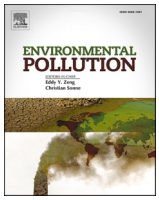
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Exposure to tire particles leachates reduces clearance rate, increases trace metal accumulation and mortality, and impairs gut microbiota in mussels (*Mytilus edulis)*

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ABSTRACT

Tire wear particles (TWPs) are a major source of microplastics in the environment, including aquatic ecosystems. Their leachates are of growing concern due to their acute toxicity on aquatic organisms. This study investigated the effects of tire leachates on mortality, clearance rate, metal accumulation, and the structure and diversity of the gut microbiota in the blue mussels (*Mytilus edulis*). Mussels were exposed for one and five weeks to different leachate concentrations (prepared from 5 g of new tire particles (TP) per liter) and a reconstituted metal solution reflecting the concentrations of the major metal constituents of the leachate stock solution. High-concentration exposure resulted in 61% cumulative mortality, compared to other treatments. Clear patterns of zinc, lead, and barium accumulation were observed, reaching 136.6 ± 17.3 mg Zn/kg in mussel tissues exposed to the highest concentration. High-concentration exposure also induced dysbiosis, characterized by an enrichment of anaerobic taxa (Fusobacteriales, Bacteroidales, and Clostridiales). Notably, Amplicon Sequence Variants (ASVs) affiliated with the order Francisellales, which includes lethal pathogens for mussels, were also detected at a higher proportion in the leachate treatments. After depuration, the clearance rate of mussels previously exposed to the highest leachate concentration decreased by 99%, and by 59% in mussels exposed to the metal solution. These results suggest that metals present in tire leachate contribute significantly to the impairment of mussel filtering activity.

1. Introduction

The widespread use of motor vehicles has resulted in the continuous production of tire wear particles (TWPs), a major yet often overlooked source of microplastic pollution in the environment. Approximately 3.1 billion tires are produced each year globally to serve more than 1.4 billion vehicles, resulting in an estimated annual emission of 0.81 kg of TWPs per capita, or roughly 6 million tons worldwide (Kole et al., 2017; Sieber et al., 2020). These particles accumulate in terrestrial environments and enter aquatic systems through atmospheric fallout, surface runoff, and wastewater effluent, representing a major fraction of total microplastic pollution (Sieber et al., 2020; Sommer et al., 2018). Despite this, the reliable quantification of TWP concentrations and rubber-derived chemicals in aquatic environments remains limited by a lack of standardized analytical methods for their determination (Anquetin et al., 2025; Zhang et al., 2023; Zhu et al., 2024). As a result, their environmental fate is not fully characterized, and the toxicological pathways through which TWPs affect marine organisms remain poorly

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understood.

Müller et al. (2022) identified 215 organic compounds in tire material, 145 of which are considered leachable, highlighting the pernicious aspect of tires as a source of complex chemical mixtures. The main ecological risks of TWPs stem from the release of these chemical additives, which leach into surrounding environments and can generate toxic transformation products (Song et al., 2025; Thomsen et al., 2024; Wagner et al., 2018). Scientific interest in tire leachates has surged following the identification of 2((4-methylpentan-2-yl)amino)-5-(phenylamino)cyclohexa-2,5-diene-1,4-dione (6PPD-quinone or 6PPD-Q), a transformation product of a tire antioxidant, as the primary toxicant causing acute mortality in spawning coho salmon (*Oncorhynchus kisutch*) in the northwestern United States (Tian et al., 2021). This discovery resolved a longstanding mystery, identifying the causal agent behind the stormwater-associated Urban Runoff Mortality Syndrome (URMS) previously documented in these fish (Scholz et al., 2011).

The potential toxic effects of tire-associated chemicals on aquatic biota have been mainly investigated through laboratory experiments in which organisms were exposed to aqueous leachates generated from artificial tire particles (TP). Under controlled experimental conditions, TP leachates have been shown to induce a range of sublethal and lethal impairments affecting physiology, survival, reproduction, and development processes in various aquatic species (Bernardini et al., 2024; Capolupo et al., 2020; Le Du-Carrée et al., 2024; Tallec et al., 2022a,b).

Bivalve species appear particularly vulnerable to tire wear toxicity. In mussels (*Mytilus galloprovincialis*), exposure to TP leachates was found to increase mitochondrial activity in gametes while reducing sperm motility, suggesting potential risks for reproductive success (Savino et al., 2025). TP leachates have also been reported to disrupt early developmental stages and induce sub-lethal effects in mussels (Capolupo et al., 2020, 2021). In the Pacific oyster (*Magallana gigas*), long-term exposure (44 days) resulted in reduced gamete quality, oxidative stress, and increased energy metabolism (Bernardini et al., 2024). Even at low concentrations of TP leachates, juvenile oysters (*Crassostrea gigas*) exhibited altered clearance and respiration rates (Tallec et al., 2022a).

In addition to organic compounds, tires release significant quantities of inorganic contaminants such as metals, which are recognized as important determinants of toxicological effects in aquatic organisms (Halle et al., 2021; Yang et al., 2022). Zinc, for example, has been identified as a major contributor to the acute toxicity (EC50) of TP leachates in daphnia (*Daphnia magna)* (Wik et al., 2009) and copepods (*Tigriopus japonicus*) (Yang et al., 2022), highlighting the need for further investigations into the specific contribution of metals to the overall tire leachate toxicity.

Previous researches indicate that the mussel gut microbiota is highly sensitive to environmental variables and diet (Griffin et al., 2023; Li et al., 2024; Pierce & Ward, 2019), making it a valuable biomarker for stress in *Mytilus edulis* (Li et al., 2019; Liu et al., 2025). Several laboratory investigations have explored the influence of plastic particles, both nano- and micro-sized, on bivalves' gut microbiota. While some studies found no significant effect of nylon fibers (Collins et al., 2023) or polystyrene particles (Yang et al., 2021) on mussel gut microbial diversity, other studies demonstrate clear dysbiosis. For instance, Xu et al. (2023) observed a decrease in microbial richness and diversity following exposure to polystyrene nanobeads. In another study, adult mussels exposed to polystyrene microspheres showed increased richness and dominance of specific microbial taxa, whereas juveniles exhibited reduced diversity, greater microbial instability, and immune impairment (Chi et al., 2025). A recent study showed that exposure to nylon microfibers altered the composition of the digestive gland microbiota (Stilo et al., 2025). Additionally, the aging of particles appears to play a role, as Li et al. (2020) demonstrated that weathered microplastics exert a more potent effect on the microbiota than their virgin counterparts.

These observations underscore that the effects of microplastics on the microbiota of filter-feeders can be modulated by factors such as species, developmental stage, and particle type. This highlights the critical importance of investigating plastic leachates, which represent a relevant exposure pathway regardless of particle shape or size. Despite extensive research on microplastics, the influence of TP leachates on mussels' gut microbial communities remains unexplored. To date, the only relevant data comes from Bernardini et al. (2024), who found no significant alterations in the oyster gut microbiota exposed to TP leachates.

The present study aimed to investigate the effects of TP leachates on mortality, clearance rate, and gut microbiota composition of adult blue mussels (*M. edulis*). Over a period of one and five weeks, mussels were exposed to four concentrations of TP leachates as well as to a reconstituted metal solution reflecting the main metal concentration of the leachate stock solution (5 g/L). Additionally, chemical analyses were conducted to characterize the leachate composition.

We hypothesized that: (1) mussels would reduce their filtration activity as a behavioral response to limit exposure to TP leachates, (2) impairment induces anoxic conditions leading to microbiota dysbiosis, which acts as a secondary stress response, and (3) leached metals would significantly contribute to the observed stress responses, including alterations in filtration activity and gut microbiota composition.

2. Materials and methods

2.1. Tire particles leachates preparation

A new car tire (Michelin Crossclimate2 205/55 R16 91 V) was purchased from a commercial store (Norauto, France). Leachates for the acute toxicity tests were generated following the protocol of Almeda et al. (2023), with minor modifications. The outer tread surface of the tire was abraded using a screwdriver with a cylindrical metal cutter. The obtained TP were sieved through a 500 μm stainless steel mesh using liquid nitrogen and a brush to aid in the sieving. We deliberately chose mechanical abrasion from a new tire to isolate the intrinsic toxicity of the tire material itself. Actual TWPs collected from roads contain numerous other contaminants (asphalt, oils, brake pad residues) (Sommer et al., 2018), which would have introduced confounding factors in identifying the specific toxic agents of the tires. TP leachates were prepared in glass bottles previously rinsed with acetone and Milli-Q water, by adding the TP to filtered (0.2 μm) and UV-treated fresh seawater (FSW) at a concentration of 5 g TP/L (corresponding to 952 ± 153 particles per mL). To count TP, 0.0015 g of TP was dispersed in 50 mL of ethanol using a magnetic stir bar for 5 min, then filtered onto a 0.7 μm GF/F filter. Particles retained on the filter were counted under a binocular microscope. The bottles were agitated for 72 h on an orbital plate at 130 rpm in a dark room at 20 °C ± 2 °C to allow for chemical leaching. Finally, the TP leachates were filtered through a GF/F 0.7 μm filter to remove particles and used immediately for toxicity experiments.

2.2. Biological material

Blue mussels (*M. edulis*) (length: 4.48 ± 0.3 cm, wet weight: 10.19 ± 1.99 g) were collected in January 2025 from a mussel farm located in Berk-plage (50°25′47.2″N 1°33′29.0″E, French Eastern English Channel coast). After collection, the mussels were directly transported to the laboratory, carefully cleaned to remove fouling organisms and adhering organic matter. They were then placed in a 250 L glass aquarium that had been meticulously rinsed with Milli-Q water and securely closed with a glass lid to limit any airborne contamination. The aquarium was maintained in a thermoregulated room at 12 ± 1 °C and under a 12 h light-dark cycle. During the 15-day acclimatization period, mussels were fed daily with *Tisochrysis lutea*, and one-third of the seawater was renewed every two days with FSW. Before initiating the exposure experiment, the mussels' shell length and wet weight were measured.

2.3. Mussel exposure to tire leachate and metals solutions

The mussel exposure experiment consisted of two exposure durations (one week and five weeks) and four concentrations of leachate solutions (10, 25, 50, and 100%), obtained by serial dilutions of the initial leachate stock solution (100%, 5 g TP/L). A control treatment consisting solely of FSW was also included. To distinguish the effects of metals from those of organic compounds, an additional exposure condition was conducted using a solution containing the four predominant metals previously measured in the 5 g TP/L leachate (0.412 mg Zn/L, 0.056 mg Ba/L, 0.022 mg Al/L, and 0.0151 mg Fe/L). Experiments were conducted in 2 L glass aquaria that had been soaked in FSW for one week, followed by cleaning with ethanol and rinsing with Milli-Q water. Twelve mussels were placed in each aquarium, which were continuously oxygenated and covered with glass plates to prevent external contamination. Twelve mussels were placed in each aquarium, with three aquaria per treatment and per time point serving as independent replicates (Fig. S1). Statistical analyses were performed using the replicates ($n = 3$). Mussels were fed with approximately 10^{10} cells of *Tisochrysis lutea* per mussel, twice a week. The same volume of *T. lutea* culture, at a known concentration, was added to all aquaria, ensuring identical feeding conditions across all replicates and treatments throughout the experiment. Throughout the five-week exposure, the water in each aquarium was completely renewed once per week using freshly prepared leachate solutions to prevent chemical degradation during the experiment. Mortality was monitored daily during the five-week exposure. Dead mussels were removed and replaced with new individuals maintained under the same conditions as the control group. This procedure ensured a constant number of mussels per aquarium and maintained the same exposure condition throughout the experiment. Newly introduced mussels were marked with a cross using a Dremel tool to facilitate their identification, as they were excluded from the analyses performed at the end of the exposure. At each sampling time point (one week and five weeks), the mussel shell size and wet weight were measured, and two mussels per tank were randomly collected for gut microbiota analysis. Intestines were meticulously dissected using sterilized scalpels and tweezers and subsequently stored at −80 °C until DNA extraction. The remaining mussels were frozen for subsequent metal bioaccumulation analysis.

2.4. Microbiota DNA extraction and sequence data processing

For each condition and replicate, the guts of two mussels were pooled together and homogenized using a sterilized plastic pestle. DNA was extracted following the manufacturer's instructions using the All-prep PowerFecal DNA/RNA Kit (Qiagen, Hilden, Germany). DNA was quantified in each sample using a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and DNA samples were subsequently stored at −80 °C. Amplicon libraries were prepared by performing a PCR amplification of 16S ribosomal RNA gene (rDNA) targeting region V3-V4 region using universal primers 341F (5′- CCTACGGGNGGCWG-CAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013), and the Phusion Plus DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). Illumina paired-end sequencing (platforme MiSeq 2 × 300 bp) was performed at the Eurofins Genomics sequencing facility (Konstanz, Germany).

A total of 4,054,007 raw sequences were obtained and processed using the QIIME2 pipeline (2023.9) for quality assessment (Bolyen et al., 2019). Subsequently, the sequences were processed with the DADA2 (Chiarello et al., 2022) pipeline R tool for denoising, paired-read merging (minimum of 12 base pairs, no mismatches allowed), and chimera removal. Forward and reverse reads were trimmed to remove primers and truncated. Sequences with ambiguous bases or exceeding expected error thresholds (<Q20) were discarded. Sequencing depth after denoising and quality filtering was 79,295 ± 9082 reads per samples.Singleton amplicon sequence variants (ASVs) were removed, and only ASVs affiliated with bacteria were retained. ASVs are exact, error-corrected DNA sequences resolved at single-nucleotide resolution without relying on arbitrary similarity thresholds, compared to traditional Operational Taxonomic Units (OTUs) (Callahan et al., 2017). The data set was subsequently rarefied (*BiodiversityR* package; Kindt and Coe, 2005) to 16,252 reads per sample, corresponding to the maximum number of reads present across samples. This approach yielded the identification of 2576 unique ASVs, as inferred by the DADA2 algorithm. The sequences were then grouped according to their index tags and ASVs were taxonomically affiliated against the SILVA database (version 132; Weingarten et al., 2019) using BLAST (Altschul et al., 1990). Sequencing data have been submitted to the NCBI sequence read archive database (SRA accession: PRJNA1444997).

2.5. Mussel clearance rates measurement

Following the five-week exposure to TP leachates or metals, three mussels per condition (one per replicate when possible) were individually transferred into a clean beaker containing 2 L of FSW for one week of depuration. During this period, the water was renewed once (after three days) and the mussels were fed every 48 h with *T. lutea*. At the end of the depuration period, water was changed, the beakers were cleaned, and each mussel was supplied with an initial algal concentration of 10^{10} cells/mussel. The decrease in algal cell concentration was monitored using a fluorometer (Trilogy, 7200-000, Turner Designs, Sunnyvale, CA, U.S.) over a 12-h period. Water samples (15 mL) were collected at 10-min intervals during the first 30 min, every 30 min for the next 2 h, and then every hour during the rest of the monitoring period (three replicate samples per time point). After homogenization of the 15 mL samples, a 1 mL aliquot was taken for fluorescence measurement, and the remaining volume was immediately returned to the corresponding aquarium.

The individual clearance rate (Cl), used as an indicator of feeding activity, was calculated from the exponential decline of algal concentration using the equation described by Riisgård and Seerup (2003):

$$Cl = aV/n,$$

where V is the volume of water, n is the number of filtering mussels, and a is the maximum declining slope of the regression line in the semi-ln plot of algal concentration versus time. The average clearance rate measured in the negative control, considered as background noise, was subtracted from the other clearance rate values. Control experiments conducted without mussels confirmed that algal sedimentation was negligible.

2.6. Chemical analyses

2.6.1. Leachate analysis

All chemical analyses were performed on leachates obtained from the stock leachate solution at 5 g TP/L.

The total trace elements were analyzed according to the protocol described by Ouddane et al. (1999). Briefly, 15 mL of water was directly collected and transferred into a plastic vial, where 100 µL of nitric acid (65%, Suprapur, Merck) was added to preserve the samples. Samples were stored at 4 °C until analysis. Trace element concentrations were analyzed by Inductively Coupled Plasma Atomic Emission Spectrometry (Agilent Technologies ICP-AES 5110, dual view, Santa Clara, CA, USA).

The method used for the analysis of organic compounds is described in the supplementary data (Text S1). Briefly, SPE was first performed to elute contaminants in a mixture of three solvents (dichloromethane, hexane and acetonitrile). After evaporation and remobilization in methanol, the samples were analyzed using a Dionex Ultimate 3000 HPLC System (ThermoFisher Scientific) coupled to an Orbitrap LTQ XL mass spectrometer.

2.6.2. Trace metal accumulation in mussel tissues

Trace metal concentrations in mussel tissues were quantified according to the protocol described by Diop et al. (2022) after one and five weeks of exposure. Briefly, mussel tissues were collected from the shell with clean ceramic tweezers and scalpels to prevent external metal contamination. Samples were transferred into pre-weighed glass vials and freeze-dried for 48 h at −100 °C. After measuring the dry weight, mussels (n = 3) were pooled and ground to a fine powder with a ceramic mortar and pestle. An aliquot of 200 mg from each sample was weighed into PTFE digestion cups and predigested at room temperature for 24 h with nitric acid (65%, Suprapur, Merck) and then digested until total decomposition on a heating block (Environmental Express HotBlock® SC100, Charleston, SC, USA) at 120 °C for 4 h. The resulting digests were diluted with ultrapure water (Milli-Q) to a final volume of 10 mL. Metal concentrations were quantified by inductively coupled plasma atomic emission spectroscopy (ICP-AES; Agilent Technologies ICP-AES 5110, dual view, Santa Clara, CA, USA). Reagent blanks were first run at intervals of every ten analyzed samples to eliminate equipment drift. All analyses were performed in triplicate to ensure reproducibility, accuracy, and precision. Procedural blanks and certified reference material (CRM) IAEA-436 (mussel tissue, International Atomic Energy Agency of Monaco) were analyzed under the same conditions as the samples. Results showed good agreement with the certified values; the measured concentrations are within 10-15% differences compared with the certified values.

2.7. Bioinformatics and statistical analyses

2.7.1. Microbiota structure analysis

All statistical analyses were performed using RStudio (version 4.5.1; Posit team, 2025). When applicable, *p*-values were adjusted using the False Discovery Rate (FDR) method to correct for multiple testing (Benjamini and Hochberg, 1995). To visualize the structure of the microbial communities and assess potential grouping patterns and temporal effects, a Principal Coordinates Analysis (PCoA) was performed based on Bray–Curtis dissimilarity on squared roots transformed data (*vegan* package; Oksanen et al., 2025). Differences in microbial community composition across conditions were tested using a permutational analysis of variance (PERMANOVA; adonis2 function from the *vegan* package). Before the interpretation, the assumption of homogeneity of group dispersions was verified using a permutational multivariate analysis of dispersion (PERMDISP; *vegan* package). To identify which specific pairs of conditions differed, post-hoc pairwise PERMANOVA tests were conducted (pairwiseAdonis). Since temporal variability was of particular interest, the dataset was split by sampling week ("Week 1″ and "Week 5″), and each time point was analyzed separately. Within each time point, an analysis of similarity (ANOSIM; *vegan*) was used to compare community structure between conditions. When global differences were significant, pairwise ANOSIM comparisons were applied to identify specific group differences. Alpha diversity indices (Observed richness, Shannon, and Simpson) were computed using *phyloseq* (McMurdie and Holmes, 2013). For each sampling time, linear mixed-effects models were fitted to test differences in alpha diversity between conditions (*lme4* package; Bates et al., 2015). When significant effects were detected, post-hoc comparisons were performed (*emmeans* package; Lenth and Piaskowski, 2025). Finally, to explore relative taxon variation, ASVs of the same order were aggregated and their abundances were compared with an analysis of variance (ANOVA; Fisher, 1925) and post-hoc Tukey test (Tukey, 1949).

2.7.2. Clearance rate and metal accumulation

Clearance rates were compared with an ANOVA (variances were homogeneous and residuals normally distributed) and post-hoc Tukey test (Tukey, 1949), using RStudio (version 4.5.1; Posit team, 2025).

GraphPad Prism (version: 10.6.1; GraphPad Software, Boston, Massachusetts, USA) was used to compare metal concentrations in mussel tissues across the different treatments for each exposure's duration. A Kruskal-Wallis test (Kruskal & Wallis, 1952) was applied to the raw data; if it resulted in a significant *p*-value (<0.05, FDR adjusted), a Dunn's multiple comparison test (Dunn, 1961) was performed to compare contaminant levels in mussels.

3. Results

3.1. Tire leachates chemical composition

A total of 34 tire-related chemicals were identified in the stock leachate solution at 5 g TP/L (Table S1). Among them, fourteen trace metals were detected, with zinc (Zn, 583 μg/L) being the most significant, followed by barium (Ba, 102.8 μg/L), iron (Fe, 37 μg/L), aluminum (Al, 15.5 μg/L), and molybdenum (Mo, 11.8 μg/L). Some metals (Fe, Al, and Mo) were present at comparable concentrations in control samples (FSW) (Table S1). Other elements were below detection limits.

Organic chemicals characterization of tire leachate composition reveals the presence of several functional categories relevant to rubber manufacturing such as the vulcanization accelerators 2-mercaptobenzothiazole (MBT), 2,2′-dithiobisbenzothiazole (MBTS), N-cyclohexylbenzothiazole-2-sulphonamide (CBS) and 1,3-diphenylguanidine (DPG); and the well-known antidegradation agents N-cyclohexyl-N′-phenyl-p- phenylenediamine (CPPD) and 1,4-benzenediamine, N-(1,3-dimethylbutyl)-N′-phenyl- (6PPD). The aged form of the last compound is also present under its oxidized compound 2-((4-methylpentan-2-yl)amino)-5-(phenylamino) cyclohexa-2,5-diene-1,4-dione (6PPD-quinone). Other antioxidants and stabilizers were detected in addition to other aromatic amines and urea compounds (Table S1).

3.2. Metals accumulation in mussels' tissues

After one week of exposure, metal accumulation was observed in mussels' tissues, with concentrations of Ba and Pb significantly increasing in proportion to the leachate concentrations (Fig. 1; Table S2). Ba, previously identified as one major element leaching from TP, had a clear accumulation pattern across leachate concentrations and was significantly higher in mussels exposed to the 50% and 100% leachate solutions compared to the control (Fig. 1). Although Pb was below the detection limit (<5 μg/L) in the tire leachate solution used in the exposure experiment, its concentration in mussel tissues increased significantly after one week in the 50% and 100% leachate treatments compared to the control. Pb concentrations also increased with exposure duration. Zn concentrations, although not significantly different among treatments, tended to rise with increasing leachate concentrations. Several other metals (Al, As, Cd, Co, Cr, Cu, Mn, Mo, Ni, V, and Fe) were also measured in mussel tissues, however their concentrations did not differ significantly from the control and showed no clear trends according to treatment conditions.

3.3. Mussels' mortality

During the first week of exposure, no mortality was recorded in any treatment. The first deaths were observed only after 17 days of exposure in the highest leachate concentration (100% = 5g TP/L). Under these conditions, the mortality rate increased from 5.6% after three weeks to a maximum of 61% after five weeks of exposure (Fig. S2). Mortality rates in the other treatments were stable and did not exceed 2.7% (control), 5.3% (25%), 5.7% (50%), and 11% (metals). All mussels exposed to 10% of the leachate solution survived the whole experiment. The mortality in the control group represents an acceptable background mortality for bivalves in laboratory conditions. Regarding the 100% treatment at 5 weeks, the toxicity was so severe that high mortality left only one viable replicate aquarium (n = 1) for some physiological analyses.

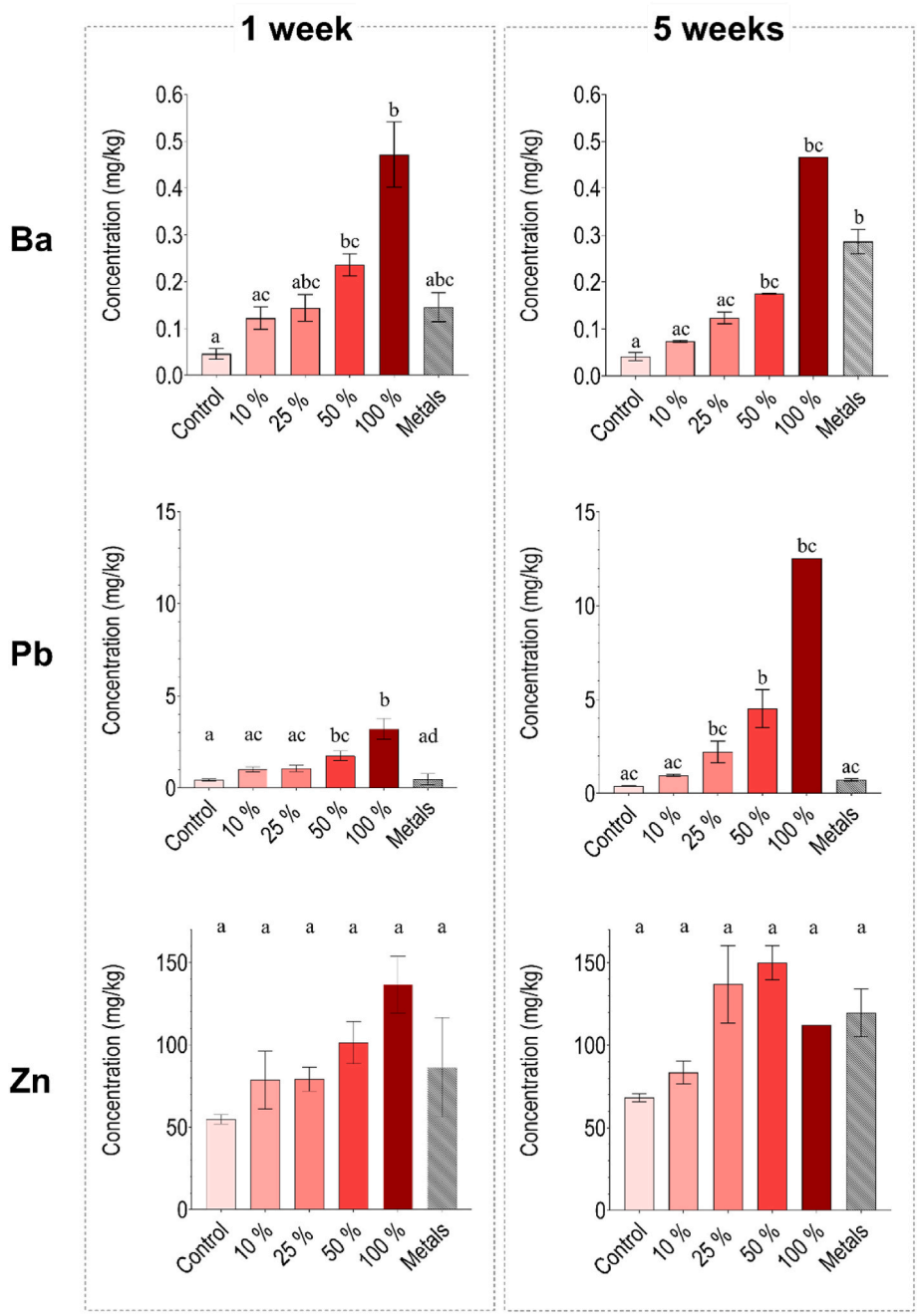


Fig. 1. Barium, lead and zinc mean concentrations in mussels' tissues according to the exposure treatment and time. Three replicates per treatment (except for treatment 100% after 5 weeks where n = 1 because of the high mortality limiting replication). Error bars = standard deviation. Different letters above bars indicate significant differences between treatments ($p < 0.05$ FDR adjusted, Dunn's test).

3.4. Clearance rates

After one week of depuration, mussels previously exposed to the highest leachate concentration (100%) showed an almost complete cessation of filtration activity, with a mean clearance rate of 0.004 ± 0.06 L/h per filtering mussel (Fig. 2, Table S3). Mussels from the control, 10%, 25%, and 50% treatments displayed similar clearance rates, ranging from 1.56 ± 0.26 to 1.87 ± 0.47 L/h/indiv.). Although not statistically significant, mussels exposed to the metals solution exhibited a lower clearance rate (0.76 ± 0.18 L/h/indiv.) compared to the control. Compared to the control group, clearance rates decreased by 99% in mussels exposed to the 100% leachate treatment and by 59% in mussels exposed to the metal treatment.

3.5. Effect on mussel's microbiota

3.5.1. Bacterial community composition and diversity

A total of 2576 ASVs were identified across the entire dataset. The highest microbial richness was observed in the gut of unexposed mussels, with 360 ± 92 ASVs recorded in individuals collected after the acclimatation period and before the experiment. The lowest richness was recorded in mussels from the control group after five weeks (141 ± 108 ASVs; see Table S4 for details). Although differences were not statistically significant, a decline in the total number of ASVs was observed over time, except for mussels exposed to 10% and 25% of the leachate solution, where diversity tended to increase after five weeks of exposure (Fig. S3). After one week of exposure, 80 ASVs were common to all

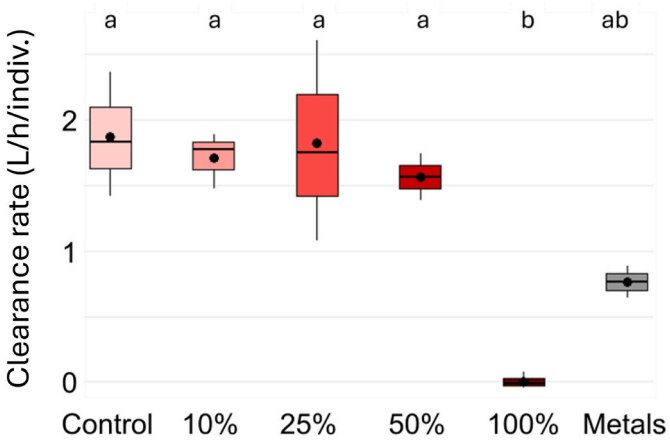


Fig. 2. Clearance rate (L h^{-1} $indiv^{-1}$) of mussels exposed to various treatments over a five-week period, followed by a one-week depuration period in clean water. Boxplots represent the median (black line), interquartile range (IQR,25th-75th percentiles) and whiskers extending to 1.5 × IQR. Black points indicate the mean clearance rate. Three replicates were used per treatment, except for the 50% and Metals treatments (n = 2), where one organism in each treatment was removed because it released gametes and ceased to filter.

treatments, including the control. Seven ASVs were common among all contaminated treatments (TP leachates and metals), and two were specific to leachate-exposed groups (Fig. S4). After five weeks, the total number of ASVs common to all treatments decreased to 52, while the number of ASVs common to contaminated treatments doubled (14 ASVs, Table S5). Equitability indices (Simpson and Shannon) were significantly affected by treatments, but only after one week of exposure, and no significant differences in mussel's microbiota diversity were detected after five weeks (Figure S5 A - B). The Simpson diversity index was lowest in mussels exposed to the metal solution compared to all other treatments, including the control. Similarly, the Shannon index was significantly lower in the metal treatment than in both the control and 25% leachate treatments.

According to PERMANOVA, the mussels' gut microbiota was significantly influenced by three main factors: Time, treatment, and their interaction (square-root transformation, p-value (FDR) ≤ 0.001, Table S6). As shown in Fig. 3, the microbial composition of the guts from mussels exposed to 100% leachate differs significantly from that of the other treatments, irrespective of the exposure time. The ANOSIM showed that treatments also significantly affected the microbiota community structure after both 1 and 5 weeks of exposure (Table S7).

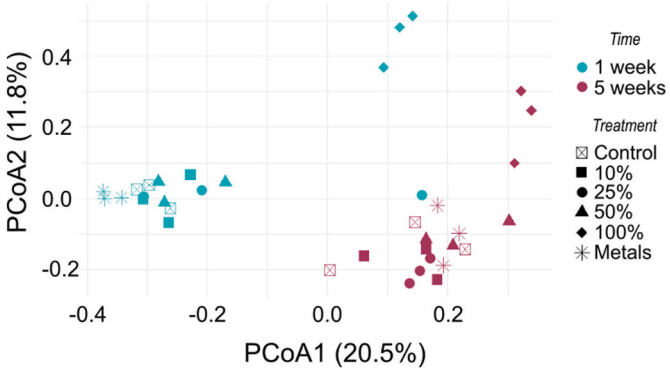


Fig. 3. Principal Coordinate Analysis (PCoA) based on Bray-Curtis distances illustrating the variation in gut microbiota composition between the control and exposed groups under different conditions of tire leachates after 1 and 5 weeks of exposure. Although the blue point (25%, week 1) lies outside the blue patch, we assume that this represents natural variability and have retained these data for subsequent analyses. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5.2. *Taxa affected by tire particles leachates*

As illustrated in Fig. 4, a total of 23 bacterial orders were identified, with relative abundances ranging from 0.4% (Chitinophagales) to 34.6% (Flavobacteriales), together accounting for 95.5% of all sequences in the dataset. The overall microbiota composition was mainly dominated by Flavobacteriales, Alteromonadales, Oceanospirillales, and Rhodobacterales. Among these orders with relative abundance >0.4%, 13 differed significantly among treatments, including four orders for which the differences were particularly pronounced. Fusobacteriales and Bacteroidales (Fig. S6) were significantly enriched in mussels exposed to the highest leachate concentration (100%) after both one and five weeks of exposure. At this concentration, the relative abundance of Clostridiales also increased after five weeks (Fig. S7). Micrococcales were strongly influenced by both the treatments and exposure duration: After one week, they were detected only in the guts of control mussels, and they were no longer detectable after five weeks (Fig. S7).

Several orders appeared to respond to leachate exposure after one week. For example, Alteromonadales were significantly more abundant in mussels exposed to the 100% leachate solution, while Oceanospirillales showed a significantly lower abundance under the same condition (Fig. S8). However, after five weeks, the relative abundance of both orders became similar across treatments. Although not statistically significant, the increasing trend in the relative abundance of Francisellales in mussels exposed to the 10%, 25%, and 50% leachate treatments over the five-week period is noteworthy (Fig. S9).

4. Discussion

The list of contaminants identified in the tire leachate solution is consistent with those reported in recent studies investigating the chemical composition and the biological effects of tire-derived compounds across a range of species (Capolupo et al., 2020; Foscari et al., 2025; Le Du-Carrée et al., 2024; Y. Li et al., 2024; Tallec et al., 2022a,b). Although the concentrations of TP used to generate leachate solutions vary widely among studies (from 0.01 g TP/L to 100 g TP/L), the contaminants detected remain largely similar. This consistency suggests that these compounds represent the most readily leachable fraction of tire constituents, detectable even at low TP concentrations (0.01 g TP/L; Tallec et al., 2022a).

In addition to organic compounds, tires are known to release

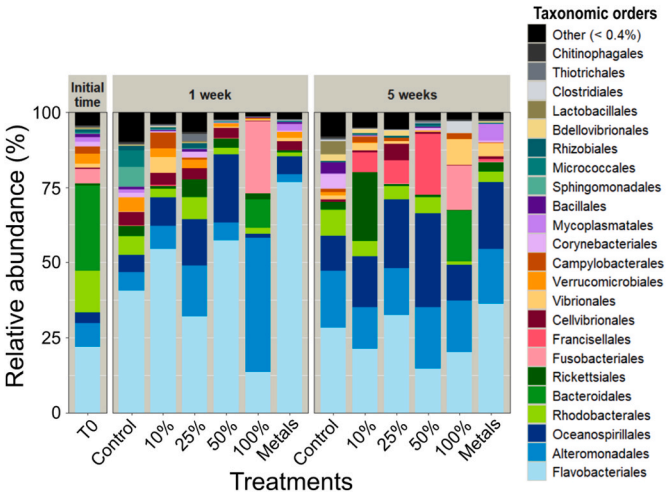


Fig. 4. Mussel's gut microbiota composition, at order level, before expositions (T0 = initial time), in the control and exposed groups under different conditions of tire leachates, after 1 and 5 weeks of exposure.

substantial quantities of inorganic contaminants, such as metals. These contaminants are considered major contributors to the toxicological effects observed in aquatic organisms (Halle et al., 2021; Yang et al., 2022). In the present study, metal accumulation in exposed mussel tissues, compared to the control group, was detected after one week of exposure. Pb and Ba concentrations were significantly increasing in proportion to leachate concentrations. Although Fe and Al were detected at significant concentrations in the leachate solution, their accumulation in mussel soft tissues was not distinguishable from natural background levels, as these elements are ubiquitously present in seawater. Conversely, despite its relatively low concentration in the leachate, Pb showed a clear accumulation pattern in tissues. Although Pb is generally considered to have a low bioaccumulation potential from seawater in isolation (van Kolck et al., 2008), its significant accumulation in mussel soft tissues in the present study may be attributed to the complex mixture of contaminants present in the leachate solution, consistent with previous observations showing enhanced Pb tissue accumulation in mussels exposed to metal mixtures (García-Navarro et al., 2017). This highlights that leachate composition alone is not sufficient to predict which metals will bioaccumulate in exposed organisms.

Consistent with previous studies (Degaffe and Turner, 2011; Yang et al., 2022), Zn was the metal detected at the highest concentrations both in the leachate solutions and in mussel tissues. Several studies have identified Zn as one of the major toxicants within TWPs leachates, contributing substantially to their overall toxicity (Yang et al., 2022). As reported by Hietanen et al. (1988), exposure to Zn induces distinct behavioral changes in mussels (*M. edulis*), including byssus detachment, mucus secretion, and cessation of valve opening.

Pb exposure has also been shown to impair physiological condition, growth, and development in the freshwater and marine mussels (Hossain et al., 2023; Fraser et al., 2018). In the present study, the maximum Pb concentration recorded in mussel tissues (12.5 mg/g of dry weight) is higher than the concentrations reported in environmental mussel tissues from polluted coastal sites, such as Dakar, Senegal (Diop et al., 2016), where they ranged from 0.37 to 1.37 mg/g of dry weight. Metal contamination and accumulation in mussel tissues (e.g., *Perna canaliculus*) are known to reduce clearance rates (Chandurvelan et al., 2015). In the present study, mussels exposed to the 100% leachate solution for five weeks displayed a substantially elevated mortality rate of 61%, compared with only 11% mortality in the metal treatment. These findings suggest that mortality is primarily driven by organic tire-derived contaminants, despite the high metal accumulation observed in mussel tissues. Nonetheless, exposure to metals alone was sufficient to alter both clearance rates and microbiota diversity.

After one week of depuration, mussels previously exposed to the highest leachate concentration (100%) exhibited a 99% reduction in clearance rate compared with the control group, while those exposed to the metal solution showed a 59% reduction. These results demonstrate that exposure to TP leachates and metal solutions negatively affects the clearance rate of blue mussels, highlighting the substantial role of metals in this response. Redpath and Davenport (1988) reported that higher Zn concentrations (470-860 μg/L) completely inhibited mussel filtration, suggesting that Zn alone may strongly impair filtration activity. This is consistent with the absence of filtration we observed in mussels exposed to the 100% leachate solution, which contained a comparable Zn concentration (583 μg/L), even after a one-week depuration period. Similar trends were reported by Thomsen et al. (2024), who documented significant reductions in the clearance rate of *M. edulis* after short-term (72 h) and long-term (3-week) exposure to TP leachate mixtures. At a leachate concentration corresponding to 1.25 g TP/L, filtration was reduced by 38% and 46% relative to the control group after 72 h and 3 weeks, respectively (Thomsen et al., 2024). The authors also observed a complete cessation of filtration activity in mussels exposed to a leachate concentration corresponding to 5 g TP/L, likely reflecting a behavioral response to limit pollutant uptake. In juvenile oysters (*Crassostrea gigas*), significant decreases in clearance rate (52%) and respiration rate (16%) after 40 h of exposure to leachate concentrations of 1, 10, and 100 μg TP/L indicate potential impairment of gill function (Tallec et al., 2022a). The present study only assessed the clearance rate after the depuration period; patterns during exposure may be more contrasted.

Contrary to what is observed after the first week of exposure, after five weeks the gut microbiota structure tends to homogenize across treatments (Fig. 3). The bacterial orders identified in the present study largely overlap with those previously reported in the gut microbiota of *M. edulis* (Ben Cheikh et al., 2024; Griffin et al., 2021; Pierce and Ward, 2019; Schill et al., 2017). However, certain orders, such as Francisellales, Corynebacteriales, Micrococcales, Bdellovibrionales, Lactobacillales, and Chitinophagales, were not observed in earlier studies. The presence of Francisellales at abundances exceeding 5% in the 10%, 25%, and 50% tire leachate exposure conditions may indicate a stress response in mussels subjected to these concentrations (Bouras et al., 2023; Garcia et al., 2024; Meyer and Itoh, 2025). Within this order, *F. halioticida* was recently isolated from *M. edulis* and has been associated with mass mortalities of mussels (*Mytilus* spp.) in Normandy, France (Bouras et al., 2023; Garcia et al., 2024). This species is also known to cause lethal infection in Yesso Scallops (*Mizuhopecten yessoensis*) (Meyer and Itoh, 2025). However, the high proportion of Francisellales was not observed in mussels exposed to the highest leachate concentration (100%, Fig. 4 and Fig. S9), suggesting that this bacterial order is unlikely to be responsible for the high mortality observed under this condition.

The acute filtration impairment observed in mussels exposed to the 100% treatment coincides with a significant increase in anaerobic bacterial orders (Fusobacteriales, Bacteroidales, and Clostridiales). The observed proliferation of anaerobic bacteria suggests a potential shift towards anoxic conditions in the gut. This might be an indirect consequence of the reduced clearance rate, although specific physiological measurements would be required to confirm this mechanism. After one week of exposure, Oceanospirillales, represented by hydrocarbon-degrading bacteria, were significantly more relatively abundant in mussels exposed to 50% leachate, following a clear gradient from the lowest to the highest leachate concentrations. In contrast, Oceanospirillales were nearly absent in mussels exposed to 100% leachate, suggesting a rapid physiological response (e.g., shell closure) that may reduce pollutant uptake and limit the proliferation of these taxa. However, after five weeks of exposure, the relative abundance of Oceanospirillales no longer differed significantly among groups, indicating that microorganisms from this order can withstand prolonged exposure to tire-leachate contaminants or a homogeneous development of this order, regardless of the treatment applied. This pattern may reflect the necessity for mussels exposed to 100% leachate to resume filtration to meet metabolic demands, resulting in unavoidable contact with polluted water (enriching Oceanospirillales or inhibiting other orders) or stress associated with the duration of the experiment.

Only a few studies have investigated the effects of TP leachates on the intestinal microbiota of aquatic organisms (Bernardini et al., 2024; Meng et al., 2025; Zhang et al., 2025). A potential dysbiosis of the intestinal microbiota of the Pacific oyster *Magallana gigas* was reported after a 44-day exposure to TP or to their leachate alone, at concentrations corresponding to 5.2 μg TP/L and 52 μg TP/L (Bernardini et al., 2024). This study showed that both particles and leachates slightly modified the structure of the oyster microbiota, suggesting that the two exposure types exert comparable impacts. In the present study, no significant shift in gut microbiota composition was observed in mussels exposed to low to moderate leachate concentrations corresponding to 0.5 - 2.5 g/L, much higher than concentrations used by Bernardini et al. (2024), suggesting a comparatively higher tolerance to this type of contamination. However, mussels exposed to the highest concentration exhibited a significant alteration of their microbiota, suggesting that tolerance thresholds can be exceeded under extreme conditions. In zebrafish (*Danio rerio*), a 15-day exposure to TP (10 mg/L) induced significant changes in intestinal microbiota composition, followed by

substantial recovery after a depuration period, demonstrating high resilience to such contamination (Meng et al., 2025; Zhang et al., 2025). Current estimates indicate that environmental concentrations of TWPs in surface waters generally do not exceed 18 mg/L, even in highly polluted systems such as urban tributaries (Sun et al., 2025). Although available data remain limited, existing evidence does not indicate immediate sanitary risks for the species previously cited, except potentially for oysters. Additionally, no data are currently available regarding TWP concentrations and their derived contaminants in shallow coastal areas, which constitute key habitats for most oyster and mussel species.

Concentrations used in the present study (corresponding to 0.5 g TP/L for the 10% treatment up to 5 g TP/L for the 100% treatment) are higher than typical environmental concentrations found in aquatic environments more like mg/L (Parker-Jurd et al., 2025; Sun et al., 2025). However, this concentration was deliberately selected to simulate acute, worst-case exposure scenarios. Such high concentrations hold ecological relevance in specific localized contexts, notably within untreated stormwater runoffs adjacent to high-traffic infrastructure during heavy precipitation events, or at the water-sediment interface where dense TWPs inevitably settle and accumulate over time. For example, TWP concentration can reach 14.5 g/L in urban snowbank (Rødland et al., 2022) and could be carried into aquatic environments when snow melts. In the Seine River, TWPs can be found up to 2.3 g/kg of sediments (Barber et al., 2024). Furthermore, from a physiological standpoint, this specific concentration was chosen based on recent findings demonstrating that 5 g/L of TWP induces a complete cessation of clearance rates in *M. edulis* (Thomsen et al., 2024). Other studies on bivalves used concentrations from 0.001 to 0.1 g/L (Tallec et al., 2022a,b) and 0.005-0.05 g/L (Bernardini et al., 2024) up to 50 g/L for *M. galloprovinciallis* gamete quality assessment (Savino et al., 2025) and 80 g/L for the study of their lysosomal membrane stability, gamete fertilization, and embryonic development (Capolupo et al., 2020). Furthermore, the two sampling time points (1 and 5 weeks) were selected to capture distinct temporal responses of mussels to TP leachate exposure. The 1-week exposure period is commonly used in ecotoxicological studies to assess early physiological and biochemical responses following contaminant uptake, including initial microbiota shifts (Li et al., 2020) and early physiological responses (Diop et al., 2022; Kazour and Amara, 2024). In marine bivalves, exposure to sudden chemical contaminants typically induces rapid behavioral defense mechanisms, notably valve closure and an immediate drop in clearance rate, to limit contaminant uptake (Ait Ayad et al., 2011; Tallec et al., 2022a; Thomsen et al., 2024). The 5-week time point was selected to investigate subchronic effects, including longer-term trace metal bioaccumulation, feeding impairment, and potential disruption or adaptation of the gut microbiota. Indeed, the toxicokinetics of trace metals such as Pb and Zn require extended exposure periods of at least one month to obtain reliable bioaccumulation responses (Jebali et al., 2014). Then, combining these two time points enables the differentiation between transient and persistent effects, as well as the identification of potential acclimation processes or progressive toxicity. Such a temporal design is particularly relevant for filter-feeding organisms such as mussels, which are continuously exposed to waterborne contaminants and may exhibit time-dependent responses to complex mixtures such as TP leachates.

5. Conclusions

In this study, we investigated the effects of TP leachate cocktail and of tire-derived metals, examined separately, on mussel mortality, clearance rates, and gut microbiota composition and diversity. Our results suggest that the high mortality observed in mussels exposed to the 100% TP leachate solution was likely driven by anoxic conditions, as supported by the increased ratio of anaerobic bacteria taxa within the gut microbiota and the observed reduction in filtration activity. Metals present in the leachate likely contributed to the decrease in microbiota diversity and could potentially have played a role in the observed decrease in filtration rates. With the development of increasingly sophisticated analytical techniques to detect tire-leachate contaminants in aquatic environments, future studies should evaluate their effects at environmentally realistic concentrations. To date, only a limited subset of the numerous tire-derived compounds can be quantified in aquatic systems. In addition, current analytical methods are insufficient to comprehensively detect and quantify tire-related organic contaminants in organism tissues. Despite these constraints, investigating their potential for bioaccumulation in consumer species such as mussels would be valuable for both health-risk assessment and regulatory decision-making purposes. The respective contributions of different groups of tire-derived contaminants to the toxic effects observed in aquatic organisms remain largely unknown. Improving our understanding of these mechanisms will be essential for developing effective mitigation strategies and informing future regulatory frameworks.

CRediT authorship contribution statement

Julie Anquetin: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jérémy Théry:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Guillaume Veillet:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Fanny Coinon:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Baghdad Ouddane:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Bayan Almasri:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Christian Rolando:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Sébastien Monchy:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Luen-Luen Li:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Zhe Lu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Rachid Amara:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Rachid Amara reports financial support was provided by French National Research Agency. Zhe Lu reports financial support was provided by Natural Sciences and Engineering Research Council of Canada (NSERC). Zhe Lu reports financial support was provided by Plastics Science for a Cleaner Future Program. 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.envpol.2026.128222.

Data availability

Data will be made available on request.

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