
TMQ_546_2	C <sub>39</sub> H <sub>36</sub> N <sub>3</sub>	<i>n.d.</i>	↗	120 ± 2	9
<i>Proposed structures:</i>					
					
6-PPD_388	PTPD_367	TMQ_546_1,2			

1) Numbers correspond to the  $m/z$  value of the molecular cation ( $[M + H]^+$ )

2) “↘” or “↗” indicate the decreasing or increasing signal intensity by >30 %; “≈” indicates the stable mean intensity ( $\leq \pm 30$  %).

3) Compared to the reached intensity at the end of the exposure phase (day 7).

(Hummel et al., 1982) and TPs formed in the course of a leaching experiment (Müller et al., 2022) due to both abiotic and biotic processes (Fohet et al., 2023; Foscari et al., 2024; Foscari et al., 2023). A total of 21 putative TPs of DPG were detected, among which 10 showed stable or even increased levels after the depuration period (Table 2). These findings suggest ongoing metabolic transformations in the mussels from unknown precursors. The identified TPs all result from hydroxylation of DPG and its derivatives, which include two isomers of  $C_{13}H_{14}N_3O$  (DPG\_228), three isomers of  $C_{19}H_{24}N_3O$  (DPG\_310), two isomers of  $C_{20}H_{20}N_5O$  (DPG\_346), as well as DPG\_226 ( $C_{13}H_{12}N_3O$ ), DPG\_234 ( $C_{13}H_{20}N_3O$ ), and DPG\_306 ( $C_{13}H_{12}N_3O_4S$ ). Particularly notable was DPG\_228\_2, a hydroxylated TP of DPG (Foscari et al., 2024), which exhibited the highest intensity with a peak area of 180,000 AU after the depuration period (Table 2, Table S4). Following this, DPG\_310\_2 showed an intensity of 20,000 AU after depuration, nearly double that of the exposure phase (day 7).

### 3.4.2. PPDs related TPs

A total of nine TPs stemming from PPD compounds was detected (Table 2, Table S5). Seven of these TPs likely originate from 6-PPD, one of which is newly identified (6PPD\_468). Additionally, one TP of DPPD, and another from PTPD were detected, which have not been previously reported. Another 6-PPD related TP is  $C_{21}H_{30}N_3O_2S$  (6PPD\_388), which was recently identified as 6-PPD-cysteine in zebra fish embryos exposed to 6-PPD (Grasse et al., 2023). Obviously, this phase-II metabolite is not only formed in (zebra) fish exposed to 6-PPD but also in other aquatic organisms such as the mussels upon exposure to TRWP. This observation supports the notion that a part of the 6-PPD of the m-TRWP was taken up by and metabolized within the mussels. 6-PPD-cysteine appears more stable in the mussel tissue than its parent compound, 6-PPD: its peak area remained constant throughout the depuration phase (Table 2). Therefore, 6-PPD-cysteine may be a promising indicator of exposure of aquatic biota to TRWP chemicals.

A novel TP of PTPD,  $C_{19}H_{15}N_2O_4S$  (PTPD\_367) was found in mussel tissue, which also is a phase-II metabolite, a sulfate adduct. This TP shows only a slight signal decrease of approximately 20 % during the depuration phase (Table 2, Table S5). This behavior can be attributed to the high levels of the parent compound PTPD remaining in the mussels even at the end of the depuration period (Table 1, Table S5), which likely contributed to the sustained intensity of the related TP.

### 3.4.3. TMQ related TPs

Technical 1,2-dihydro-2,2,4-trimethylquinoline (TMQ) consists of a mix of oligomers, mainly dimers, trimers and tetramers (Zhang et al., 2020, 2020). Some of these oligomers were detected in the exposed mussels, namely two dimers and two trimers (Table S5) with a substantial signal intensity (10,000–45,000 AU). All of these TMQ oligomers decreased in intensity during the depuration phase, suggesting that they were probably released with the m-TRWP from the mussels. On the contrary, the mean intensity of two isomeric TPs of the TMQ dimer ( $C_{39}H_{36}N_3$ , TMQ\_546\_1/2) remained stable during the depuration (Table 2, Table S5). TMQ\_546 may have been formed within the tire through the addition of pyrene, a compound known to be abundant in tire materials (Aatmeeyata and Sharma, 2010). Subsequently, TMQ\_546 isomers may have been taken up from the m-TRWP. Therefore, they are further suitable candidates as indicators of exposure to tire-chemicals.

## 4. Conclusions

The transfer of tire-related chemicals into mussels upon exposure to TRWPs and the subsequent biotransformation in the mussels was studied to learn about the uptake of tire-related chemicals and to identify potential biomarkers of exposure of marine mussels.

- The test material m-TRWP produced from CMTT by milling with sand was found to be suitable for exposure experiments with mussels

to TRWP. It may also be a more realistic test material for other exposure experiments with aquatic biota.

- Of the 21 quantified compounds, DPG was found to be predominant in mussel tissue upon exposure to m-TRWP. DCH, 2-MTBT, PTPD, DPPD and 6-PPDQ followed in concentration.
- Transfer of chemicals from m-TRWP to mussel was affected by compounds stability and polarity. Transfer was strongest for PTPD, DTPD and 6-PPDQ and was orders of magnitude lower for 6-PPD. Data suggest that uptake from incorporated TRWP into mussel tissue is of relevance.
- Several tire-related compounds (DPG, DCH, 6-PPDQ) rapidly decreased in concentration during the depuration phase with 10 to 20 % remaining after 7 days. Thus, these compounds may be suitable biomarkers of TRWP exposure.
- Suspect and non-target screening for transformation products in mussel tissue identified 37 putative TPs of tire-related chemicals, mostly originating from DPG, such as isomers of DPG\_228, DPG\_310 and DPG\_346. Many of these TPs remained or continued to increase during the depuration phase. Among them were phase-II metabolites of DPG, 6-PPD, PTPD and TMQ. These TPs may serve as biomarkers of exposure to tire-related chemicals.

Future field studies may use both, the biomarkers of exposure to TRWP and the biomarker of exposure to tire-related chemicals to learn more about the exposure of mussels in riverine as well as in marine environment.

## CRedit authorship contribution statement

**Aurelio Foscari:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Dorte Herzke:** Writing – review & editing, Visualization, Methodology, Conceptualization. **Riham Mowafi:** Methodology, Investigation. **Bertina Seiwert:** Writing – review & editing, Validation, Methodology, Investigation, Conceptualization. **Bavo De Witte:** Writing – review & editing, Methodology, Investigation. **Daan Delbare:** Writing – review & editing, Methodology, Investigation. **Gustavo Blanco Heras:** Writing – review & editing, Methodology, Investigation. **Jesús Gago:** Writing – review & editing, Methodology, Investigation. **Thorsten Reemtsma:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Thorsten Reemtsma reports financial support was provided by Federal Ministry for Education and Research (BMBF). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marpolbul.2025.118311>.

## Data availability

Data will be made available on request.

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