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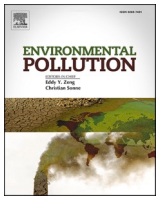

Life stage-specific effects of tire particle leachates on the cosmopolitan planktonic copepod *Acartia tonsa*☆

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ABSTRACT

Tire wear particles (TWP) are a major source of microplastics in the aquatic environment and the ecological impacts of their leachates are of major environmental concern. Among marine biota, copepods are the most abundant animals in the ocean and a main link between primary producers and higher trophic levels in the marine food webs. In this study, we determined the acute lethal and sublethal effects of tire particle leachates on different life stages of the cosmopolitan planktonic copepod *Acartia tonsa*. Median lethal concentration (LC_{50}, 48 h) ranged from 0.4 to 0.6 g L^{-1} depending on the life stages, being nauplii and copepodites more sensitive to tire particle leachates than adults. The median effective concentration (EC_{50}, 48 h) for hatching was higher than 1 g L^{-1}, indicating a relatively low sensitivity of hatching to tire particle leachates. However, metamorphosis (from nauplius VI to copepodite I) was notably reduced by tire particle leachates with an EC_{50} (48 h) of 0.23 g L^{-1} and the absence of metamorphosis at 1 g L^{-1}, suggesting a strong developmental delay or endocrine disruption. Leachates also caused a significant decrease (10–22%) in the body length of nauplii and copepodites after exposure to TWP leachates (0.25 and 0.5 g L^{-1}). We tested a battery of enzymatic biomarkers in *A. tonsa* adult stages, but a sublethal concentration of 50 mg L^{-1} of tire particle leachates did not cause a statistically significant effect on the measured enzymatic activities. Our results show that tire particle leachates can negatively impact the development, metamorphosis, and survival of planktonic copepods. More field data on concentrations of TWPs and the fate and persistence of their leached additives is needed for a better assessment of the risk of tire particle pollution on marine food webs.

1. Introduction

Up to 12.7 million metric tons of plastics are estimated to enter the ocean yearly (Jambeck et al., 2015) and microplastics have been ubiquitously found in all marine compartments (Cózar et al., 2014; Isobe et al., 2021). Plastics contain a variety of additives intentionally added to the polymers to give them specific properties and improve their functionality and durability. Examples of these additives are flame-retardants, plasticizers, antioxidants, dyes, antimicrobial coatings, and UV stabilizers. Plastic additives are commonly not chemically bound to the polymer matrix and, depending on their solubility, they leach into water when entering the aquatic environment. These additives as parent compounds (e.g., polybrominated flame-retardants, ortho-phthalates, bisphenol A, nonylphenol, biocides) or their transformation products (e.g., N-(1, 3-Dimethylbutyl)-N′-phenyl-p-phenylenediamine-quinone, known as 6PPD-quinone) can be hazardous to marine organisms (e.g., Gunaalan et al., 2020; Lithner et al., 2012; Paluselli & Kim, 2020; & Tian et al., 2021). Therefore, the effects of plastic additive leachates on marine ecosystems are of major environmental concern.

Tire wear particles (TWPs) are now considered a major source of microplastics in the environment, contributing over 50% of total microplastic emissions in some European countries (Baensch-Baltruschat et al., 2020; Boucher & Friot, 2017; Kole et al., 2017; Ly & Sayegh, 2023; Rødland et al., 2022; Wagner et al., 2018). TWPs, typically smaller than 100 μm (Kreider et al., 2010), are formed through the mechanical abrasion of tires with road surfaces when driving. It has been estimated that approximately 6 million tons of TWP are globally emitted every year (Khan et al., 2019; Sheng et al., 2021). Large tire particles are transported by road runoff to the aquatic systems whereas small TWPs become airborne and enter the oceans by atmospheric deposition

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(Evangeliou et al., 2020; Wagner et al., 2018). TWP particles contain a complex mixture of chemicals at high concentrations (Müller et al., 2022). Once TWPs enter the aquatic systems, dozens of potentially toxic additives leach from the particles (Foscari et al., 2023; Müller et al., 2022) and can cause ecological impacts after runoff events (Tian et al., 2021).

Copepods are the most abundant animals in the ocean (Humes, 1994) and a main link between primary producers and higher trophic levels in the marine food webs (Mauchline, 1998; Yang et al., 2022). Copepods typically dominate the zooplankton biomass and contribute to the global biochemical processes in the ocean like nutrient recycling and carbon sequestration by the biological pump (Verity & Smetacek, 1996). The effects of tire wear particle pollution on this important zooplankton group can result in alterations to marine ecosystem productivity and functions. Planktonic copepods are dioic and reproduce sexually. The postembryonic development of copepods is generally characterized by six naupliar stages, namely nauplius I to nauplius VI (NI to NVI), and five copepodite stages, namely copepodite I to copepodite V (CI to CV) (Marchus & Wilcox, 2007). The metamorphosis occurs from NVI to CI. Copepod nauplii are the main prey of many fish larvae (Last, 1980) and their abundance determines the recruitment of commercially important fish species (Castonguay et al., 2008).

Little is still known about the toxicity of TWP leachates on marine zooplankton. Li et al. (2023) showed that stormwater runoff from roads and TWP leachates were acutely lethal to freshwater zooplankton. Available information on the effects of TWP leachates on marine plankton is limited to a few species of phytoplankton (Capolupo et al., 2000; Page et al., 2022; Turner & Rice, 2010) and copepods (Bournaka et al., 2023; Halle et al., 2021; Halsband et al., 2020; Yang et al., 2022) and sea urchin larvae (Rist et al., 2023). As far as we know, there are no any published studies on the effects of tire particle leachates on naupliar and copepodites stages despite the importance of these stages in copepod population recruitment and marine food webs. Toxicity endpoints can cover different biological levels. For instance, enzymatic biomarkers are useful tools to understand the response of organisms to pollution and toxicity mechanisms (Gonçalves et al., 2021). Exposure to pollution can activate detoxification mechanisms (Regoli & Giuliani, 2014) and negatively affect biochemical processes causing alteration in the energetic metabolism and oxidative stress (Gonçalves et al., 2021; Villarroel et al., 2009). However, little is still known about the enzymatic response of marine planktonic copepods to plastic leachates and additives (Ensibi & Daly Yahia, 2017; Glippa et al., 2018).

The general aim of this study was to investigate the acute effects of tire particle leachates on the life stages of planktonic copepods. We used *Acartia tonsa* as a model species, an abundant and cosmopolitan species of marine calanoid copepod. The specific hypotheses of this study were: 1) Early life stages are more sensitive to TWP leachates due to the smaller size and higher surface-to-volume ratio, 2) tire particle leachates cause reduced hatching success, growth, and metamorphosis success as well as changes at the biomolecular level (enzymatic activities). These hypotheses were tested by addressing the following specific objectives: 1) determination of the life stage-specific lethal toxicity of tire particle leachates; 2) assessment of the acute impact of tire particle leachates on the hatching, metamorphosis success, body length, and enzymatic activities. To the best of our knowledge, this is the first study on the acute effects of tire particle leachates in copepod nauplii, key components of the marine food web as grazers and as the main prey of many species of fish larvae. Therefore, the results obtained here are relevant to evaluating the potential impact of TWP pollution on the planktonic food web.

2. Methodology

2.1. Experimental organisms

The calanoid copepod *A. tonsa* was obtained from the stock cultures at the EOMAR lab (ULPGC) established from specimens provided by DTU AQUA (strain DFH-ATI). *A. tonsa* is a species recommended by the International Organization of Standardization (ISO, 1999) for the assessment of acute toxicity of marine pollutants. The cultures of *A. tonsa* were kept in 30 L buckets filled with 0.1 μm filtered seawater (FSW), 35 ‰ salinity, with constant aeration, and at a temperature of 20 °C. *A. tonsa* cultures were fed the cryptophyte *Rhodomonas salina ad libitum* (>20000 cells mL^{-1}) 3 times weekly. The *R. salina* cultures were grown in B1 medium (Hansen, 1989) at 20 °C, a salinity of 35 ‰, an irradiance of ca. 80 μE m^{-2} s^{-1}, and a 12:12 h day: night photoperiod. The *R. salina* cultures were diluted 3 times per week with B1 medium under a laminar flow hood to avoid contamination.

To start a new cohort for the toxicity tests, we first separated adults of *A. tonsa* from the stock cultures using a 200 μm mesh sieve. The adults (sex ratio = 0.5) were placed in a 2 L glass beaker with FSW and food (*R. salina*) for 24 h. After 24 h, the adults were separated again with a 200 μm mesh sieve and placed back into the stock cultures, and the <200 μm water fraction containing the eggs was placed in a jar. After that, the eggs were collected using a 40 μm sieve, concentrated, and rinsed with a high-pressure sprayer to break detritus and obtain cleaner egg samples. The eggs were placed into a beaker with 100 mL of FSW. Then, two aliquots (1–2 mL) were taken with an automatic pipette and placed in Petri dishes to count the eggs under the stereomicroscope. Finally, the collected eggs (from approx. 13000-150000 eggs depending on the experiment) were transferred to acid-washed glass containers (20–30 L depending on the experiment) with FSW and gently aeration. The nauplii were fed *R. salina ad libitum* (<20000 cells mL^{-1}) to allow non-limited food conditions for the growth of the copepod cohort.

2.2. Leachate extraction

A new car tire tread (Imperial 145/70-13 71T- Snowdragon HP-Vinterdæk) was used to generate tire particles and their leachates. To obtain tire particles, the tread of the tire was cut into strips and then micronized by grinding the strips with a stainless-steel pneumatic milling cutter (Page et al., 2022). The micronized tire particles were sieved through a 250 μm certified steel sieve and the fraction <250 μm was stored in a glass bottle. The protocol proposed by Almeda et al. (2023) was followed to obtain leachates for aquatic toxicity testing. Briefly, tire particles <250 μm were placed in 600 mL acid-washed glass bottles with autoclaved FSW at a concentration of 1 g L^{-1}. The bottles were closed with screw caps with a polytetrafluoroethylene (PTFE) protected seal without headspace/air. Then the bottles were placed for 72 h in a roller (15 rpm) in a temperature-controlled incubator at 20 °C in the dark. After 72 h, the bottle content was filtered in a glass vacuum filtration system using glass-fiber filters (Whatman GF/F filters 0.7 μm pore size). The obtained leachate solution (stock solution, 100%) was stored in glass bottles at −20 °C and later used in the experiments. The target chemical analysis of some relevant compounds in the leachates was conducted as described in Rist et al. (2023). The concentrations of the detected PAHs, flame retardants, and metals in the leachates are provided in Rist et al. (2023) and can be found in the Supplementary Information Table S1.

2.3. Toxicity tests

2.3.1. . Bioassays to estimate the lethal and sublethal effects at the organismal level

A total of six toxicity tests were conducted with five different life stages of the life cycle of *A. tonsa*: eggs, mid nauplii (N3–N4), late nauplii (N5–N6), copepodites (C3–C5), and adults (Table 1). The experiment conducted with adults (sex ratio = 0.5) was done twice (Table 1). Each life stage was collected from the corresponding cohort, concentrated with a sieve (40 μm mesh for eggs and nauplii, 100 μm for copepodites, and 200 μm for adults), and placed in a beaker with 100 mL of FSW. Two aliquots were taken from the concentrate to quantify the number of organisms and estimate the required volumes to have 20 individuals per

Table 1
Summary of the bioassays including the used life stages, their average size in μm (body length for nauplii and prosome length for copepodites and adults), their age (days after hatching), and the endpoints investigated in each test. *Average size of nauplii. SD: standard deviation.

Bioassay	Life stage	Age (days)	Size ± SD	Endpoints
1	Eggs/early nauplii (NI-II)	0–2	*122.6 ± 4.3	Hatching success Nauplii mortality Body length
2	Mid nauplii (N3–N5)	4	195.1 ± 34.3	Mortality Body length
3	Late nauplii (N5–N6)	6	212.8 ± 25.3	Total mortality Metamorphosis N6–C1 Body length
4	Copepodites (C3–C4)	12	505.4 ± 52.4	Mortality Prosome length
5	Adults (C6)	21	774.7 ± 59.7	Mortality
6	Adults (C6)	15	689.9 ± 61.6	Mortality
7	Adults (C6)	19	678.5 ± 67.5	Enzymatic activities

replicate. Then, aliquots from the concentrate were added to Petri dishes and the number of individuals was adjusted to 20 per replica under the microscope.

Twenty individuals of their respective developmental stages (Table 1) were placed in 34 mL glass bottles containing 25 mL of the test solutions. All the experiments were conducted without food. In each toxicity test, the specific life stage was exposed to different leachate dilutions (100%; 50%; 25%; 12.5%; 6.25%), a negative control (only FSW), and a positive control (Nickel chloride, 0.4 mg Ni L^{-1}). All treatments were conducted in triplicates. The leachate dilutions were prepared by serial dilution of the stock leachate solution (100%, 1 g L^{-1}) in autoclaved FSW. Therefore, the equivalent concentrations in g of particles L^{-1} of the used dilutions are 1, 0.5, 0.25, 0.125 and 0.0625 g L^{-1}. As recommended in Almeda et al. (2023), a solid-to-liquid ratio of 1 g L^{-1} was used in the stock solution to prepare dilutions covering a range of potential effects, from no effect to >50% effect to allow precise calculation of median lethal concentration (LC_{50}) or median effective concentration (EC_{50}). Nickel chloride was used as a positive control (toxicity reference) based on the standardization methods recommended by Gorbi et al. (2012) for early nauplii of *A. tonsa*. To prepare the positive control exposure solution, 10 mg of nickel chloride powder (SIGMA-ALDRICH, 654507) were diluted in autoclaved FSW (0.1 μm) and, from this solution, a second dilution was prepared to have a final nickel concentration of 0.4 mg Ni L^{-1}.

Before adding the organisms to the experimental bottles, both the leachate stock solution and the controls complied with the following criteria: a temperature of 20 °C, a salinity of 35 ‰, a pH of 7.9, and oxygen saturation. The temperature was assessed using a digital thermometer, salinity was measured using a refractometer, and pH was recorded using a Grison (GLP 21) pH meter. The leachate stock solution and the controls were bubbled for 15 min with glass pipettes acting as an "air outlet" connected to a silicone tube for the aquarium pump to achieve oxygen saturation (Almeda et al., 2023). Finally, the individuals (20 per bottle) were added with a glass pipette to the experimental bottles with the test solutions. The bottle caps were not fully closed to allow gas exchanges during the exposure. Finally, the bottles were placed in an incubator at 20 °C for 48 h in dark and static conditions.

After the exposure period, the content of each bottle was poured into a Petri dish to determine the mortality using a stereomicroscope. In all bioassays, dead individuals were counted and removed with a glass pipette. Then, the rest of the sample was placed in a falcon tube and fixed with Lugol's solution (1%) for further counting and length measurements using the microscope. Mortality (%) was estimated as the proportion of dead individuals from the total amount of individuals found in each bottle, and in the case of nauplii where decomposition occurs fast, the initial number of individuals was used. To estimate the sublethal effect on growth, stereomicroscope images were taken to measure the total body length for nauplii and prosome length for copepodites and adults using Leica Application Suite V4.12 (LAS V4.12). Additionally, hatching success was estimated in the experiment with eggs, and metamorphosis success (N6–C1) in the experiment with late nauplii (Table 1).

2.3.2. Bioassay to estimate the acute effects of tire particle leachate at the biomolecular level

A new cohort with approximately 137000 eggs of *A. tonsa* was used to get enough biomass of copepods for the analyses of biomarkers (>20 mg wet weight per sample). Several batches of eggs were harvested as described above and stored in 15 mL Falcon tubes with autoclaved FSW without air at 4 °C in the dark. The eggs were placed in a 30-L glass container with FSW and aeration to start the cohort. After 24 h, *R. salina* was added to the cultures as food 3 times per week. After two and a half weeks, the adult copepods were used for the biomarker experiment. In this bioassay, we used a control (only FSW) and one experimental treatment (leachates, 50 mg L^{-1}), with triplicates for both treatments. The leachates were obtained as described above but using a solid-to-liquid ratio of 50 mg L^{-1}. This concentration was chosen because is a sublethal concentration within the range of predicted concentrations of TWP in surface waters (0.03–56 mg L-1, Wik and Dave, 2009) and estimates of TWP discharged from surface water drainage (12–179 mg L^{-1}, Parker-Jurd et al., 2021 and references therein). Copepod abundance in the cohort was quantified by counting the number of copepods in 3 subsamples of culture collected with a 200 μm filter. Then, a volume of culture of 2.7 L was filtered to have a concentration of 3000 individuals per bottle/replicate. The collected copepods were concentrated in 100 mL of FSW and added to 2.3 L glass bottles. Then we add 2 L of the test solutions to the glass bottles. The exposure nominal concentration in the leachate treatment would be equivalent to 47.6 mg L^{-1}. Leachates and FSW were prepared as described above the other bioassays; quality control and incubation conditions were similar to the organism-level effect tests. Quality control in terms of temperature, salinity, oxygen, pH, and incubation conditions was done as described above for the toxicity tests. After 48 h of exposure, we mixed the bottles and took an aliquot of 20 mL from each bottle with an automatic pipette to check the status of the copepods in a Petri dish under the stereomicroscope. Mortality was very low (<%5) without a significant difference between the control and experimental treatment. Then, the entire content of each bottle was concentrated in a 200-μm sieve and place in a beaker with 100 mL of FSW and then filtered through a 50 μm mesh placed in a conical strainer, concentrated, and carefully collected with a laboratory stainless-steel spatula. Finally, the samples were then placed in microtubes, weighed, and stored at −80 °C for further biomarker analyses.

A battery of enzymatic biomarkers was tested: electron transport system (ETS), catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), lactate dehydrogenase (LDH), Citrate Synthase

(CS), Acetylcholinesterase (AChE). The samples were homogenized in a microtube for 45 s in a sonicator (Vibra-cell) in 1 mL of 0.1M phosphate buffer (0.1M Na2HPO4, 0.1 mM KH2PO4, 75 μM MgSO4 · 7H2O, 1.5% PVP and 2% Triton X-100), at pH = 8.5 as described by (Owens & King, 1975). Homogenates (1 mL) were centrifuged at 4000 rpm at 4 °C for 10 min and the supernatant was used for the enzymatic analysis. After that, an aliquot of the remaining supernatant was stored at −20 °C for further protein analysis. Duplicates for the control treatment and triplicates for the experimental treatment were analyzed. The detailed methodology to estimate the enzymatic activity of each enzymatic biomarker can be found in Supporting Information (S.I. Methods, text S1). The enzymatic activities were performed at 25 °C and standardized by protein content (mg). The unit for all activities was μmol min^{-1} mg^{-1} of protein (= IU mg^{-1} of protein), except for ETS which was nmol O_2 min^{-1} mg^{-1} of protein. The analyses of ETS, CAT, and protein were followed on a Cary series UV-VIS spectrophotometer, and the analyses of GST, AChE, CS, LDH, and SOD were followed on a BMG-FLUOstar Omega microplate reader.

3. Data and statistical analyses

Data on the mortality (%) in relation to the leachate dilution (%) after 48 h of exposure was fitted to the following sigmoid model:

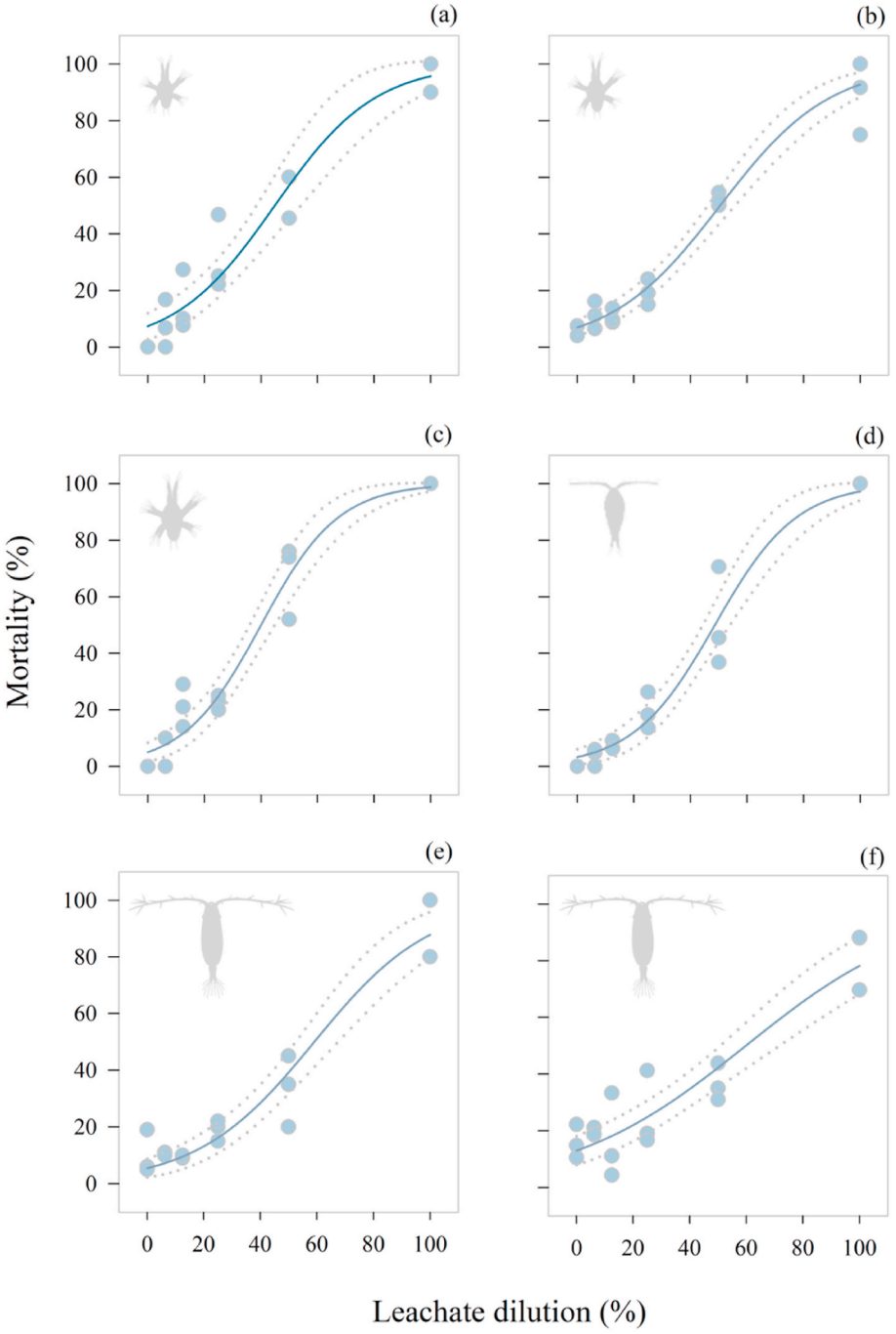

Fig. 1. Mortality (%) of different life stages of the copepod *A. tonsa* after 48 h of exposure to a range of TWP leachate dilutions (%). Stages are (a) = N1-2; (b) = N3-4; (c) = N5–N6; (d): copepodites II-III; (e) and (f) = adults. The stock solution (100%) was prepared at a solid-to-liquid ratio of 1 g L^{-1}. The continuous lines are the fitted curves based on Eq. (1). The dotted curves indicate the 95% confidence bands. The estimated model parameters are presented in Table 2.

$$M = 100/\left(1 + e^{-(C-LD_{50})/b}\right) \quad \text{Eq..(1)}$$

where M is the mortality (%), C is the TWP leachate solution (%), LD_{50} is the median lethal dilution (%) and b is the slope.

To evaluate the lethal effect of the tire particle leachates as a function of size (body/prosome length), we plotted the obtained LD_{50} vs the initial length of each life stage. In the case of the egg test, the size of the nauplii in the control treatment was used for the relationship.

Data on hatching or metamorphosis success in relation to TWP leachate solution (%) after 48 h of exposure were fitted to the following sigmoid model:

$$Y = Y_0 \Big/ \left(1 + \left(C/ED_{50}\right)^b\right) \quad \text{Eq. (2)}$$

where, Y is the hatching or metamorphosis success (%), Y_0 is the hatching or metamorphosis success (%) in the absence of leachates (i.e. in the control), C is leachate dilution (%), ED_{50} is the median effective dilution (here defined as the leachate solution in % required to reduce the hatching or metamorphosis by half compared to the control, Y_0), and b is the slope.

The estimated LD_{50} (%) and ED_{50} (%) were expressed, respectively, in their equivalent median lethal concentration (LC_{50}) and median effective concentration (EC_{50}) in g L^{-1} for comparison with other toxicity studies and environmental concentrations of TWP.

Statistical analyses were conducted with Sigmaplot v.12. Statistically significant differences in mortality, hatching, metamorphosis success, and length ($p \leq 0.05$) among treatments were assessed using one-way analysis of variance (ANOVA). The assumptions of normality and homogeneity of variances were tested with the Shapiro-Wilks Test and the Levene Test, respectively. When data did not follow a normal distribution or show heterogeneity of variances, the non-parametric test, Kruskal-Wallis was used. Post hoc Dunnet's or Dunn's test was used to compare the control with the experimental treatments. A non-parametric Wilcoxon signed-rank test was used to determine statistically significant differences between treatments ($p \leq 0.05$) for the biomarker data.

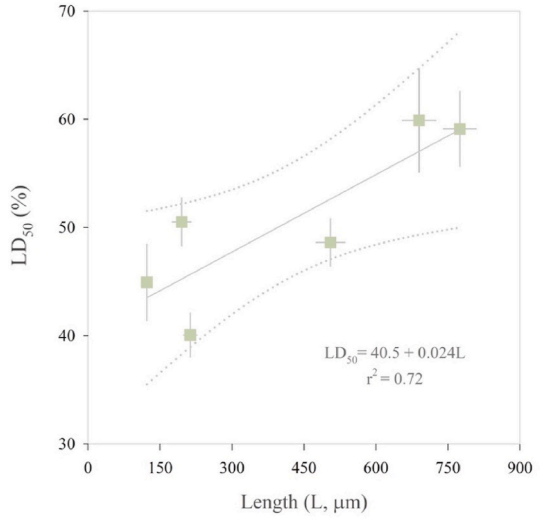

Fig. 2. Median lethal dilution (LD_{50}, %) as a function of body/prosome length for the studied life stages of *A. tonsa*. The vertical errors bars are standard error of the LD_{50} coefficients estimated with eq. (1) and the horizontal error bars the standard deviation on the length (Table 1). The continuous line is the fitted linear regression. Equation shown in the graph. The dotted curves indicate the 95% confidence bands.

4. Results

4.1. Life stage-specific lethal effect of TWP leachate in A. tonsa

Exposure to leachates for 48 h caused mortality in all the stages, with lethality increasing with increasing leachate concentration (Fig. 1). The naupliar mortality in the positive control was 49.5%, perfectly according to the criteria proposed by Gorbi et al. (2012) for standardized methods for toxicity tests with *A. tonsa*. Significant differences in mortality of early nauplii were found between the control and the 100%, 50%, 25%, leachate dilution treatments ($p < 0.05$) (Fig. 1a). In the rest of the bioassays (Fig. 2 b-f), the mortality in the two highest leachate solutions (100% and 50%; $p < 0.05$) were significantly higher than in the control. The sigmoidal model fitted well to the data ($r^2 = 0.87–0.97$; Table 2) and the estimated coefficients were statistically significant ($p < 0.05$). The estimated LD_{50} ranged from 40.1 to 59.9 % with equivalent LC_{50} of 0.401 and 0.599 g L^{-1}, respectively (Table 2). The relationship between LD_{50} and the length of studied life stages was positive but moderately correlated (Fig. 2). The LD_{50} did not exhibit a clear pattern of correlation with the size of the naupliar stages, but adults were more tolerant to TWP leachates than nauplii and copepodites (Table 2).

4.2. Effects on hatching and metamorphosis success

The hatching success (%) decreased moderately with increasing TWP leachate concentration, with a minimum hatching of 52% at the highest leachate concentration tested (100%) (Fig. 3a). Significant differences in hatching were found between the control treatment and the 25% and 100% leachate dilutions ($p < 0.05$); The EC_{50} (48h) for hatching was higher than 1 g L^{-1} (Fig. 3a, Table 3). Metamorphosis from N6 to C1 was observed in the bioassays 2 (mid nauplii) and 3 (late nauplii; Table 1) but the % of copepodites the bioassay 2 was very low (<10%) in all the treatments including the control. In the bioassay with late nauplii, metamorphosis success (%) was high in the control treatment (average: 65%) (Fig. 3b). The metamorphosis success decreased notably after exposure to increasing concentration of leachates, being completely absent at the 100 % leachate dilution (Fig. 3b). A statistically significant difference in metamorphosis success was observed between the 100%, 50% and 25% treatments and the control ($p < 0.05$) (Fig. 3b). The estimated EC_{50} for metamorphosis (48 h) was 0.28 g L^{-1} (Table 3).

Table 2
Parameters from the sigmoidal model (Eq. (1)) relating *A. tonsa* life-stage specific mortality to TWP leachate dilution (%) after 48 h of exposure (Fig.2). LD_{50}: medial lethal dilution (%), b = slope, SE: standard error, r^2: coefficient of determination, t and p values for the estimated LD_{50}; *LC_{50}: estimated median lethal concentration expressed in **g** TWP L^{-1}.

Bioassay	Stage	$LD_{50} \pm$ SE (%)	$b \pm SE$	r^2	t	p	*$LC_{50} \pm$ SE (g L^{-1})
1	N1–N2	44.9 ± 3.6	17.7 ± 2.7	0.94	6.56	<0.0001	0.449 ± 0.036
2	N4–N5	50.5 ± 2.2	19.4 ± 1.8	0.97	22.57	<0.0001	0.505 ± 0.022
3	N5–N6	40.1 ± 2.1	13.6 ± 1.6	0.96	8.48	<0.0001	0.401 ± 0.021
4	C3–C4	48.6 ± 2.2	14.2 ± 1.9	0.97	7.63	<0.0001	0.486 ± 0.022
5	Adults	59.1 ± 3.5	20.7 ± 2.5	0.94	8.25	<0.0001	0.591 ± 0.035
6	Adults	59.9 ± 4.8	31.5 ± 4.1	0.87	12.4	<0.0001	0.599 ± 0.048

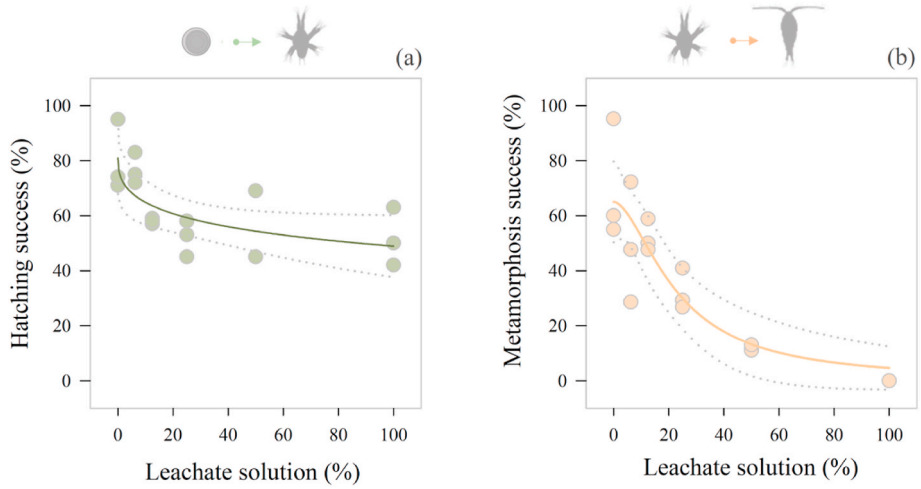

Fig. 3. Effect of TWP leachates on hatching (a) and N6–C1 metamorphosis success (b) in *A. tonsa* after 48 h of exposure. The continuous lines are the fitted curves based on Eq. (2). The dotted curves indicate the 95% confidence bands Table 3.

Table 3
Parameters from the sigmoidal logistic model (Eq. (2)) relating hatching and metamorphosis success to leachate dilutions. ED50 is the median effective dilution (%), b = slope, Y0 is the hatching or metamorphosis success (%) in the absence of leachates (i.e., in the control), SE: standard error, R^2: coefficient of determination, t and p values for the estimated ED_{50}; LC_{50}: estimated median lethal concentration expressed in g TWP L^{-1}. (*) Note that the model does not predict the ED_{50} well for hatching since the reduction in hatching did not reach 50% compared to the control; we indicate that ED_{50} is higher than the maximum tested concentration (1 g L^{-1}).

Endpoint	Stage	LD_{50} ± SE (%)	*Yo* ± SE (%)	*b* ± *SE*	r^2	*t*	*p*	LC_{50} ± SE (g L^{-1})
Hatching	Egg	273* ± 286	81 ± 6.1	0.4 ± 0.2	0.53	2.03	0.061	>1
Metamorphosis	N6–C1	22.8 ± 5.6	65 ± 6.9	1.73 ± 0.6	0.80	4.05	0.001	0.288 ± 0.056

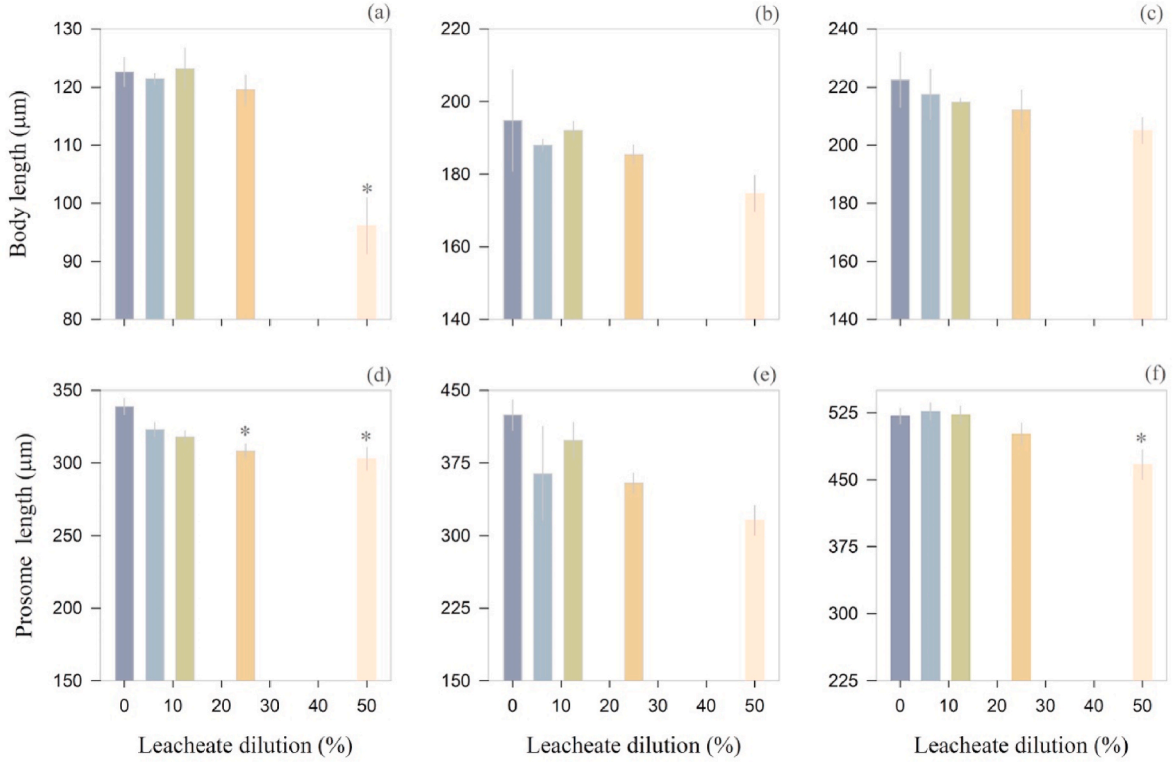

Fig. 4. Effect of TWP leachates (%) on the mean body length of nauplii (a–c) and prosome length (d–f) of copepodites after 48 h of exposure. The asterisk indicates a statistically significant difference compared to the control ($p < 0.05$). Errors bars are the standard deviations.

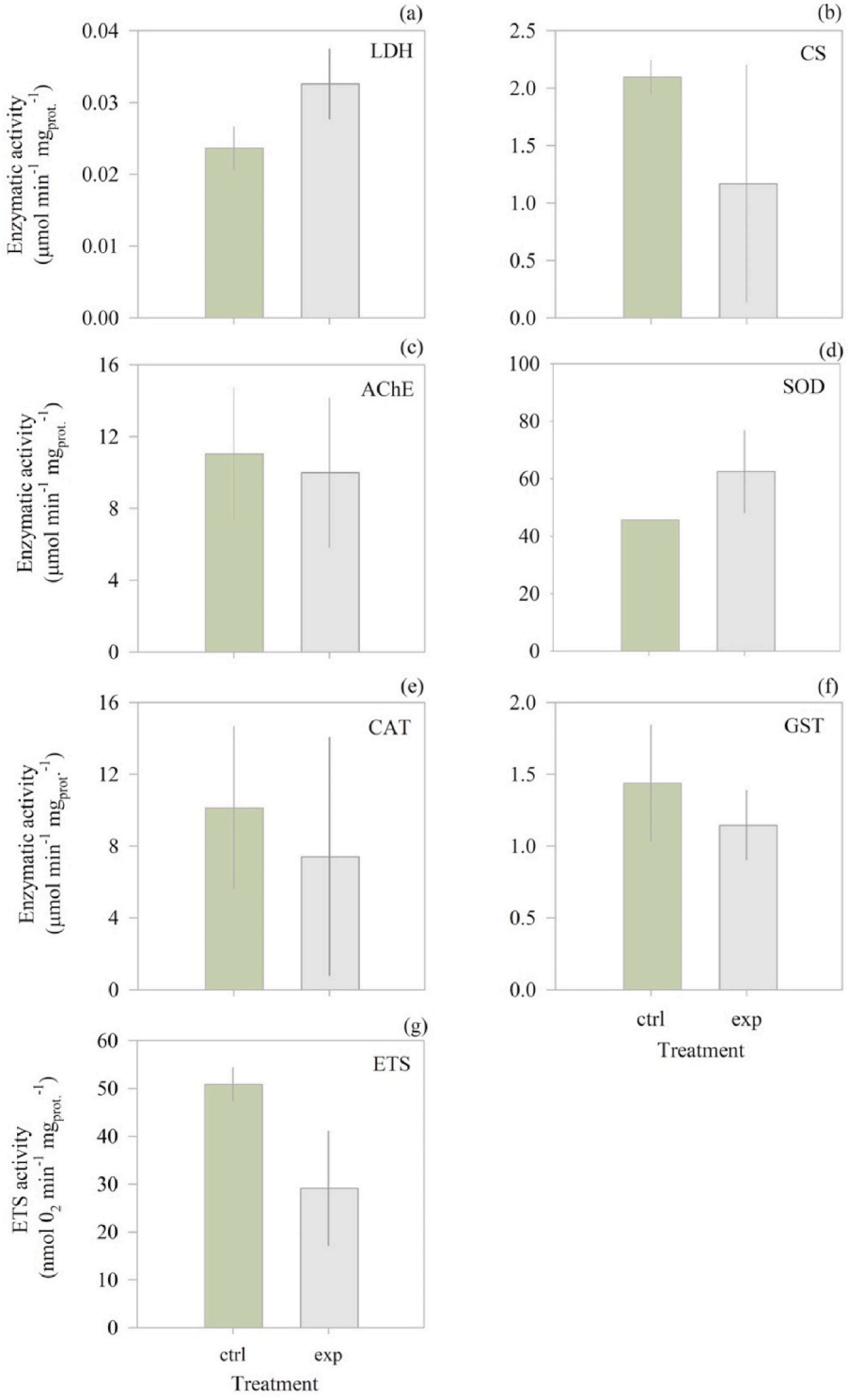

Fig. 5. Effect of TWP leachates on the enzymatic activities of *Acartia tonsa* : (a) Lactate dehydrogenase (LDH), (b) Citrate Synthase (CS), (c) Acetylcholinesterase (AChE) , (d) superoxide dismutase (SOD), (e)catalase (CAT), (f) glutathione S-transferase (GST), (g) Electron Transport System (ETS). Enzymatic activities are expressed in µmol min^{-1} mg^{-1} of protein (=IU mg^{-1} of protein) except for ETS which is expressed in nmol $O_2 min^{-1}$ mg^{-1} of protein. Ctrl = control treatment; exp = leachates from 50 mg TWP L^{-1}. Errors bars are the standard deviations.

4.3. *Effects on growth rates (body length) and enzymatic activities*

We observed a clear tendency of reduction in body size with increasing leachate concentration in nauplii and copepodites (Fig. 4). Significant differences in the mean body size of early nauplii were observed in the 50% dilution with a decrease in length of 22% compared to the control (Fig. 4a). A statistically significant decrease of approximately 10% in mean prosome size was observed for copepodites (Fig. 4d and f).

The activities of all the tested enzymes were successfully measured in *A. tonsa* adults (Fig. 5, Table S2). However, exposure to TWP leachates from a solid-to-liquid ratio of 50 mg L^{-1} did not cause any statistically

significant effect on the tested enzymatic activities ($p > 0.05$). The activity of AChE enzymewas quite similar between treatments (Fig. 5c). Some non-significant tendencies were observed; for instance, the measured activity of ETS, CS, CAT, and GST enzymes was higher in the control than in leachate treatment, whereas LDH and SOD showed the opposite trend (Fig. 5, Table S2).

5. Discussion

5.1. Lethal effect of tire particle leachates on A. tonsa life stages

Our results show that tire particle leachates negatively affect all life stages of a cosmopolitan and ecologically relevant marine planktonic copepod in estuaries and coastal areas. This is in line with other studies showing that tire rubber leachates are acutely toxic to marine plankton (Bournaka et al., 2023; Capolupo et al., 2020; Halsband et al., 2020; Page et al., 2022). Our results on the lethal effect of tire particle leachates on adult stages of *A. tonsa* ($LC_{50} = 0.591$ and $= 0.559$ g L^{-1}), Table 1) are very close to those found by Bournaka et al. (2023) for the same exposure period (LC_{50}, 48 h, $= 0.54$g L^{-1}). The toxicity of tire particle leachates on plankton varies depending on the car tire type and the leachate extraction methodology (Wik & Dave, 2006). Thus the direct comparisons of species sensitivities should be done cautiously. But, based on the limited available data, the tolerance of *A. tonsa* to rubber particle leachates seems to be lower than for other marine copepods (Bournaka et al., 2023; Halsband et al., 2020) and freshwater zooplankton (Wik & Dave, 2006). This indicates that *A. tonsa* is a sensitive species to TWPs that can be potentially used as a good bioindicator of pollution impacts on the coastal pelagic food web.

As hypothesized, early life stages were found to be more sensitive to the tire leachates than adults. Similarly, other studies have also found that than naupliar stages are more affected by pollutants than adult stages (e.g., Sunda et al. (1987), Araújo-Castro et al. (2006); Huang et al., 2006; Saiz et al., 2009), making copepod nauplii suitable as biological models for ecotoxicity testing due to their sensitivity and ecological relevance. The lower tolerance of nauplii to pollutants can be related to larger surface to body volume, which leads to a higher sorption of dissolved toxicants, and to a thinner exoskeleton compared to adults (Forget et al., 1998; Jeong et al., 2016)). Other factors can also explain the higher sensitivity of early larval stages of aquatic organisms compared with adults, such as underdeveloped homeostatic mechanism and immature detoxification pathways, and higher carbon-specific metabolic demands (Mohammed, 2013). However, the differences in sensitivity to leachates between adults ($LC_{50} = 0.59$–0.60 g L^{-1}) and the earlier developmental stages ($LC_{50} = 0.40$–49 g L^{-1}) were relatively low. This suggests that other factors and not only the surface-volume ratio and exoskeleton thickness influence the uptake of dissolved pollutants and their toxic effects on planktonic copepods. For instance, it is possible that defense/detoxification mechanisms are already present in the early stages of copepods, increasing the tolerance of nauplii to pollutants despite their smaller sizes compared to adults (Ensibi & Daly Yahia, 2017; Li et al., 2023). More research is needed to better understand the differences in sensitivity among zooplankton life stages to pollutants.

5.2. Effects of tire particle leachates on hatching, metamorphosis, and growth of A. tonsa

Data on the sublethal effects of TWP and their leachates on marine planktonic copepods are very scarce. In this study, eggs were exposed directly to the tire particle leachates and the hatching was negatively affected, but not drastically. Koski et al. (2021) did not find any effects of tire and crumb rubber particles on the hatching success of *Acartia tonsa* and *Temora longicornis* when eggs were exposed for 24 h. The high tolerance of copepod embryos, allowing hatching in polluted waters, could be related to the chitin shells of copepod eggs that offer some protection against the pollutants. This suggests that hatching success is not the most sensitive endpoint to assess the toxicity of pollutants to copepods. However, we found that once hatched, early naupliar stages lose the protective chitin shell and become vulnerable to the toxicity leachates, showing reduced survival and growth rates as hypothesized here. Several studies have reported a developmental delay in copepods and other marine invertebrates caused by different pollutants (Almeda et al., 2014, 2016; Grenvald et al., 2013; Rist et al., 2023). Since our exposure tests were conducted in the absence of food, the observed decrease in nauplii and copepodite growth after exposure to TWP leachates could be related to alterations in the mobilization of lipid reserves and altered allocation of energy. Among the studied endpoints, TWP leachates have the strongest toxicity on the metamorphosis from nauplii to copepodites. This process involved the most drastic morphological change in the life cycle of copepods, which could partly influence the high sensitivity of this endpoint to toxicants. Although the hormone regulation of metamorphosis in copepods is still not fully understood, several studies suggest that this process is controlled by the "juvenile hormones" in conjunction with the actions of ecdysteroids, as known from insects and other crustaceans (Cheong et al., 2015; Laufer et al., 1988; Rodríguez et al., 2007). Certain pollutants, including additives from conventional plastics and synthetic rubber, can cause endocrine disruption in crustaceans (Rodríguez et al., 2007). Andersen et al. (2001) found inhibition of *A. tonsa* metamorphosis (from nauplii to the copepodite stage) after exposure to endocrine disrupters, including p-octylphenol, which is used in the production of p-tert-octylphenol based resins for the manufacturing of tires and other rubber materials. It has been also reported that *Acartia tonsa* is highly sensitive to brominated flame retardants, especially PBDEs, which significantly reduced metamorphosis success. Among brominated flame retardants, BDE-99 and BDE-100, have been found to be ecdysteroid antagonists *in vitro* and can be considered endocrine disruptors in arthropods (Wollenberger et al., 2005). Additionally, it has been documented that exposure to polystyrene nanoparticles in the crustacean *Macrobrachium nipponense* caused inhibition of molting-associated and growth-related genes (Li et al., 2022). More research is needed to evaluate what chemicals present in the leachates of TWP can act as endocrine disruptors in planktonic copepods, and the molecular mechanisms behind the toxic action of synthetic rubber additives.

5.3. Effects of tire particle leachates on the enzymatic activities of A. tonsa

Enzymatic biomarkers are commonly used in benthic invertebrates (e.g., mussels) and fish to assess the effects of pollution at the biomolecular level and organisms' response to pollutants (Alberdi, 2019; Le Du-Carrée et al., 2021a; Lénia et al., 2016; Schmitz et al., 2015). Great variability exists regarding how contaminants trigger enzymatic responses in different organisms (Alberdi, 2019; Le Du-Carrée et al., 2021b). Alterations in enzymatic activities in zooplankton can serve as crucial indicators, shedding light on the repercussions of contaminants within ecosystems (Minutoli et al., 2002a; Minutoli and Fossi, 2002b). Notably, fluctuations in acetylcholine esterase (AChE) levels across various zooplankton species have been linked to the presence of environmental contaminants (Minutoli and Fossi, 2002b, Minutoli et al., 2007). Furthermore, the responsiveness of enzymes associated with redox balance in copepods has demonstrated a correlation with contamination from a diverse array of pollutants. For instance, Han et al. (2014) exposed *Tigriopus japonicus* to different concentrations of the water-accommodated fraction (WAF) of crude oil for 24h, and found that antioxidant enzymes as GST and CA, among others, increase their activity in a concentration-dependent manner. Soloperto et al. (2022) also found a significant increase in the GST activity in the copepod *Calanus finmarchicus* after 24 and 72h of exposure to WAF solution. Similarly, an increase in the GST activity and ROS level has been reported when the cyclopoid copepod *Paracyclopina nana* after 24 h of exposure to different concentrations of the pollutant 2,2′,4,

4′-tetrabromodiphenyl ether, known as BDE-47 (Lee et al., 2016). It has been also documented that the activities of the enzymes GST and SOD and *reactive oxygen species (ROS)* significantly increase when *T. japonicus* was exposed to the antimicrobial agent triclosan (TCS) for 12 and 14 h (Park et al., 2017). In our study, we successfully measured the different enzymatic activities in *A. tonsa* adults. However, we cannot provide conclusions on the effect of TWP leachates at the molecular level since we did not find a significant difference in the enzymatic activity between treatments The lack of significant differences can be explained by the relatively low leachate concentration tested, the variability among replicates, or/and the low sensitivity as a biomarker of the tested enzymatic activities for *A. tonsa*. To our knowledge, there are no studies in the literature reporting the effects of TWP leachates on the enzymatic biochemistry of copepods. More studies with longer exposure time and multiple concentrations are required to go further in the understanding of the biomolecular response of copepods to TWP leachates.

5.4. The chemical composition of tire particle leachates

Tires are commonly made of synthetic rubber polymers (mostly styrene-butadiene-rubber and butadiene rubber, also halobutyl rubber) in combination with natural rubber and high amounts of diverse additives including filling agents, vulcanization agents, flame retardants, antioxidants, and antiozonants (Page et al., 2022; Wagner et al., 2018; Wik & Dave, 2009). Heavy metals and dozens of potentially toxic organic chemicals have been detected in tire particle leachates (Capolupo et al., 2020; Foscari et al., 2023; Halsband et al., 2020; Jiang et al., 2023; Müller et al., 2022; Page et al., 2022). This indicates that the entry of TWP into aquatic ecosystems results in the release of a potent cocktail of organic additives and metals with potential effects on marine organisms. In the leachates used in this study, we found several potential toxicants such as flame retardants (e.g., Tris (2-chloroisopropyl)phosphate), polyaromatic hydrocarbons (naphthalene, pyrene) and metals (e.g., zinc) (S.I. Table 1). Several studies have indicated that Zn can be one of the main contributors to the toxicity of TWP leachates to plankton (Bournaka et al., 2023; Capolupo et al., 2020; Halsband et al., 2020; Rist et al., 2023). Also, organic compounds used as additives in tire rubber (e.g., alkylphenols like 4-tert-butylphenol and the antioxidant N-(1, 3-dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD) are known to be toxic to aquatic organisms (Gray & Metcalfe, 1999; K. Li et al., 2023). Further studies on single additive toxicity, chemical fractionation of leachates, and effect-directed analysis are needed to identify which compounds are the main ones responsible for the observed toxicity and/or the "cocktail effect" to marine planktonic organisms.

5.5. Ecological risk of tire particle pollution

Based on the current literature, concentrations ranging from less than 25 to 100,000 mg of TWP per liter of water can cause acute effects of TWP on aquatic biota, while long-term effects have been reported at concentrations ranging from 10 to 3600 mg of TWP per liter of water (Page et al., 2022; Wagner et al., 2018; Wik & Dave, 2009). It is important ant to note that TWPs and their additives undergo transformation in the environment due to physico-chemical factors. A reduction of their toxicity has been observed within a sort weathering time, while other studies have reported a considerable increase in toxicity after weathering (Tian et al., 2021, Liu et al., 2022; Fohet et al., 2023). Therefore, to fully understand the impact of TWPs it is important to consider their environmental transformation and how this affects the toxicity of their leachates (Tamis et al., 2021). More research with leachates from field collect tyre wear particles is needed to better understand the effects of TWP pollution on aquatic systems.

Field data on concentrations of TWPs are still limited; reported/predicted concentrations of TWPs in aquatic systems can range from a few milligrams per liter in the water column (up to 56 mg L^{-1}) to hundreds of grams per Kg in the sediments (155 g kg^{-1} DW) (Wagner et al., 2018; Wik & Dave, 2009). It has been estimated that the mass of tire wear discharge from surface water drainage range from 12 to 179 mg L^{-1} (Reddy and Quinn, 1997; Kumata et al., 1997, 2000; 2002; Wik and Dave, 2009; Zeng et al., 2004; Parker-Jurd et al., 2021). Although the reported concentrations of TWP in surface waters are below the median lethal and effective concentrations found here, concentrations in the sediments are much higher, and TWP on the surface of the sediments/soil can leach their toxic chemicals into the water. Additionally, long-term sub-lethal toxicity of TWP leachate could occur at lower concentrations than the acute effects investigated in this study. Therefore, coastal shallow areas and estuaries, the habitat of *A. tonsa,* situated near roads are more susceptible to run-offs, making these aquatic ecosystems more vulnerable to the ecological effects of TWP pollution.

6. Conclusions

Acute exposure to TWP leachates caused lethality in all life stages of *A. tonsa*, as well as harmful effects on critical life cycle processes, such as reduced hatching, growth rate, and metamorphosis success. Nauplii and copepodites were the most sensitive stages to tire particle leachates. The metamorphosis from nauplii to copepodites was especially affected by the exposure to tire particle leachates, suggesting endocrine disruption. We did not observe statistically significant effects on enzymatic activities at the studied exposure concentration. Although field data on tire leachates are still limited, our findings suggest that TWP pollution can have a potential negative impact on the planktonic food web in coastal areas, particularly after road runoff events and in sites affected by the drainage of urban stormwater after torrential rainfalls.

CRediT authorship contribution statement

Wilma Moreira: Formal analysis, Investigation, Writing – original draft. **Olalla Alonso:** Investigation, Writing – review & editing. **Antonio Paule:** Investigation, Writing – review & editing. **Ico Martínez:** Formal analysis, Investigation, Supervision, Writing – review & editing. **Jessy Le Du-Carreé:** Formal analysis, Investigation, Supervision, Writing – review & editing. **Rodrigo Almeda:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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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.2023.123256.

References

Alberdi, J.E., 2019. Uso de biomarcadores de contaminación en *Scyliorhinus canicula* (Linnaeus, 1758). Revisión bibliográfica.

Almeda, R., Bona, S., Foster, C., Buskey, E.J., 2014. Dispersant Corexit 9500A and chemically dispersed crude oil decreases the growth rates of meroplanktonic barnacle nauplii (*Amphibalanus improvisus*) and tornaria larvae (*Schizocardium* sp.). Marine Environ. Res. 2014, 212–217.

Almeda, R., Harvey, T.E., Connelly, T.L., Baca, S., Buskey, E.J., 2016. Influence of UVB radiation on the lethal and sublethal toxicity of dispersed crude oil to planktonic copepod nauplii. Chemosphere 152, 446–458.

Almeda, R., Gunaalan, K., Alonso-López, O., Vilas, A., Clérandeau, C., Loisel, T., Nielsen, T.G., Cachot, J., Beiras, R., 2023. A protocol for lixiviation of micronized plastics for aquatic toxicity testing. Chemosphere 333, 138894. https://doi.org/10.1016/j.chemosphere.2023.138894.

Andersen, H.R., Wollenberger, L., Halling-Sørensen, B., Kusk, K.O., 2001. Development of copepod nauplii to copepodites—A parameter for chronic toxicity including endocrine disruption. Environ. Toxicol. Chem. 20, 2821–2829.

Araújo-Castro, C.M.V., Souza-Santos, L.P., Costa, M.F., 2006. Avaliação da Toxicidade dos sedimentos dos Porto de Suape utilizando o copépodo bentônico *Tisbe biminiensis* nos períodos seco e chuvoso de 2003. Coletâneade Trabalhos Técnicos. 1ed. Recife: Editora Universitária da UFPE 1, 47–60.

Baensch-Baltruschat, B., Kocher, B., Stock, F., Reifferscheid, G., 2020. Tyre and road wear particles (TRWP) - a review of generation, properties, emissions, human health risk, ecotoxicity, and fate in the environment. Sci. Total Environ. 733, 137823 https://doi.org/10.1016/j.scitotenv.2020.137823.

Boucher, J., Friot, D., 2017. Primary Microplastics in the Oceans: A Global Evaluation of Sources. IUCN International Union for Conservation of Nature. https://doi.org/10.2305/IUCN.CH.2017.01 en.

Bournaka, E., Almeda, R., Koski, M., Page, T.S., Mejlholm, R.E.A., Nielsen, T.G., 2023. Lethal effect of leachates from tyre wear particles on marine copepods. Mar. Environ. Res. 191, 106163 https://doi.org/10.1016/j.marenvres.2023.106163.

Capolupo, M., Sørensen, L., Jayasena, K.D.R., Booth, A.M., Fabbri, E., 2000. Chemical composition and ecotoxicity of plastic and car tire rubber leachates to aquatic organisms. Water Res. 169, 115270 https://doi.org/10.1016/j.watres.2019.115270.

Castonguay, M., Plourde, S., Robert, D., Runge, J.A., Fortier, L., 2008. Copepod production drives recruitment in a marine fish. Can. J. Fish. Aquat. Sci. 65 (8), 1528–1531. https://doi.org/10.1139/F08-126.

Cheong, S.P.S., Huang, J., Bendena, W.G., Tobe, S.S., Hui, J.H.L., 2015. Evolution of Ecdysis and metamorphosis in arthropods: the rise of regulation of juvenile hormone. Integr. Comp. Biol. 55 (5), 878–890. https://doi.org/10.1093/icb/icv066.

Cózar, A., Echevarría, F., González-Gordillo, J.I., Irigoien, X., Úbeda, B., Hernández-León, S., Palma, Á.T., Navarro, S., García-de-Lomas, J., Ruiz, A., Fernández-de-Puelles, M.L., Duarte, C.M., 2014. Plastic debris in the open ocean. Proc. Natl. Acad. Sci. USA 111 (28), 10239–10244. https://doi.org/10.1073/pnas.1314705111.

Ensibi, C., Daly Yahia, M.N., 2017. Toxicity assessment of cadmium chloride on planktonic copepods Centropages ponticus using biochemical markers. Toxicol Rep 4, 83–88. https://doi.org/10.1016/j.toxrep.2017.01.005.

Evangeliou, N., Grythe, H., Klimont, Z., Heyes, C., Eckhardt, S., Lopez-Aparicio, S., Stohl, A., 2020. Atmospheric transport is a major pathway of microplastics to remote regions. Nat. Commun. 11 (1), 3381. https://doi.org/10.1038/s41467-020-17201-9.

Fohet, L, Andanson, JM, Charbouillot, T, Malosse, L, Leremboure, M, Delor-Jestin, F, Verney, V., 2023. Time-concentration profiles of tire particle additives and transformation products under natural and artificial aging. Sci Total Environ 859 (Pt 1), 160150. https://doi.org/10.1016/j.scitotenv.2022.160150.

Forget, J., Pavillon, J.F., Menasria, M.R., Bocquené, G., 1998. Mortality and LC50Values for several stages of the marine CopepodTigriopus brevicornis(müller) exposed to the metals arsenic and cadmium and the pesticides atrazine, carbofuran, dichlorvos, and malathion. Ecotoxicol. Environ. Saf. 40 (3), 239–244. https://doi.org/10.1006/eesa.1998.1686.

Foscari, A., Schmidt, N., Seiwert, B., Herzke, D., Sempéré, R., Reemtsma, T., 2023. Leaching of chemicals and DOC from tire particles under simulated marine conditions. Front. Environ. Sci. 11 https://doi.org/10.3389/fenvs.2023.1206449.

Glippa, O., Engström-Öst, J., Kanerva, M., Rein, A., Vuori, K., 2018. Oxidative stress and antioxidant defense responses in Acartia copepods in relation to environmental factors. PLoS One 13 (4), e0195981. https://doi.org/10.1371/journal.pone.0195981.

Gonçalves, A.M.M., Rocha, C.P., Marques, J.C., Gonçalves, F.J.M., 2021. Enzymes as useful biomarkers to assess the response of freshwater communities to pesticide exposure – a review. Ecol. Indicat. 122, 107303 https://doi.org/10.1016/j.ecolind.2020.107303.

Gorbi, G., Invidia, M., Savorelli, F., Faraponova, O., Giacco, E., Cigar, M., Buttino, I., Leoni, T., Prato, E., Lacchetti, I., Sei, S., 2012. Standardized methods for acute and semichronic toxicity tests with the copepod *Acartia tonsa*. Environ. Toxicol. Chem. 31 (9), 2023–2028. https://doi.org/10.1002/etc.1909.

Gray, M.A., Metcalfe, C.D., 1999. Toxicity of 4-tert-octylphenol to early life stages of Japanese medaka (*Oryzias latipes*). Aquat. Toxicol. 46 (2), 149–154. https://doi.org/10.1016/S0166-445X(98)00126-X.

Grenvald, J.C., Nielsen, T.G., Hjorth, M., 2013. Effects of pyrene exposure and temperature on early development of two co-existing Arctic copepods. Ecotoxicology 22 (1), 184–198. https://doi.org/10.1007/s10646-012-1016-y.

Gunaalan, K., Fabbri, E., Capolupo, M., 2020. The hidden threat of plastic leachates: a critical review on their impacts on aquatic organisms. Water Res. 184, 116170 https://doi.org/10.1016/j.watres.2020.116170.

Halle, L.L., Palmqvist, A., Kampmann, K., Jensen, A., Hansen, T., Khan, F.R., 2021. Tire wear particle and leachate exposures from a pristine and road-worn tire to *Hyalella azteca*: comparison of chemical content and biological effects. Aquat. Toxicol. 232, 105769 https://doi.org/10.1016/j.aquatox.2021.105769.

Halsband, C., Sørensen, L., Booth, A.M., Herzke, D., 2020. Car tire crumb rubber: does leaching produce a toxic chemical cocktail in coastal marine systems? Front. Environ. Sci. 8 https://doi.org/10.3389/fenvs.2020.00125.

Hansen, J., 1989. The red tide dinoflagellate *Alexandrium tamarense*: effects on behaviour and growth of a tintinnid ciliate. In MARINE ECOLOGY PROGRESS SERIES Mar. Ecol. Prog. Ser 53.

Han, J., Won, E.J., Hwang, D.S., Shin, K.H., Lee, Y.S., Leung, K.M., Lee, S.J., Lee, J.S., 2014 Jul. Crude oil exposure results in oxidative stress-mediated dysfunctional development and reproduction in the copepod Tigriopus japonicus and modulates expression of cytochrome P450 (CYP) genes. Aquat Toxicol. 152, 308–317. https://doi.org/10.1016/j.aquatox.2014.04.027. Epub 2014 May 9. PMID: 24813263.

Huang, Y., Zhu, L., Liu, G., 2006. The effects of bis(tributyltin) oxide on the development, reproduction and sex ratio of calanoid copepod *Pseudodiaptomus marinus*. Estuarine. Coastal and Shelf Science 69 (1–2), 147–152. https://doi.org/10.1016/j.ecss.2006.04.010.

Humes, A.G., 1994. How many copepods? Hydrobiologia 292–293 (1), 1–7. https://doi.org/10.1007/BF00229916.

ISO, 1999. Water quality — determination of acute lethal toxicity to marine copepods (Copepoda, Crustacea). International standard 14669.

Isobe, A., Azuma, T., Cordova, M.R., Cózar, A., Galgani, F., Hagita, R., Kanhai, L.D., Imai, K., Iwasaki, S., Kako, S., Kozlovskii, N., Lusher, A.L., Mason, S.A., Michida, Y., Mituhasi, T., Morii, Y., Mukai, T., Popova, A., Shimizu, K., et al., 2021. A multilevel dataset of microplastic abundance in the world's upper ocean and the Laurentian Great Lakes. Microplastics and Nanoplastics 1 (1), 16. https://doi.org/10.1186/s43591-021-00013-z.

Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., Narayan, R., Law, K.L., 2015. Plastic waste inputs from land into the ocean. Science 347 (6223), 768–771. https://doi.org/10.1126/science.1260352.

Jeong, C.-B., Won, E.-J., Kang, H.-M., Lee, M.-C., Hwang, D.-S., Hwang, U.-K., Zhou, B., Souissi, S., Lee, S.-J., Lee, J.-S., 2016. Microplastic size-dependent toxicity, oxidative stress induction, and p-JNK and p-p38 activation in the monogonont rotifer (*Brachionus koreanus*). Environ. Sci. Technol. 50 (16), 8849–8857. https://doi.org/10.1021/acs.est.6b01441.

Jiang, J.-R., Chen, Z.-F., Liao, X.-L., Liu, Q.-Y., Zhou, J.-M., Ou, S.-P., Cai, Z., 2023. Identifying potential toxic organic substances in leachates from tire wear particles and their mechanisms of toxicity to Scenedesmus obliquus. J. Hazard Mater. 458, 132022 https://doi.org/10.1016/j.jhazmat.2023.132022.

Khan, F.R., Halle, L.L., Palmqvist, A., 2019. Acute and long-term toxicity of micronized car tire wear particles to Hyalella azteca. Aquat. Toxicol. 213, 105216 https://doi.org/10.1016/J.AQUATOX.2019.05.018.

Kole, P.J., Löhr, A.J., Van Belleghem, F., Ragas, A., 2017. Wear and tear of tyres: a stealthy source of microplastics in the environment. Int. J. Environ. Res. Publ. Health 14 (10), 1265. https://doi.org/10.3390/ijerph14101265.

Koski, M., Søndergaard, J., Christensen, A.M., Nielsen, T.G., 2021. Effect of environmentally relevant concentrations of potentially toxic microplastic on coastal copepods. Aquat. Toxicol. 230, 105713 https://doi.org/10.1016/j.aquatox.2020.105713.

Kreider, M.L., Panko, J.M., McAtee, B.L., Sweet, L.I., Finley, B.L., 2010. Physical and chemical characterization of tire-related particles: comparison of particles generated using different methodologies. Sci. Total Environ. 408 (3), 652–659. https://doi.org/10.1016/j.scitotenv.2009.10.016.

Kumata, H., Takad, H., Ogura, N., 1997. 2-(4-Morpholinyl)benzothiazole as an Indicator of Tire-Wear Particles and Road Dust in the Urban Environment, pp. 291–305. https://doi.org/10.1021/bk-1997-0671.ch019.

Kumata, H., Sanada, Y., Takada, H., Ueno, T., 2000. Historical trends of N -Cyclohexyl-2-benzothiazolamine, 2-(4-morpholinyl)benzothiazole, and other anthropogenic contaminants in the urban reservoir sediment core. Environ. Sci. Technol. 34 (2), 246–253. https://doi.org/10.1021/es990738k.

Kumata, H., Yamada, J., Masuda, K., Takada, H., Sato, Y., Sakurai, T., Fujiwara, K., 2002. Benzothiazolamines as tire-derived molecular markers: sorptive behavior in street runoff and application to source apportioning. Environ. Sci. Technol. 36 (4), 702–708. https://doi.org/10.1021/es0155229.

Last, J.M., 1980. The food of twenty species of fish larvae in the west-central North Sea. Fisheries Research Technical Report. Ministry of Agriculture, Fisheries and Food, Directorate of Fisheries Research 60, 1–44. Lowestoft.

Laufer, H., Borst, D.W., Foley, T.A., Landau, M., 1988. Ecdysteroid titres in vitellogenic Libinia emarginata. J. Insect Physiol. 34 (7), 615–617. https://doi.org/10.1016/0022-1910(88)90068-6.

Lee MC, Puthumana J, Lee SH, Kang HM, Park JC, Jeong CB, Han J, Hwang DS, Seo JS, Park HG, Om AS, Lee JS. BDE-47 induces oxidative stress, activates MAPK signaling pathway, and elevates de novo lipogenesis in the copepod Paracyclopina nana. Aquat Toxicol. 2016 Dec;181:104-112. doi: 10.1016/j.aquatox.2016.10.025. Epub 2016 Oct 25. PMID: 27825063.

Le Du-Carrée, J., Cabon, J., Morin, T., Danion, M., 2021a. Immunological and metabolic effects of acute sublethal exposure to glyphosate or glyphosate-based herbicides on

juvenile rainbow trout, Oncorhynchus mykiss. Sci. Total Environ. 784 https://doi.org/10.1016/j.scitotenv.2021.147162.
Le Du-Carrée, J., Morin, T., Danion, M., 2021b. Impact of chronic exposure of rainbow trout, *Oncorhynchus mykiss*, to low doses of glyphosate or glyphosate-based herbicides. Aquat. Toxicol. 230 https://doi.org/10.1016/j.aquatox.2020.105687.
Lénia, R., Sara, N., Marco, L., Luís, A., Sergio, L., 2016. European lobster larvae (*Homarus gammarus*) under an acidification scenario: addressing biochemical, development and behaviour responses. Front. Mar. Sci. 3 https://doi.org/10.3389/conf.FMARS.2016.04.00004.
Li, Y., Liu, Z., Jiang, Q., Ye, Y., Zhao, Y., 2022. Effects of nanoplastic on cell apoptosis and ion regulation in the gills of Macrobrachium nipponense. Environ. Pollut. 300, 118989 https://doi.org/10.1016/j.envpol.2022.118989.
Li, K., Su, H., Xiu, X., Liu, C., Hao, W., 2023. Tire wear particles in different water environments: occurrence, behavior, and biological effects—a review and perspectives. Environ. Sci. Pollut. Control Ser. 30 (39), 90574–90594. https://doi.org/10.1007/s11356-023-28899-7.
Z. Liu, Y. Sun, J. Wang, J. Li, H. JiaIn vitro assessment reveals the effects of environmentally persistent free radicals on the toxicity of photoaged tire wear particlesEnviron. Sci. Technol., 56 (2022), pp. 1664-1674.
Lithner, D., Nordensvan, I., Dave, G., 2012. Comparative acute toxicity of leachates from plastic products made of polypropylene, polyethylene, PVC, acrylonitrile–butadiene–styrene, and epoxy to *Daphnia magna*. Environ. Sci. Pollut. Control Ser. 19 (5), 1763–1772. https://doi.org/10.1007/s11356-011-0663-5.
Ly, A., Sayegh, Z. El, 2023. Effect of truck tyre tread design on tyre-terrain interaction using advanced computational techniques. Int. J. Heavy Veh. Syst. 30 (3), 365–379. https://doi.org/10.1504/IJHVS.2023.132995.
Marchus, N.H., Wilcox, J.A., 2007. Guide to the Meso-Scale Production of the Copepod *Acartia Tonsa* Item Type Monograph. http://hdl.handle.net/1834/20023.
Mauchline, J. (John), 1998. The Biology of Calanoid Copepods. Academic Press.
Minutoli, R., Fossi, M.C., Guglielmo, L., 2002a. Potential use of biomarkers in zooplankton as early warning signals of ecotoxicological risk in the marine food chain. Mar. Ecol. 23, 291–296.
Minutoli, R., Fossi*, M.C., 2002b. Evaluation of acetylcholinesterase activity in several zooplanktonic crustaceans. Marine Environmental Research 54, 799–804.
R. Minutoli , M. C. Fossi , A. Granata , S. Casini & L. Guglielmo (2007).Use of biomarkers in zooplankton for assessment of the 'health status' of marine and brackish environments: a short overview, Chemistry and Ecology, 23:6, 471-477.
Mohammed, Azad., 2013. Why Are Early Life Stages of Aquatic Organisms More Sensitive to Toxicants than Adults?' New Insights into Toxicity and Drug Testing. InTech. https://doi.org/10.5772/55187.
Müller, K., Hübner, D., Huppertsberg, S., Knepper, T.P., Zahn, D., 2022. Probing the chemical complexity of tires: identification of potential tire-borne water contaminants with high-resolution mass spectrometry. Sci. Total Environ. 802, 149799 https://doi.org/10.1016/j.scitotenv.2021.149799.
Owens, T.G., King, F.D., 1975. The measurement of respiratory electron-transport-system activity in marine zooplankton. Mar. Biol. 30 (1), 27–36. https://doi.org/10.1007/BF00393750.
Page, T.S., Almeda, R., Koski, M., Bournaka, E., Nielsen, T.G., 2022. Toxicity of tyre wear particle leachates to marine phytoplankton. Aquat. Toxicol. 252, 106299 https://doi.org/10.1016/j.aquatox.2022.106299.
Paluselli, A., Kim, S.-K., 2020. Horizontal and vertical distribution of phthalates acid ester (PAEs) in seawater and sediment of East China Sea and Korean South Sea: traces of plastic debris? Mar. Pollut. Bull. 151, 110831 https://doi.org/10.1016/j.marpolbul.2019.110831.
Park JC, Han J, Lee MC, Seo JS, Lee JS. Effects of triclosan (TCS) on fecundity, the antioxidant system, and oxidative stress-mediated gene expression in the copepod Tigriopus japonicus. Aquat Toxicol. 2017 Aug;189:16-24. doi: 10.1016/j.aquatox.2017.05.012. Epub 2017 May 26. PMID: 28575748.
Parker-Jurd, F.N.F., Napper, I.E., Abbott, G.D., Hann, S., Thompson, R.C., 2021. Quantifying the release of tyre wear particles to the marine environment via multiple pathways. Mar. Pollut. Bull. 172, 112897 https://doi.org/10.1016/j.marpolbul.2021.112897.
Reddy, C.M., Quinn, J.G., 1997. Environmental chemistry of benzothiazoles derived from rubber. Environ. Sci. Technol. 31 (10), 2847–2853. https://doi.org/10.1021/es970078o.
Regoli, F., Giuliani, M.E., 2014. Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Mar. Environ. Res. 93, 106–117. https://doi.org/10.1016/j.marenvres.2013.07.006.
Rist, S., Le Du-Carrée, J., Ugwu, K., Intermite, C., Acosta-Dacal, A., Pérez-Luzardo, O., Zumbado, M., Gómez, M., Almeda, R., 2023. Toxicity of tire particle leachates on early life stages of keystone sea urchin species. Environ. Pollut. 336, 122453 https://doi.org/10.1016/j.envpol.2023.122453.
Rødland, E.S., Lind, O.C., Reid, M.J., Heier, L.S., Okoffo, E.D., Rauert, C., Thomas, K.V., Meland, S., 2022. Occurrence of tire and road wear particles in urban and peri-urban snowbanks, and their potential environmental implications. Sci. Total Environ. 824, 153785 https://doi.org/10.1016/j.scitotenv.2022.153785.
Rodríguez, E.M., Medesani, D.A., Fingerman, M., 2007. Endocrine disruption in crustaceans due to pollutants: a review. Comp. Biochem. Physiol. Mol. Integr. Physiol. 146 (4), 661–671. https://doi.org/10.1016/j.cbpa.2006.04.030.
Saiz, E., Movilla, J., Yebra, L., Barata, C., Calbet, A., 2009. Lethal and sublethal effects of naphthalene and 1,2-dimethylnaphthalene on naupliar and adult stages of the marine cyclopoid copepod *Oithona davisae*. Environ. Pollut. 157 (4), 1219–1226. https://doi.org/10.1016/j.envpol.2008.12.011.
Schmitz, H.A., Maher, W.A., Taylor, A.M., Krikowa, F., 2015. Effects of cadmium accumulation from suspended sediments and phytoplankton on the Oyster *Saccostrea glomerata*. Aquat. Toxicol. 160, 22–30. https://doi.org/10.1016/j.aquatox.2014.12.019.
Sheng, Y., Liu, Y., Wang, K., Cizdziel, J.V., Wu, Y., Zhou, Y., 2021. Ecotoxicological effects of micronized car tire wear particles and their heavy metals on the earthworm (*Eisenia fetida*) in soil. Sci. Total Environ. 793, 148613 https://doi.org/10.1016/J.SCITOTENV.2021.148613.
Soloperto, S., Altin, D., Hallmann, A., Skottene, E., Hansen, B.H., Jenssen, B.M., Ciesielski, T.M., 2022. Oil-mediated oxidative-stress responses in a keystone zooplanktonic species, Calanus finmarchicus. Sci. Total Environ. 806, 151365.
Sunda, W.G., Tester, P.A., Huntsman, S.A., 1987. Effects of cupric and zinc ion activities on the survival and reproduction of marine copepods. Mar. Biol. 94 (2), 203–210. https://doi.org/10.1007/BF00392932.
Tamis, J.E., Koelmans, A.A., Dröge, R., Kaag, N.H.B.M., Keur, M.C., Tromp, P.C., Jongbloed, R.H., 2021. Environmental risks of car tire microplastic particles and other road runoff pollutants. Microplastics and Nanoplastics 1 (1), 10.
Tian, Z., Zhao, H., Peter, K.T., Gonzalez, M., Wetzel, J., Wu, C., Hu, X., Prat, J., Mudrock, E., Hettinger, R., Cortina, A.E., Biswas, R.G., Kock, F.V.C., Soong, R., Jenne, A., Du, B., Hou, F., He, H., Lundeen, R., et al., 2021. A ubiquitous tire rubber–derived chemical induces acute mortality in coho salmon. Science 371 (6525), 185–189. https://doi.org/10.1126/science.abd6951.
Turner, A., Rice, L., 2010. Toxicity of tire wear particle leachate to the marine macroalga, *Ulva lactuca*. Environ. Pollut. 158 (12), 3650–3654. https://doi.org/10.1016/j.envpol.2010.08.001.
Verity, P., Smetacek, V., 1996. Organism life cycles, predation, and the structure of marine pelagic ecosystems. Mar. Ecol. Prog. Ser. 130, 277–293. https://doi.org/10.3354/meps130277.
Villarroel, M.J., Sancho, E., Andreu-Moliner, E., Ferrando, M.D., 2009. Biochemical stress response in tetradifon exposed *Daphnia magna* and its relationship to individual growth and reproduction. Sci. Total Environ. 407 (21), 5537–5542. https://doi.org/10.1016/j.scitotenv.2009.06.032.
Wagner, S., Hüffer, T., Klöckner, P., Wehrhahn, M., Hofmann, T., Reemtsma, T., 2018. Tire wear particles in the aquatic environment - a review on generation, analysis, occurrence, fate and effects. Water Res. 139, 83–100. https://doi.org/10.1016/j.watres.2018.03.051.
Wik, A., Dave, G., 2006. Acute toxicity of leachates of tire wear material to *Daphnia magna*—variability and toxic components. Chemosphere 64 (10), 1777–1784. https://doi.org/10.1016/j.chemosphere.2005.12.045.
Wik, A., Dave, G., 2009. Occurrence and effects of tire wear particles in the environment – a critical review and an initial risk assessment. Environ. Pollut. 157 (1), 1–11. https://doi.org/10.1016/j.envpol.2008.09.028.
Wollenberger, L., Dinan, L., Breitholtz, M., 2005. Brominated flame retardants: activities in a crustacean development test and in an ecdysteroid screening assay. Environ. Toxicol. Chem. 24 (2), 400–407. https://doi.org/10.1897/03-629.1.
Yang, K., Jing, S., Liu, Y., Zhou, H., Liu, Y., Yan, M., Yi, X., Liu, R., 2022. Acute toxicity of tire wear particles, leachates and toxicity identification evaluation of leachates to the marine copepod, *Tigriopus japonicus*. Chemosphere 297, 134099. https://doi.org/10.1016/j.chemosphere.2022.134099.
Zeng, E.Y., Tran, K., Young, D., 2004. Evaluation of potential molecular markers for urban stormwater runoff. Environ. Monit. Assess. 90 (1–3), 23–43. https://doi.org/10.1023/B:EMAS.0000003564.24169.86.