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Cocktail effects of tire wear particles leachates on diverse biological models: A multilevel analysis

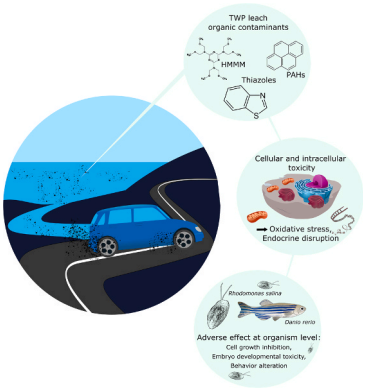
Jessy Le Du-Carrée [a,*], Clara Kempkens Palacios [b], Anna Rotander [b], Maria Larsson [b], Andi Alijagic [b,c,d], Oleksandr Kotlyar [b,e], Magnus Engwall [b], Viktor Sjöberg [b], Steffen H. Keiter [b], Rodrigo Almeda [a]

[a] University of Las Palmas de Gran Canaria: Las Palmas de Gran Canaria, Spain
[b] Man-Technology-Environment Research Center (MTM), Biology, Örebro University, SE-701 82 Örebro, Sweden
[c] Inflammatory Response and Infection Susceptibility Centre (iRiSC), Faculty of Medicine and Health, Örebro University, SE-701 82 Örebro, Sweden
[d] Faculty of Medicine and Health, School of Medical Sciences, Örebro University, SE-701 82 Örebro, Sweden
[e] Centre for Applied Autonomous Sensor Systems (AASS), Mobile Robotics and Olfaction Lab (MRO), Örebro University, SE-701 82 Örebro, Sweden

HIGHLIGHTS

- TWP leachate cocktail toxicity examined across organizational levels.
- Organic compounds like PAHs and thiazoles leach into seawater from TWP.
- Effects on organisms and cells: Altered algae growth, fish embryo development, cytotoxicity.
- Toxic mechanisms imply oxidative stress and endocrine disruption.
- Emphasizes the need for further studies on toxicity drivers.

GRAPHICAL ABSTRACT



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ABSTRACT

Tire wear particles (TWP) stand out as a major contributor to microplastic pollution, yet their environmental impact remains inadequately understood. This study delves into the cocktail effects of TWP leachates, employing molecular, cellular, and organismal assessments on diverse biological models. Extracted in artificial seawater and analyzed for metals and organic compounds, TWP leachates revealed the presence of polyaromatic hydrocarbons and 4-tert-octylphenol. Exposure to TWP leachates (1.5 to 1000 mg peq L^{-1}) inhibited algae growth and induced zebrafish embryotoxicity, pigment alterations, and behavioral changes. Cell painting uncovered pro-apoptotic changes, while mechanism-specific gene-reporter assays highlighted endocrine-disrupting potential, particularly antiandrogenic effects. Although heavy metals like zinc have been suggested as major players in TWP leachate toxicity, this study emphasizes water-leachable organic compounds as the primary causative agents of

* Corresponding author.
E-mail address: jessy.leducarree@ulpgc.es (J. Le Du-Carrée).

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observed acute toxicity. The findings underscore the need to reduce TWP pollution in aquatic systems and enhance regulations governing highly toxic tire additives.

1. Introduction

In recent years, plastic additives have been recognized as one of the major threats resulting from plastic pollution in humans and ecosystems [37,87]. However, risk assessments associated with these contaminants are still in their early stages, hindered by the high numbers of compounds used as additives in the plastic industry and their diverse chemical nature [23]. Among plastic pollution sources, road-generated tire rubber microparticles (tire wear particles, TWP) are now considered a global emerging concern. Tire wear particles (TWP) emitted by car traffic tend to accumulate near roads, and their primary pathway to enter marine environments includes water treatment systems effluent, surface runoff, and atmospheric fallout. In the EU, TWP enters surface water at a rate estimated at 1.3 t a^{-1}, with the majority being retained in freshwater systems. However, up to around 15% could be exported to marine systems [99,63,74], and coastal areas situated near accumulation sites are particularly susceptible to acute TWP pollution following events such as stormwater runoff. Moreover, chemical compounds leach from these particles, contaminating surrounding water. Molecular markers like p-phenylenediamine quinones (PPD-Qs) have been consistently found in both freshwater and marine environments, indicating contamination [109]. Therefore, it is imperative to improve the toxic evaluation of TWP leachates in both freshwater and marine model species. Previous studies have highlighted adverse effects caused by additives at environmentally relevant concentrations [91], yet knowledge gaps persist regarding the various exposure modes and mechanisms of action contributing to these effects [6,34].

The evaluation of the risk associated with TWP is intricate due to the varied additive formulations used. The leaching of TWP yields complex mixtures containing bioactive chemical compounds, each with unique physio-chemical properties and interactions, culminating in a cocktail effect on exposed organisms. However, as highlighted by Kim et al. [47], a significant proportion of studies investigating the effects of TWP on organisms lack chemical characterization of the product. Most studies that conducted chemical analyses primarily targeted zinc as a potential causative agent of toxicity. Recent research by Tamis et al. [91] identified 35 organic micropollutants and 22 polycyclic aromatic hydrocarbons (PAHs) released from tires into the environment, with particular concern raised for Benzo(a)pyrene, Fluoranthene, and Mercaptobenzothiazole due to their environmental toxicity. Other studies employing organism testing have identified various candidate compounds responsible for induced toxic effects, including zinc, 4-methylaniline, benzothiazole, naphthalene, and arylamines [15,18,73]. It is imperative to consider the contributions of a wide array of organic compounds beyond heavy metals or PAHs [24]. For instance, para-phenylenediamines (PDs), ubiquitous tire additives, have demonstrated toxic impacts on fish due to their bioavailability [38]. Additionally, the transformation product of a specific PD, the 6PPD-quinone, has been demonstrated responsible for decades of salmon mortality events associated with stormwater runoffs [93]. However, the recent work of Dudefoi et al. [28] shows that the effect of the 6PPD-quinone might be species-specific, and depending on the considered model, toxic interactions of several compounds, including some cited above, have to be taken into account.

Due to the complexity of the chemical mixtures arising from TWP leachates, it remains challenging to attribute their toxicity to specific mechanisms of action. The latter may vary depending on the organism, the composition of the mixture, including the nature and concentration of leachates, and their degree of weathering. Furthermore, considering the specific physiology and sensitivity associated with various life stages is essential to unveil the molecular mechanisms underlying the toxicity of these pollutants. For instance, car tires have been found to contain plasticizers known for their endocrine-disrupting properties [5]. TWP have been demonstrated to inhibit algae growth, reduce the survival of copepods, and significantly affect mussel and sea urchin embryonic development to a greater extent than conventional plastics [15]. Organisms with simpler physiologies may primarily be affected by processes such as oxidative stress, as demonstrated by Shin et al. [85] across several levels of biological organization on a rotifer species exposed to TWP. More complex organisms could be impacted by compounds exhibiting bio-activity even at low concentrations through specific mechanisms of action, as demonstrated in a study on zebrafish employing a top-down approach (i.e., impact on the individual to molecular mechanisms; [17]). Furthermore, the risk of TWP for human health has been primarily assessed regarding genotoxicity or inhalation exposure, with limited studies available. Controversial results highlight the need for further clarification. Additionally, the potential risks associated with TWP exposure through the food chain remain unknown [6,51].

The present study aims to evaluate the impact of leachates from TWPs on a diverse range of biological models and endpoints. *In vivo* toxicity tests included a growth inhibition bioassay using microalgae (*Rhodomonas salina*) and embryotoxicity and behavioral tests with zebrafish (*Danio rerio*). At the cellular level, in vitro tests using various cell lines measured hormonal and aryl hydrocarbon (Ah) receptor-mediated responses and changes in the morphological phenotypes as early markers of exposure. Specifically, the Cell painting technique assessed the effects on different subcellular compartments, while reporter gene assays were used to examine Ah receptor, estrogenic, and anti-androgenic activities induced by the exposure to leachates. This comprehensive study, including novel approaches, will enhance the understanding of toxicity associated with TWP pollution across environments and species.

2. Materials and methods

2.1. General experimental design

The experimental design of the present study is illustrated in Figure 1, and the specific details are provided in the following subsections. Briefly, we first obtained leachates of TWPs in artificial seawater (ASW-TWP). Second, chemicals in the leachate were concentrated by solid phase extraction (SPE). Later, SPE extracts underwent a series of biotests to evaluate their toxicity. The identification of organic compounds within the extracts was performed using gas chromatography-mass spectrometry (GC-MS). Additionally, metals in both water and SPE extracts were detected using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

2.2. Leachate preparation

Tire wear particles (TWP) were obtained by micronizing an unused car tire, specifically, the Imperial 145/70–13 71 T-Snowdragon HP model. The process to obtain the leachate from microplastic for aquatic toxicity testing followed the protocol described by Almeda et al. [4].

In brief, the outer layer of the tire was initially cut into small pieces using a stainless-steel knife and subsequently micronized using a pneumatic milling cutter. The resulting particles were then subjected to dry-sieving using ISO-certified stainless-steel sieves to isolate size fractions smaller than 250 μm.

To conduct the leaching process, artificial seawater (ASW) with a salinity of 25.2% was utilized as the leaching medium. The artificial

seawater recipe includes the following components with their respective amounts (g/l): NaF (0.003), $SrCl_2 \cdot 6\ H_2O$ (0.024), $Na_2B_4O_7 \cdot 10\ H_2O$ (0.0475), KBr (0.100), KCl (0.700), $CaCl_2 \cdot 2\ H_2O$ (1.47), Na_2SO_4 (4.00), $MgCl_2 \cdot 6\ H_2O$ (10.78), NaCl (24.50), and $NaHCO_3$ (0.200). The particles were incubated in glass bottles, each containing a concentration of 1 g peq L^{-1} (1 g of plastic equivalent L^{-1}) while being placed on a rotary wheel (Heidolph Schwabach, Germany) rotating at 20 rpm for a duration of 72 h at 21 ˚C.

Upon completion of the leaching process, the particles were separated from the leachate solutions using Whatman® glass microfiber filters with a pore size of 0.7 μm (VWR Stockholm, Sweden) connected to a vacuum filtration unit. All the equipment used in this step was meticulously rinsed in the following order: ethanol, n-hexane, and dichloromethane, in that order to prevent potential chemical contamination. The obtained leachate solution will be referred to as the "ASW-TWP extract."

The leachates were subsequently subjected to solid-phase extraction (SPE) using solid-phase extraction glass cartridges (Waters®, Sweden, Oasis HLB 5 cm^3, 200 mg of sorbent per cartridge, 60 μm) in order to concentrate the organic chemical compounds. Before loading the leachates, the cartridges were washed with 10 mL of hexane, 10 mL of dichloromethane, 10 mL of methanol, and 15 mL of ultrapure water (LC-MS grade). The leachates were extracted at a flow rate of approximately 2 drops s^{-1}.

Finally, the sorbents were washed with 5 mL of ultrapure water and dried using vacuum suction for 30 min. The compounds trapped in the sorbents were subsequently eluted with 10 mL of dichloromethane: hexane (1:1) and 10 mL of dichloromethane:acetone (1:1). Solvents in the extracts were then evaporated under a gentle stream of nitrogen, yielding approximately 1 mL of hexane. A portion of this solution was dedicated to chemical analysis and spiked with an internal standard solution, PAH-mix 9, containing deuterated 16 US EPA PAHs (97.1–98.8%, Labor Dr. Ehrenstrofer-Schäfers, Augsburg, Germany). Extracts were evaporated into 100 μl of toluene, and D12-perylene (Sigma-Aldrich, Stockholm, Sweden) was added as recovery standard. The other portion, intended for biotests, was solvent exchanged to 25 μL of dimethyl sulfoxide (DMSO) using a gentle stream of nitrogen gas. These two portions will be referred to as the "SPE-TWP extract." Both of them were subsequently stored at − 20˚C. The same procedure was performed using only ASW as a procedure blank. The extract obtained from this procedure blank will be designated as the "SPE-ASW extract." The unit of concentration employed to characterize this leachate is μg peq L^{-1}. The expression "of plastic equivalent" (peq) describes the quantity of particle-free leachate extracted from a specified amount of particles. This unit provides a standardized measure that equates the leachate concentration to its equivalent amount of plastic.

2.3. Chemical analysis

To determine the organic chemical composition of SPE-ASW and SPE-TWP extracts, the analysis was conducted using a Gas Chromatograph (GC) coupled with an Orbitrap Mass Spectrometer (Q Exactive GC Orbitrap, Thermo Scientific, Bremen, Germany) equipped with a DB5-MS column (length 30 m, inner diameter 0.25 μm, stationary phase 0.25 μm). These analyses, including the suspect screening analyses, followed the procedure described by Dubocq and Wang [27]. In short, suspect screening was performed using the HRMS spectral library published by Price et al. [76] combined with an in-house library using the parameters: Retention index tolerance (50), *m/z* tolerance (0.01 Da) and EI similarity cut-off (70%) [76]. Representative spectra from the aligned peak list were also exported in msp format and matched against the NIST14 library. To assign confidence to the data, the system proposed by Koelmel et al. [50] was used. Here, level 1 implies confirmed identification (retention time, EI spectra, and reference masses) using in-house library, level 2: Probable structure or close isomer using external libraries (RI match, [molecular ion], and EI match to exact mass library or including metrics incorporating accurate mass information), level 3: Tentative candidate (EI accurate mass spectral match or EI match with metrics incorporating accurate mass) using external library; alternatively, RI match with accurate mass fragment matches.

Inductively coupled plasma mass spectrometry (ICP-MS Agilent 7500cx) was employed to determine the metal composition, following the procedure described by Zeiner et al. [108]. Before ICP-MS analysis, liquid-liquid extraction was performed on SPE-ASW and SPE-TWP extracts to resolubilize metals in ASW.

2.4. In vivo toxicity tests

2.4.1. Bioassay with marine microalgae (R. salina)

The acute toxicity of leachate of TWPs, after both water and SPE extraction, was examined using the microalgal model [92], *R. salina*. This marine microalga belongs to the phylum Cryptophyta [72] and is a suitable model for studying primary producers with significant ecological importance [2]. Previous studies have evidenced its sensitivity to environmental contaminants [36]. Experiments were conducted using a non-axenic monoculture of *R. salina* grown in autoclaved artificial seawater and fed three times a week with B1 medium. Before experiments, an aliquot of culture was filtered in a stainless steel 50 μm filter to remove algae aggregates.

The *R. salina* biotest was conducted in a 48-well plastic microplate

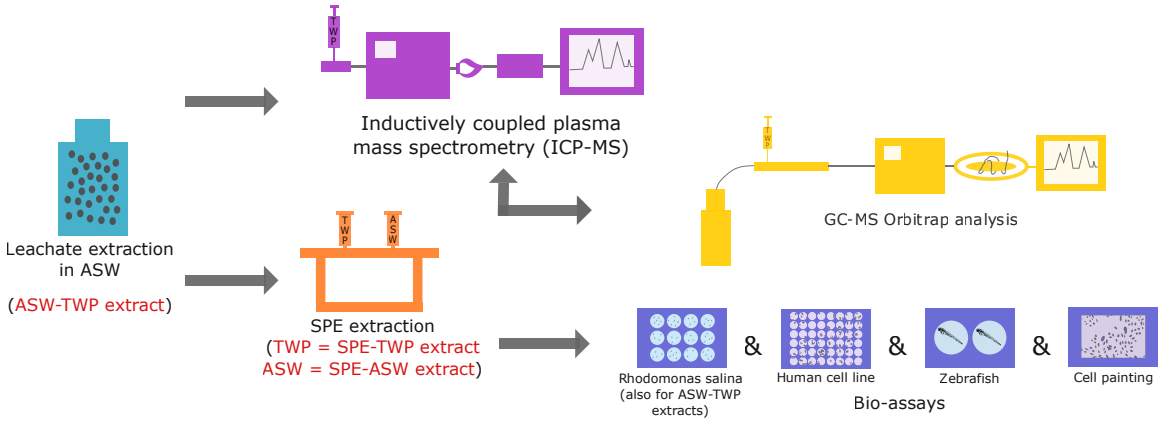


Fig. 1. Graphical representation of the experimental design. Following tire wear particles (TWP) leaching in seawater, organic compounds extracted through solid phase extraction (SPE) undergo chemical analysis and are subjected to various biotests. GC, gas chromatography; MS, mass spectrometry; SPE, solid phase extraction; ASW, artificial seawater. Information on the preparation of ASW-TWP, SPE-TWP, SPE-ASW extracts (in red) are available in section 2.2.

(TPP® tissue culture test plate). The outer border of the plate was filled with autoclaved ASW to avoid any edge effect [62]. All the wells were filled with 1.4 mL with the different exposure solutions. Contaminant dilutions were prepared in an intermediate plastic well plate with a larger capacity. SPE extracts in DMSO were initially diluted to the equivalent of 100% ASW-TWP extract. Then, both ASW-TWP and SPE-TWP extracts were serially diluted to the following concentrations: 100%, 75%, 50%, 25%, 12.5%, and 6.25%. ASW and the ASW-TWP extract were used as blanks for biotests of ASW-TWP and SPE-TWP extracts, respectively. In each 5 mL of pooled concentration mix was added: 5 μL of B1 medium and the appropriate volume ($< 20\mu$L) of filtered cellular suspension to reach a final algae concentration of 1500 cells mL^{-1} in the 48-well plate.

2.4.2. Developmental and behavioral test with zebrafish (Danio rerio)

Fish maintenance and eggs production. Adult zebrafish (*Danio rerio*, strain AB; ZFIN ID: ZDB-GENO-960809–7) obtained from the Karolinska Institute (Stockholm, Sweden) and maintained at the research facilities of the Man-Technology-Environment Research Centre (MTM) at Örebro University were used for breeding. The maintenance and breeding of the fish followed the description published by Nilén et al. [69]. Fish were kept at a 14 h:10 h light: dark cycle in a recirculating system where water exchange, as well as conductivity, pH, and temperature, were automatically controlled and regulated by a ProfiLux 4 controller (GHL Advanced Technology, Kaiserslautern, Germany). Carbonate hardness, nitrates, nitrites (EasyTest stripes, JBL, Germany), and ammonia (Tetra, Germany) contents were monitored once a week. The water temperature was kept at 27 °C ± 1°C. Fish were fed twice a day either with flakes (TetraRubin®, Tetra) or freshly prepared artemia (Ocean Nutrition, Canada). The laboratory animal facilities at Örebro University (#5.2.18.–12707/17) hold permission to use (#5.12.18–12628-17) and breed (#5.2.18–12630-17) laboratory animals. These permits are affiliated with the Swedish Board of Agriculture, Jönköping, Sweden. Moreover, the facilities hold ethical permission (#ID1166) affiliated with the same board in Linköping, Sweden.

Fish embryo toxicity test as monitoring of normal development. Fish embryo toxicity tests (FET) were conducted in accordance with OECD Technical Guideline no. 236 [71], following the modified protocol as detailed in Nilén et al. [68,69]. SPE-ASW and SPE-TWP extracts were examined at five concentrations (ranging from 5.14 to 0.32 μg peq mL^{-1}), prepared through a 1:2 serial dilution. Each test plate comprised three controls: positive (4 mg DCA), negative (ISO-water), and solvent (0.05% DMSO) controls.

Behavioral analyses. The influence of SPE-ASW and SPE-TWP extracts on the swimming behavior of 96 hpf zebrafish larvae was investigated at five sub-lethal concentrations, spanning from 1.2 to 0.075 μg peq mL^{-1}. Larval photo-motor response (LPMR) and vibrational stimulus (VS) tests were conducted using DanioVision (Noldus Information Technology, Wageningen, Netherlands), following the methodology outlined by Nilén et al. [68].

2.5. In vitro tests

2.5.1. Image-based profiling by cell painting assay

Cell Painting assay was performed by using U-2 OS cells (Sigma-Aldrich) that were cultured in DMEM-F12 +GlutaMAX (Gibco, San Diego, CA) supplemented with 7.5% (v/v) fetal bovine serum (FBS; Sigma), 10 U mL^{-1} penicillin and 10 μg mL^{-1} streptomycin (P/S; Gibco) and 1x non-essential amino acids (NEAA; Gibco) and kept at 37 °C with 5% CO2. Cell Painting assay was performed as described by Bray et al. [12], with slight modifications described by Alijagic et al. [3]. In brief, cells were seeded into 96-well black-walled microplates with optically clear flat-bottom (PhenoPlates; PerkinElmer) in a volume of 100 μL. After 24 h, cells were exposed to eight different concentrations of TWP extracts in triplicates. Experiments were performed three times. Exposures were randomized across plates to reduce potential position effects, and outer wells were excluded from the analysis to avoid plate edge-effects. The cells were exposed for 24 h to final concentrations set at 0.1875, 0.375, 0.75, 1.5, 3, 6, 12, and 24 μg mL^{-1}. After exposure, the cell medium was discarded, and cells were stained according to the protocol described in Alijagic et al. [3]. Cell images were obtained using the high-throughput imaging platform InCell 2200 HTS system (GE Healthcare; Uppsala, Sweden). Morphological features were extracted using the image analysis software CellProfiler v. 4.2.1 (www.cellprofiler.org; [89]). A detailed description of the data analysis can be found in Alijagic et al. [3]. The self-developed Python scripts, available upon request, were used for the heatmap construction.

2.5.2. Reporter gene bioassay (CALUX)

Three cell-based assays were used to quantify the bioactivity of the TWP leachate. The DR-CALUX® assay described by Aarts et al. [1] was used to measure AhR activation. The ERα-CALUX® and anti--AR-CALUX® bioassays were used to quantify estrogen receptor (ER) activation and androgen receptor (AR) inhibition, respectively.

DR-CALUX assay. In a 96-well plate, the SPE-TWP extract was tested at six concentrations (four-fold serial dilution, 1:4, v/v in culture medium) along with a 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) standard (0–300 pmol L^{-1}) and added in triplicate wells. The final DMSO concentration was 0.4% in all wells. The protocol described by Larsson et al. [56] was followed for the DR-CALUX® Assay.

ERα and anti-AR-CALUX assays. Using U2-OS cells, ERα-CALUX® and anti-AR CALUX® assays followed procedures by Sonneveld [86], van der Burg et al. [13], with modifications by Titaley et al. [95]. SPE-TWP extract was tested at five concentrations in both assays (four-fold serial dilution, 1:4, v/v in culture medium) along with 17-β-estradiol (E2) standard (0–100 pmol L^{-1}) in ERα-CALUX® and flutamide standards (0–10 μmol L^{-1}) in anti-AR-CALUX®. Dihydrotestosterone (DHT) at 300 pmol L^{-1} was added as a competing agonist in the cell culture medium used for serial dilutions in the anti-AR-CALUX® assay. The final DMSO concentration was 0.1% in ERα-CALUX® and 0.2% in anti-AR-CALUX®.

Calculation. Following Titaley et al. [95], bio-equivalents (bio-eq) for DR-CALUX® (bio-TEQs, TCDD), ERα-CALUX® (bio-EEQs, Estradiol), and anti-AR-CALUX® (bio-FEQ, flutamide) were calculated from the concentration-response curves by correlating the luciferase induction capacities of the sample extracts with those of the standards.

2.6. Data processing and statistical analyses

Concentration-response curves for reporter gene bioassays were performed using a sigmoidal (variable slope) curve fitting equation (GraphPad Prism® 5.01 software).

For data associated with experiments involving algae and zebrafish, the R software [77] was employed for data analysis, and figure generation was conducted using the ggplot2 package [103]. Quantitative datasets underwent tests for normality (Shapiro-Wilk) and homoscedasticity (Levene's test for parametric data and Fligner-Killeen for non-parametric data). When normality and homoscedasticity were confirmed, one-way ANOVA tests were employed to compare means, followed by a post-hoc Dunnett test [30]. In cases of normal but heteroscedastic data, modified one-way ANOVA tests (Welch's test) [101] were utilized for comparing means, followed by a post-hoc Tamhane--Dunnett test [70]. For non-normal data, a Kruskal-Wallis test was employed to compare means, followed by a post-hoc Dunn test [29].

3. Results

3.1. Chemical composition

Numerous organic compounds were detected in the TWP leachate extracted on SPE columns and subsequently analyzed using gas chromatography coupled with Orbitrap mass spectrometry. Data obtained from the TWP extract were corrected with the data from the ASW control extract. In total, 537 features were detected at levels exceeding those of the blanks by 20 times. Among them, 9 hits were consistent with the HRMS library (see Table 1), and 18 hits corresponded to entries in the NIST14 library (see Table 1), and 18 hits corresponded to entries in the NIST14 library (see Table A1; with a match factor of 700 or above, or 600 or above for RI match). Matches with HRMS libraries were categorized as confidence level 2 (probable structure), while hits matching with the NIST library were categorized as confidence level 3 (tentative structure).

Various metals and elements were identified in the leachate extracts, with major elements (concentration > 20 ppb in ASW) depicted on the left graph and minor elements (concentration < 20 ppb in ASW) on the right graph in Figure 2. Most of the metals hadn't been significantly enriched by the TWP leachate and are inherent to the composition of the artificial seawater (i.e., added during the preparation). However, barium and zinc were significantly released during the lixiviation process and are respectively 37 and 11 times more concentrated in the ASW-TWP than in the ASW. Measurements in SPE extracts revealed that the solid-phase extraction significantly reduced the concentration of most metals. Only aluminum, barium, iron, and lithium were extracted in a significant proportion compared to their initial concentration in the water extract.

3.2. Toxicity of the ASW-TWP and SPE-TWP extracts to R. salina

ASW-TWP and SPE-TWP extracts induced concentration-dependent growth inhibition of *R. salina* (Figure 3). The relative $EC_{50-24\,h}$ values were similar between both extracts: $EC_{50-24\,h}$= 48.43% ± 8.10% for ASW-TWP extract and $EC_{50-24\,h}$= 46.82% ± 7.92% for the pure leachate equivalent for SPE-TWP extract. The highest concentration of leachate induced up to 88% and 99% mean growth inhibition for the water and SPE extracts, respectively, with no significant difference between extraction methods ($W = 9, p > 0.05$). Significant overall growth inhibition was found for both extracts ($p = 0.02$, $df = 5$, $\chi^2 = 12.96$ and $p = 0.03$, $df = 5$, $\chi^2 = 12.35$ for ASW-TWP and SPE-TWP extracts, respectively). Due to the limited statistical power ($2 < n < 3$), only a significant growth inhibition was found for the pure ASW-TWP extract compared to its respective control condition.

3.3. Developmental and behavioral effects on zebrafish

The pre-experiment aimed to select a leachate concentration that did not induce severe sub-lethal changes. Concentrations of 2.5 and 5 $\mu g\ mL^{-1}$ did not result in mortality but did produce visible sub-lethal effects in 30% and 60% of the specimens, potentially impacting subsequent behavioral measurements. These effects included pericardial edemas, reduced blood flow throughout the cardiovascular system, and scoliosis. At a leachate concentration of 1.2 $\mu g\ mL^{-1}$, fewer than 10% of the fish exhibited such effects, making it the chosen concentration for subsequent testing.

3.3.1. Morphological analysis

The hatching rate was evaluated at 48, 72, and 96 hpf (Table 2). In the ASW controls, few fish hatched at 48 hpf, with the majority hatching at 72 hpf, and all eggs were fully hatched at 96 hpf for all the tested concentrations of ASW control leachate. Eggs exposed to concentrations of TWP leachate greater than 0.3 $\mu g\ mL^{-1}$ tended to exhibit delayed hatching, as only half to a third hatched at 72 hpf. Hatching success at 96 hpf was only slightly reduced in the exposed fish compared to the control group.

During the pre-experiment, embryos exposed to TWP leachate had less pigmentation than the controls. Qualitatively, the evaluation revealed a concentration-dependent decrease in pigmentation in the exposed larvae, while all fish in the control group presented normal pigmentation. Figure 5 B shows the scoring scale used in the present study. Figure 5 A demonstrates the concentration-dependent decrease in pigmentation of the embryos exposed to TWP leachate with significant differences between controls and larvae of the two highest concentration treatments ($p = 0.027$, $df = 4$, $\chi^2 = 10.97$; note that tank replicates, $n = 3$, have been use for statistical comparison). A major lack of pigmentation (LPI) was observed in the embryos exposed to 1.2 $\mu g\ mL^{-1}$ of leachate, specifically in the eyes and specific body regions. Most of them had no pigment cells on the dorsal part of the body and significantly reduced pigmentation in the eyes. The LPI was evident at concentrations of exposure greater than 0.3 $\mu g\ mL^{-1}$.

3.3.2. Larval photo-motor response (LPMR) and tapping tests

The artificial seawater leachate (ASW) showed no effect on zebrafish behavior for the concentrations between 0.075 to 1.2 $\mu L\ mL^{-1}$. Therefore, the highest concentration of SPE-ASW extracts, representative of the TWP leachate extraction, was used in the subsequent tests as a negative control.

During the LPMR test, zebrafish larvae at 96 h exhibited different movement patterns in the control conditions. In the dark, embryos moved twice as much under light conditions, increasing the distance from approximately 50 cm in the light phase to 100 cm in the dark phase (Figure 4 A.). These patterns of activity were attenuated in all leachate treatments and were almost absent at the highest test concentration. A concentration-dependent trend of reduced swimming activity during the dark phase was observed and reached statistical significance from 0.15 $\mu g\ mL^{-1}$ in comparison to the ASW control.

During the tapping test, the swimming distances covered by larvae were compared within different conditions of chemical exposure, specifically before (BT) and after the first (AT1), fourth (AT4), and eighth

Table 1
Organic compounds detected in the SPE-TWP extract (at 1 g peq L^{-1}, $n = 1$) analyzed by gas chromatography coupled with Orbitrap mass spectrometry using the High-Resolution MS/MS Spectral Library (HRMS). Retention Time (RT), Retention Index (RI), Quantification mass (Qu mass).

RT	RI	Qu mass	Compound	Formula	Match Factor	R. Match Factor	Library	RI Library	Mass Error
7.50	1230	135.0136	Benzothiazole	C7H5NS	0.87	0.87	In-house HRMS	1232	− 1.5
9.41	1347	84.0808	o-Nicotine	C10H14N2	0.80	0.87	In-house HRMS	1349	0.4
10.90	1440	158.0962	2,2,4-trimethyl-1 H-quinoline	C12H15N	0.93	0.85	In-house HRMS	1449	− 0.6
13.29	1599	135.0804	4-tert-Octylphenol	C14H22O	0.86	0.87	In-house HRMS	1602	0.7
13.39	1607	181.0013	2-(Methylthio)benzothiazole	C8H7NS2	0.99	0.98	In-house HRMS	1610	0.6
15.91	1788	178.0777	Phenanthrene	C14H10	0.81	0.96	External HRMS	1833	1.1
19.43	2047	204.0569	4 H-Cyclopenta[def]phenanthren-4-one	C15H8O	0.77	0.95	In-house HRMS	2043	1.2
20.50	2119	202.0776	Fluoranthene/Pyrene	C16H10	0.86	0.92	External HRMS	2103	0.7
25.87	2445	177.0883	Hexamethoxy methyl melamine	C15H30N6O6	0.96	0.94	In-house HRMS	2448	0.0
28.01	2569	177.0884	Hexamethoxy methyl melamine isomer	C15H30N6O6	0.96	0.98	In-house HRMS	2448	0.6

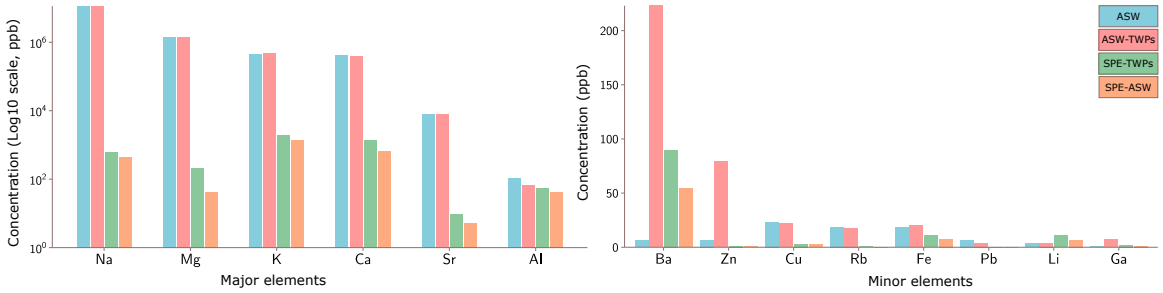


Fig. 2. Concentration (in ppb, $n = 1$) of major (concentration > 20 ppb in ASW; left), and minor (concentration < 20 ppb in ASW; right) elements and metals in ASW, and ASW-TWP, SPE-ASW, and SPE-TWP extracts at 1 g peq L^{-1}, measured using inductively coupled plasma mass spectrometry.

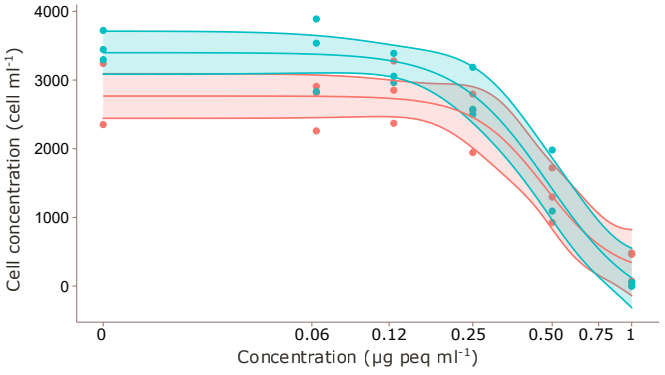


Fig. 3. Cellular concentration of *Rhodomonas salina* (mean in cells mL^{-1}, $2 < n < 3$) as a function of leachate concentration (in μg peq L^{-1}) after 24 h incubation for ASW-TWP (water leachate) and SPE-TWP extracts (post solid phase extraction).

(AT8) tapping events (Figure 4 A). At BT, as described previously in the LPMR test, zebrafish larvae exhibited a tendency towards reduced swimming distances. This trend became more pronounced and reached statistical significance at concentrations of 0.6 and 1.2 μL mL^{-1}, showing a reduction in swimming activity by − 21% and − 47%, respectively, compared to the control. After the first tapping (AT1) within each exposure condition, larvae significantly increased their swimming distances compared to BT. Control larvae increased their swimming activity by 56% at AT1 compared to BT. In contaminated conditions, this increase was more substantial and concentration-dependent, ranging from + 113% at 0.075 μL mL^{-1} to + 274% at 1.2 μL mL^{-1}. For the fourth and eighth tappings (AT4, AT8), the ASW control exhibited only a slightly increased swimming activity compared to BT (ranging from + 18% to + 22%), with no significant difference. However, in all contaminated treatments, swimming activity remained significantly higher after AT4 and AT8 compared to BT (with differences ranging between + 57% to + 223%) Fig. 5.

3.4. Reporter gene bioassays: ERα-CALUX, DR-CALUX, anti-AR-CALUX

The leachate extract from TWP (i.e., the SPE-TWP extract) induced estrogenic activity, exhibiting a concentration-dependent response in the ERα-CALUX® bioassay (Figure 6. B). The Bio-EEQ based on the EC_{25} was calculated as 435 pg g peq^{-1}. No estrogenic response was detected in the control (i.e., the ASW extract).

Furthermore, the SPE-TWP extract revealed a dioxin-like effect in the DR-CALUX® bioassay, as illustrated by the concentration-response curve in Figure 6.A. The Bio-TEQ based on the EC_{25} was determined as 45.2 pg g peq^{-1}. The response from the ASW extract was below the quantifiable limit.

Additionally, the SPE-TWP extract was observed to elicit an androgen receptor antagonistic activity in the anti-AR-CALUX® bioassay, and the calculated bio-FEQ value (IC25) was 1440×10^5 pg g peq^{-1} (Figure 6. C). No inhibition was detected in the ASW control.

3.5. High content screening (HCS) of the morphological phenotypes by cell painting assay

To assess the impact of the SPE-TWP extract on single-cell morphological phenotypes, the HCS with a non-targeted Cell Painting assay was employed [12]. For this purpose, the commonly used U-2 OS cell line was exposed to different concentrations of SPE-TWP extract for 24 h. At the highest exposure concentration (12 mg mL^{-1}), pronounced effects were observed in each cell compartment, including nuclei (DNA), actin/Golgi/plasma membrane (AGP), endoplasmic reticulum (ER), cytoplasmic RNA/nucleoli (RNA), and mitochondria (Mito). Notably, TWP exposure induced an evident collapse of the cytoskeleton (AGP channel), changes in the nuclear morphology (DNA), a decline in detectable RNAs, and an increase in the mitochondrial intensity (Figure 7. A). Morphological alterations were less evident in images per se when extract concentration was reduced (± 6 mg mL^{-1}). Therefore,

Table 2
Hatching frequencies of zebrafish (mean ± standard deviation in % per tank triplicate) at 48, 72, and 96 h post-fertilization (hpf), exposed to different concentrations (in μg peq μL^{-1}) of SPE-ASW and SPE-TWP extracts. 'n' corresponds to the total number of fish in the tank triplicates.

									ASW						
Concentration	0075			0,15			0,3			0,6			1,2		
hpf	48	72	96	48	72	96	48	72	96	48	72	96	48	72	96
Hatching (%)	4 ± 8	98 ± 3	100 ± 0	4 ± 8	94 ± 11	100 ± 0	4 ± 8	96 ± 8	100 ± 0	0 ± 0	95 ± 5	100 ± 0	4 ± 8	95 ± 4	100 ± 0
n	45	45	45	45	45	45	45	45	45	40	40	40	44	44	44
									TWP						
Concentration	0075			0,15			0,3			0,6			1,2		
hpf	48	72	96	48	72	96	48	72	96	48	72	96	48	72	96
Hatching (%)	5 ± 5	98 ± 3	96 ± 3	0 ± 0	93 ± 7	100 ± 0	0 ± 0	59 ± 40	92 ± 13	0 ± 0	75 ± 2	100 ± 0	0 ± 0	55 ± 25	94 ± 7
n	48	48	48	45	45	45	44	44	44	44	44	44	47	47	47

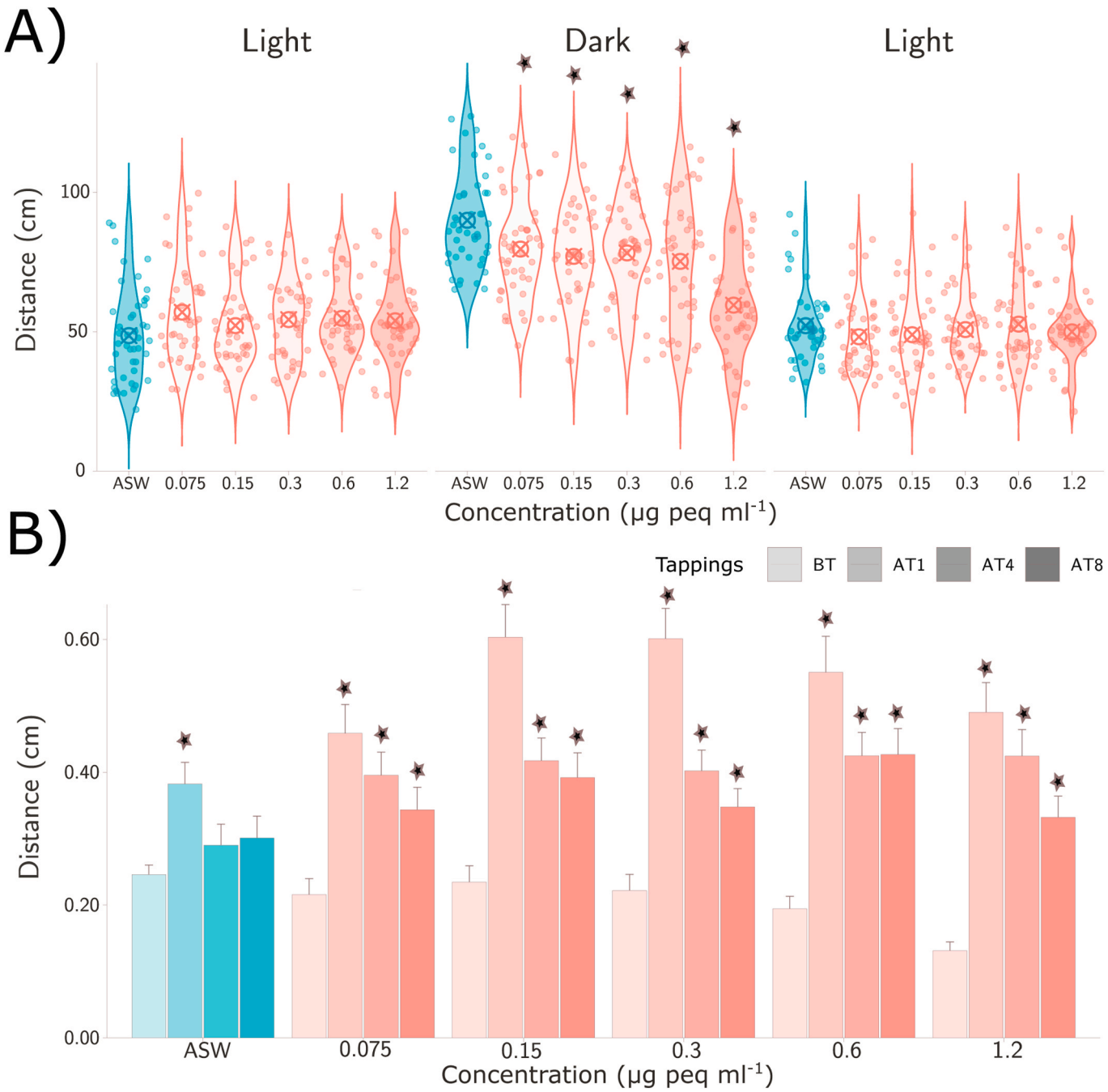


Fig. 4. Zebrafish movement distance relative to TWP leachate exposure concentration during A. the larval photo-motor response test. Each data point represents the absolute distance (in cm) moved by an individual during each respective light phase (i.e., first light period, dark, second light period), while the cross-circle symbol illustrates the mean value ($40 \leq n \leq 46$); B. the tapping test. Each bar represents mean ± standard error ($35 \leq n \leq 45$). BT: swimming distance before tapping. AT1, AT4, and AT4: swimming distance after the first, fourth, and eighth tapping events, respectively. Significant differences from the control mean tested at each respective light phase (A) or exposure concentration (B) are indicated with "* " ($p < 0.05$). ASW: Artificial Seawater control (i.e., the SPE-ASW extract).

CellProfiler, an automated image analysis software, was employed to extract more than 3000 single-cell morphological features, and the obtained data were summarized in the feature-clustered heatmaps (Figure 7. B). Image-based profiling revealed that SPE-TWP extract exposure induced changes in a wide range of morphological features across different channels/cell compartments. Additionally, the majority of changed features exhibited concentration-dependent responses. The most affected features were related to the texture of different cell compartments, especially mitochondria and RNA. At the single-feature level, a prominent increase was observed in the case of Cells_Correlation_K_AGP_ER, Cells_Granularity_13_Mito, Cells_Granularity_14_DNA, Cells_Granularity_14_ER, Cells_Correlation_K_AGP_ER. In addition to the reported TWP concentrations, additional lower concentrations were tested. However, no significant alterations in cell morphological phenotypes were detected (data not shown).

4. Discussion

4.1. Chemical composition of TWP leachate

The seawater leachate from TWP revealed a variety of organic chemical compounds grouped into categories such as amines, azoles,

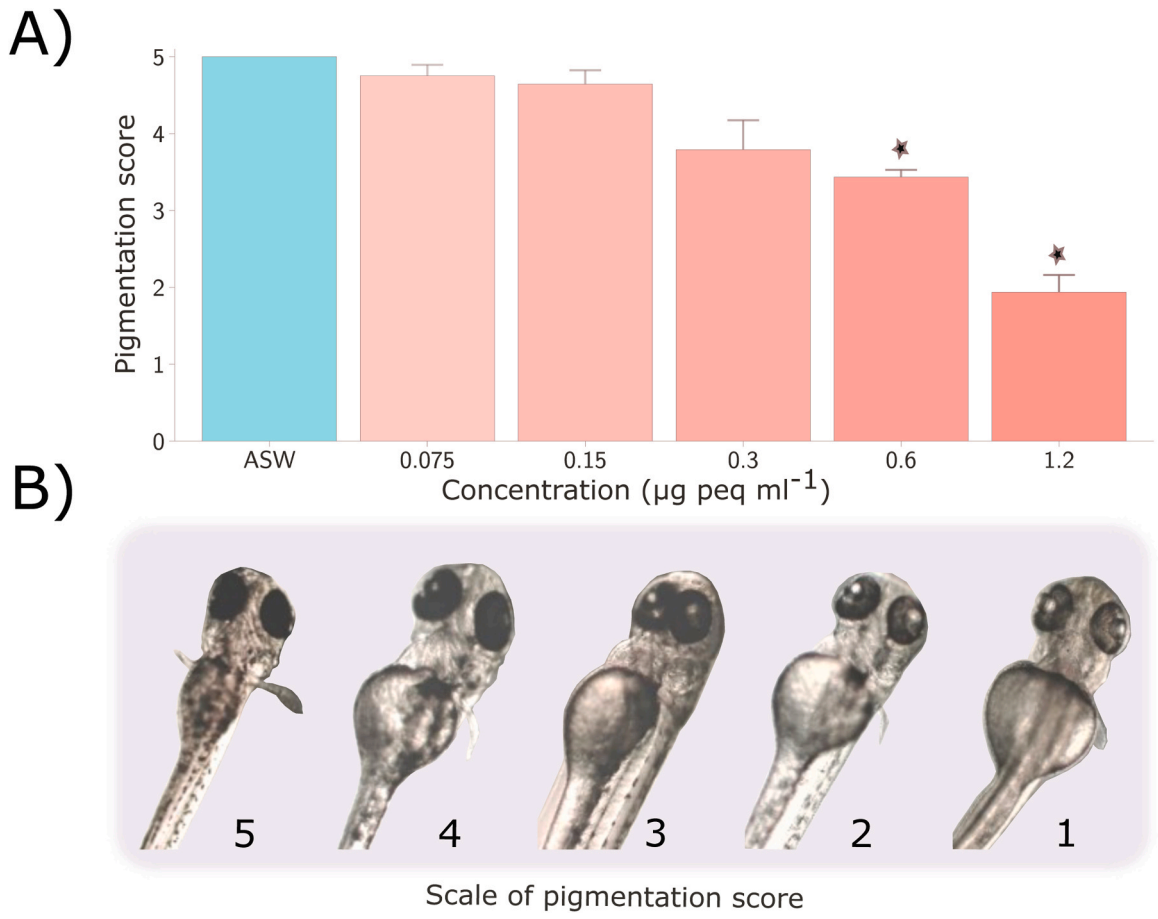
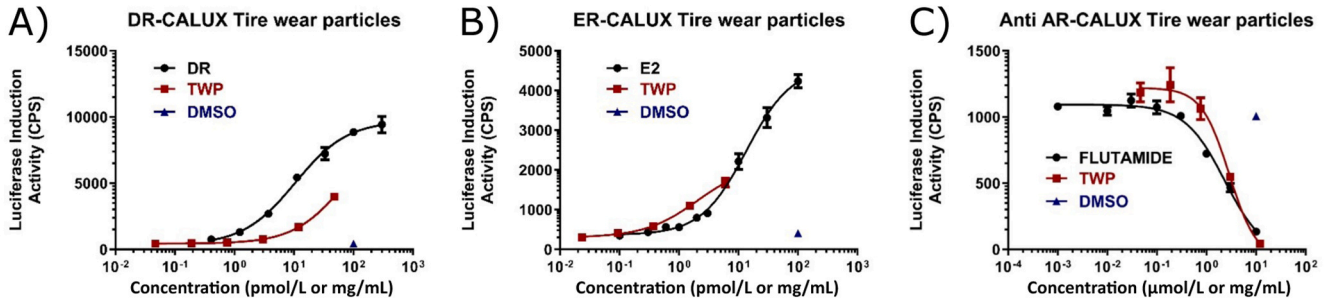
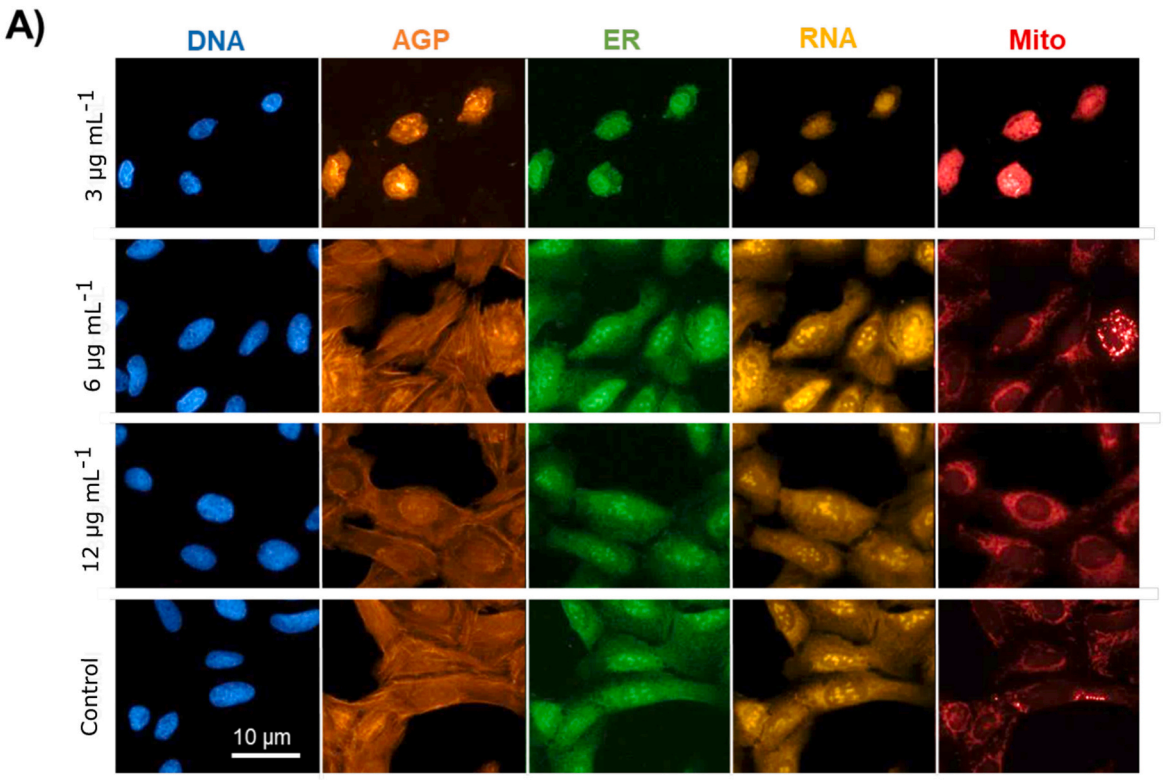


Fig. 5. A. Mean pigmentation scores of the zebrafish larvae tanks in relation to the concentration of leachate exposure (blue bars represent the ASW control while red bars represent the TWP exposed conditions; mean ± standard error, n = 3). B. Scale of pigmentation score measured from 5 (most pigmented) to 1 (least pigmented). Significant differences from the control mean are indicated with "* " ($p < 0.05$).



Calux assay	DR	ERα	Anti-AR
Bio-eq ($pg\ g\ peq^{-1}$)	TEQ EC25	EEQ EC25	FEQ IC25
TWP	45.2	435	1440×10^5

Fig. 6. Concentration response curves for the reporter gene bioassays following SPE-TWP extract exposure (mean ± sd, $n = 3$): A. estrogen-responsive chemical-activated luciferase gene expression (ER-α-CALUX®) assays after 24 h of exposure to 17β-estradiol (E2) or SPE-TWP extract; B. dioxin-responsive chemical-activated luciferase gene expression (DR-CALUX®) assays after 24 h of exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or SPE-TWP extract; C. anti-AR-mediated luciferase gene expression (anti-AR-CALUX®) assay after 24 h of exposure to flutamide or SPE-TWP extract. The table represents mean bio-equivalent concentrations (Bio-eq, pg g peq^{-1}: Bio-TEQ, EEQ, FEQ) for the SPE-TWP extract. Bioactivities are expressed in equivalences of the standard reference compound in each respective assay. No responses were measured in the DMSO negative control.



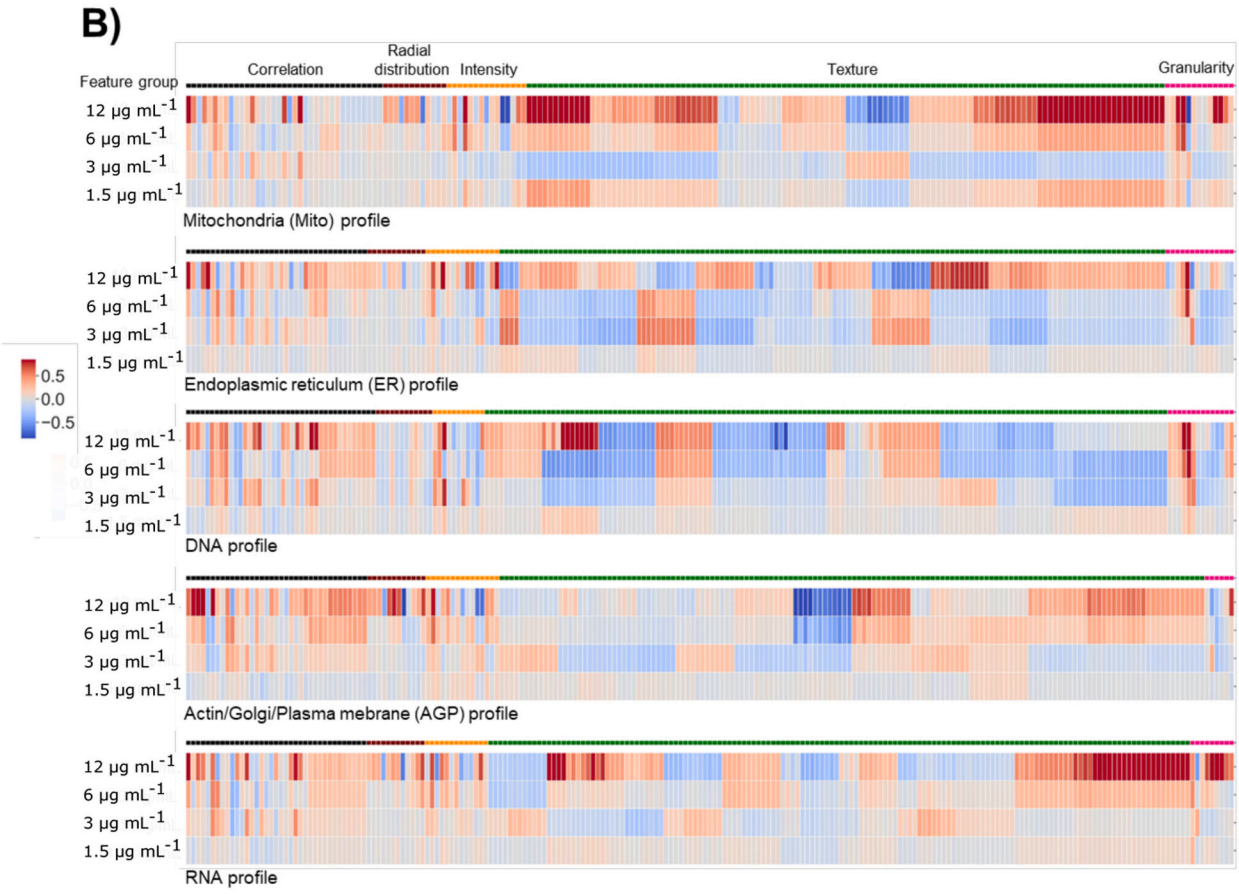


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Fig. 7. Morphological phenotypes of U-2 OS cells unveiled by the high-content screening (HCS) Cell Painting assay. A. Representative images depict control cells and cells exposed to varying concentrations (3, 6, and 12 μg mL^{-1} peq) of SPE-TWP extract. Cells were live-labeled for mitochondria (Mito) and, after fixation and permeabilization, labeled with fluorescent probes for nuclei (DNA), actin/Golgi/plasma membrane (AGP), endoplasmic reticulum (ER), and RNA/nucleoli (RNA). Images were captured at a 20 × magnification. B. Heatmaps summarizing differences in morphological effects in the analyzed channels/cell compartments. Features were clustered into several feature groups, including correlation, radial distribution, intensity, texture, and granularity. Columns in the heatmap represent individual morphological features. The colors represent the fold increase or decrease of each measured morphological feature in comparison to DMSO control. Data were derived from single-cell profiles distributed across three microplates/biological replicates. Rows correspond to individual concentrations in μg peq mL^{-1}, with exposure concentrations arranged in descending order from top to bottom.

polycyclic aromatic hydrocarbons (PAHs), and phenolic compounds, commonly used to manufacture and enhance tire performance. However, these compounds may exhibit intentional (e.g., anti-fungal) or unintentional biological activities, raising concerns for environmental and human safety [39,45].

Several of the detected compounds, including benzothiazole, 2-(methylthio)benzothiazole (MTBT), hexamethoxy methyl melamine (HMMM), 4-tert-octylphenol, phenanthrene, 4 H-cyclopenta[def]phenanthren-4-one, and fluoranthene and pyrene, have been identified as micropollutants and PAHs originating from tires [91]. Notably, 1,4-Benzenediamine, N-(1,3-dimethylbutyl)-N'-phenyl-, also known as 6PPD, was detected and serves as a precursor of 6PPD-quinone, a significant marker indicating TWP contamination across ecosystems, particularly affecting fish toxicity [10].

Other compounds, such as amines, often found in tire wear particle leachates due to their use as plastic stabilizers, have been associated with both aquatic and human toxicity [88]. Additionally, the antioxidant additive 2,2,4-trimethyl-1 H-quinoline, commonly used in tires, lacks sufficient ecotoxicological data [67].

While metals are frequently implicated in tire leachate toxicity, the bio-assays discussed in this study focused on seawater leachate extracts after removing metals via SPE extraction. Hence, the observed toxicity likely stems from water-leachable organic compounds rather than metals initially present in the seawater leachate.

The tire used in this study was new, but real-world factors like speed, braking force, and road conditions can alter leachate composition [11], potentially impacting toxicity [94]. However, our study, employing consistent material across various toxicity bioassays, serves as a basis for evaluating tire particle toxicity. Further research on weathered tire particles and leachate will complement these findings.

4.2. Toxicity of TWP leachate on a microalgae model

Leachate from TWP exhibited acute toxicity to *R. salina*, whether directly following seawater lixiviation or post-SPE extraction. This suggests that TWP pollution may have adverse effects on phytoplankton communities at the tested concentrations. Page et al. [73] documented an inhibition of *R. salina* growth caused by TWPs leachate in seawater ($EC_{50-24\,h}$ of 0.39 g peq L^{-1}) at similar concentrations than observed here. The authors predominantly linked this toxicity to the elevated zinc concentration in the leachate, as opposed to detected organic compounds like naphthalene, which had concentrations well below commonly reported ecotoxicological thresholds. Zinc has been proposed as one of the primary factors contributing to the toxicity of TWP leachates on both macro and microalgae [106,66,96]. However, the present study showed consistent toxicity in both water and SPE leachates, with the latter lacking zinc, suggesting that organic compounds are the likely contributors to the toxicity of TWP on *R. salina*. Oxidative stress has been highlighted as a crucial factor in algal growth inhibition induced by organic compounds leached from TWP [44]. In this study, authors reported cyclic amines as the most potent among 13 other leachable organic substances. Additionally, various organic compounds identified in the present leachate, including benzothiazole [14] and PAHs [9], may also contribute to the observed toxicity to algae. On the other hand, pollutants like 4-Tert-Octylphenol that might express specific effects, such as endocrine disruption via interaction with biological structures absent in microalgae, may also exhibit toxicity via other mechanisms.

4.3. Toxicity of TWP leachates on zebrafish embryo-larval stages

Zebrafish has been selected as one of the in vivo model organisms for this study due to its established reputation as one of the premier fish models for assessing the potential effects of chemicals on fish, encompassing both marine and freshwater species, as well as human health [55]. In the early stages of fish development, immature biological systems undergo finely regulated processes, rendering them particularly sensitive to environmental stressors, including chemical contaminants [20]. The existing literature highlights the potential of leachates from TWP to induce adverse outcomes in fish's early stages, embryos, and larvae. These outcomes include malformations, impaired eye development, reduced growth, heartbeat, and hatching success [19,18,17]. In the present study, zebrafish embryos exposed to the TWP leachate exhibited no mortality. However, they displayed sublethal negative effects such as malformations, edemas, and reduced blood flow at concentrations ranging from 2 to 5 mg peq L^{-1}. Even at lower concentrations of 1.2 g peq L^{-1}, developmental alterations were observed, and hatching success was notably reduced, with concentrations as low as 0.3 g peq L^{-1}. Previous research on fish species models has documented lower hatching success associated with other developmental impairments [18], but negative effects at the organism level are generally observed only at high, non-environmentally relevant concentrations ranging from single to hundreds of g peq L^{-1} [17,19].

The variations in toxicity observed in the present study compared to previous findings could be attributed to variations in micronization, leaching protocols, and the specific tire model used. For instance, McIntyre et al. [65] demonstrated that environmentally realistic concentrations of 320 mg peq L^{-1} of TWP leachate, reflecting roadway runoff, caused mortality in coho salmon. In contrast, chum salmon remained insensitive to the same concentration, highlighting species-specific sensitivities.

Exposed larvae in the present study displayed a noticeable lack of pigmentation, even discernible at concentrations as low as 0.3 g peq L^{-1}. This observation aligns with findings from diverse studies on chemical exposures to fish, including TWP leachates, as exemplified by Chibwe et al. [18], who documented deficiencies in eye and body pigmentation in exposed fathead minnow embryos. In a study on zebrafish, Chang et al. [17] reported impaired eye development, affecting pigmented structures, which hindered larval swim activity and phototactic response induced by TWP leachate.

Behavioral analysis is a powerful integrative tool known for its ability to reflect changes in biochemical and physiological aspects at higher organizational levels, with high sensitivity [48]. In the present investigation, in the larval photo-motor response test, non-exposed larvae exhibited a common behavioral pattern observed in healthy zebrafish characterized by higher swimming activity in the dark and rest during the light periods [7]. Exposure to TWP leachate induced a concentration-dependent alteration of this pattern, leading to hypoactivity during dark periods. This observed change potentially indicates impairment in predator avoidance, likely attributed to visual defects or neurotoxic effects, in line with the findings reported by Chang et al. [17]. Furthermore, during the tapping test, chemically exposed larvae revealed a concentration-dependent overreaction to vibrational stimuli. Therefore, it seems that TWP leachate does not compromise the

avoidance reaction of zebrafish, in contrast to what has been observed with other neurotoxic environmental contaminants [32]. However, this result might suggest higher levels of fear or anxiety induced by the mixture [79]. Furthermore, an increased anxiety might have affected short-term habituation in response to vibrational stimuli in zebrafish. maintaining hyperactivity after eight tapping events. Similar changes in both startle and habituation responses in zebrafish exposed to various environmental pollutants have been previously demonstrated by Faria et al. [33]. In conclusion, both behavioral tests conducted in the present study suggest an elevated predation risk. Vibrational tests indicate impacts on habituation and an increased startle response due to anxiety, potentially leading to exhaustion, but no significant impairment in the lateral line. Conversely, the LPMR test suggests potential visual defects that could diminish predator avoidance.

Various chemicals detected in the leachate could be responsible for these effects. Benzothiazoles and arylamines have been identified as potential causative agents in the developmental impairments induced by TWP leachate in fathead minnows [18]. The 6-PPD detected in the leachate and its derivative, the 6-PPD-Q, also has been demonstrated to impact zebrafish embryo development and induce myopia [10]. Additionally, three PAHs (fluorene, pyrene, and phenanthrene) have been linked to reduced eye pigmentation in zebrafish embryos and a subsequent decrease in swimming activity [25]. Notably, these effects were associated with disruptions in thyroid signaling, underscoring the intricate mechanisms.

4.4. Disruption of signaling pathways

TWP leachates, implicated in the disruption of signaling pathways [111], were found to exhibit estrogenic, AhR, and anti-androgenic activities, as reported by Eriksson et al. [31] using CALUX® bioassays. A similar pattern was observed in the present study, where estrogenic and AhR activities were measured at approximately one order of magnitude lower. This discrepancy may arise from intentionally focusing solely on leachable chemicals in the present investigation and the content of TWP chemicals probably differ between particles from different tires. Notably, most TWP compounds didn't leach into seawater under the applied protocol [73]. In contrast, similar anti-androgenic activity was reported (FEQ IC25 341–904 $\mu g\ g^{-1}$ vs 144 $\mu g\ g^{-1}$ in the present study). Chemicals with higher solubility could probably possess more anti-androgenic than estrogenic or AhR activities. Solubility of the TWP additives influence the lixiviation in aqueous media [39], possibly resulting in different toxic outcomes [113]. For instance, water under environmentally relevant conditions likely did not extract Ah receptor-active compounds from rubber mulches, as detected by an AhR-CALUX® assay [46].

Chemical analysis of the TWP leachate revealed estrogen-disrupting PAHs such as phenanthrene, fluoranthene, and pyrene, potentially explaining observed endocrine-disrupting activities in cell assays. These compounds can elicit AhR-mediated activity, estrogenicity, and anti-androgenicity, interacting with nuclear or cytosolic receptors, and are associated with cellular toxicity and carcinogenicity [97,112].

The phenolic compound 4-tert-octylphenol (tOP), included during the tire rubber manufacturing process, was detected in the leachate. tOP induces hormonal responses, acting as an estrogen receptor agonist and antagonizing androgen and progesterone receptors within the micromolar range, potentially explaining results in CALUX assays [98]. Krüger et al. [53] found anti-androgenic effects of tOP, which, combined with other endocrine-active additives, activated both aryl hydrocarbon and androgen receptors.

While alkylphenol derivatives are well-known for their estrogenic potential, other compounds released from TWP, such as benzothiazole derivatives (BTs), have been proven to exhibit weak but consistent estrogenic activity in the micro to millimolar range[16]. Using a CALUX cell bioassay for toxicant identification, He et al. [40] demonstrated that tire extract contained AhR-active PAHs and two AhR agonists BTs, 2-mercaptobenzothiazole and MTBT, also detected in the present leachate. Despite their low affinities for estrogenic receptors, the extent of their contribution to the endocrine-disrupting potential of environmental mixtures depends on the effective concentration reaching within exposed biota. Additionally, their potential for synergistic interactions with other EDCs is a critical factor that warrants more attention in ecotoxicology.

4.5. Effects on cell morphology: non-targeted indices of toxicity

Effects of TWP extracts on cell morphology were assessed using Cell Painting, a high-content image-based assay for cell morphological profiling [12]. The morphology of cells closely mirrors their state, emphasizing the dynamic functional requirements determining future cell behavior [82]. Moreover, the Cell Painting assay is intended to be unbiased and not targeted to a particular effect of interest, being useful in detecting early morphological markers upon exposure to chemicals or particles even before the exposure reaches cytotoxic levels [3,78]. Effects disclosed by Cell Painting profiling upon exposure to the highest TWP concentration indicated pro-apoptotic cell changes. Some of the common signs were cell rounding (Figure 7. A, top panel) and change in several features linked to mitochondrial toxicity, endoplasmic reticulum stress, disruption of the cytoskeleton, and, most probably, increased lysosomal activity [54,81]. Even if changes at the lower TWP concentrations became more subtle and not evident in images per se, Cell Painting profiling disclosed that similar morphological feature clusters were changed (Figure 7. B). Importantly, this finding revealed that even lower concentrations of TWP may lead to the development of toxicity phenotypes.

Moreover, parallel morphological effects on several cell compartments indicated that multiple mechanisms of action orchestrate the effects of chemicals found in the TWP extracts. However, limited information is available about the mechanisms that identified TWP chemicals use to exert its effects. For example, BT identified in this study presents very scarce information regarding its toxicity [61]. Interestingly, the pro-apoptotic phenotype observed in the Cell Painting assay may be attributed to tOP, as its endocrine-disrupting properties are also known to induce mitochondria-mediated apoptosis [110]. Another possible explanation behind the observed effects is the detection of several PAHs, including phenanthrene and fluoranthene/pyrene. PAHs are well-known inducers of oxidative stress that may lead to mitochondrial and DNA damage and carcinogenesis [58]. Additionally, HMMM's mechanism of toxicity is almost entirely unknown. Overall, observed Cell Painting results may be explained by the cocktail effect that identified TWP chemicals induced upon exposure.

4.6. Mechanisms of toxicity

While adverse effects on communities and ecosystems significantly impact human well-being and environmental health, understanding molecular and cellular toxic mechanisms is crucial in ecotoxicology for comprehensive risk assessment, prediction, and mitigation. In the case of complex mixtures like TWP leachates, it is fundamental to consider the toxic mechanisms of individual components and their interactions.

4.6.1. Toxicity pathways: examine the mechanisms and pathways behind TWP toxicity

The present data demonstrates the toxicity exhibited by TWP leachates across various model organisms, each with differing complexities. These findings underscore the hypothesis that multiple mechanisms of action involving one or several toxic compounds, independently or in combination, may vary depending on the organism under consideration.

Maintaining redox balance is vital for biological homeostasis. Oxidative stress, triggered by excess reactive oxygen species (ROS) from xenobiotics like plastic additives, disrupts this balance [75]. In TWP

leachates, oxidative stress is a key toxicity driver, evidenced by increased ROS, altered antioxidant enzymes, and resulting damage in exposed organisms [11,85]. Effects include behavioral shifts and modified immune defenses [59,85]. While chemicals in leachates like BTs, alkylphenols, and PAHs are linked to oxidative stress, understanding their precise toxic mechanisms requires consideration of related pathways and cascading effects [60,97]. Assessing oxidative stress biomarkers may unveil broader cellular stress, necessitating additional specific biomarkers to discern chemical contributions to overall toxicity [80].

Limited information suggests that compounds in TWP leachate may directly inhibit enzymes in vivo systems [22], as hinted by data available for specific chemicals like PAHs [42,84] and potentially BTs [83]. Thus, enzymatic inhibitions through direct interactions between enzymes and certain organic chemicals present in tire leachates could play a role in their toxic mechanisms of action.

PAHs exhibit diverse toxicity mechanisms, including genotoxic effects like covalent DNA adduct formation and non-genotoxic effects such as nuclear receptor interaction, disrupting signaling pathways and cell functions [97,112]. Conversely, tOP primarily disrupts endocrine pathways through nuclear receptor activation, though other mechanisms like endoplasmic reticulum Ca^{2+} pump inhibition have been suggested [53,98,41]. The specific toxic mechanisms of 2,2,4-trimethyl-1 H-quinoline remain unclear, yet compounds with quinoline rings are often associated with lysosomal enzyme inhibition or protein synthesis disruption. Notably, they may induce retinal pigment epithelium-specific toxicity by binding with melanin, concentrating in the retinal pigment epithelium, potentially compromising its integrity [102]. For compounds like hexamethoxy methyl melamine, which are environmentally ubiquitous with proven toxicity, data on their mechanisms are still under exploration [26].

4.6.2. Cocktail effects: toxic interactions among TWP contaminants

This study, along with others, demonstrates that tire particles can generate complex chemical mixtures in aquatic environments, soil, and air [39,45]. While certain studies have identified single main drivers of TWP leachates toxicity, such as zinc [107] or 6-PPD [94], others have suggested the potential involvement of several compounds [104,105,96]. While zinc may play a significant role in the toxicity of TWP leachates in specific contexts, this study, in alignment with others, underscores the importance of considering organic pollutants leaching from this material [91]. Toxic interactions in some of the compounds detected in the leachate have already been discussed, such as for additive toxicity among PAHs mixtures [57] or more-than-additive effects between metals and PAHs [35]. Other authors described potential toxicity synergism with the carbon black of the TPW [85]. However, knowledge regarding interactions between individual toxicants in TWP leachates mixtures is still in its infancy, and further studies using approaches such as Toxicity Identification and Evaluation (TIE) or Effect-Directed Analysis (EDA) have the potential to enhance understanding. Additional toxicity testing focusing on the primary drivers of toxicity is crucial for identifying potential additives or synergistic mechanisms among them.

4.7. Risks for ecosystems and human health

4.7.1. Ecotoxicological impact

Tires are recognized as a significant source of microplastic pollution [99,49,51], with the contribution of particles smaller than 10 μm (PM10) potentially underestimated [8]. TWP may exhibit different transport behavior than lighter microplastics, accumulating in marine sediment near urban areas [63]. Leaching from sediment particles could elevate tire additive concentrations in aquatic environments. Tire wear may represent PM10 emission factors of 4.0–13 mg km^{-1} $vehicle^{-1}$ for light-duty vehicles, with this fraction size accounting for only 10% of the emission [90]. With up to 3.5 billion light vehicles worldwide by the middle of this century [64], each traveling 12500 km annually [21], the global light vehicle fleet could potentially produce approximately $5 * 10^5$ tons per year. Additionally, TWP are recognized as sources of environmental pollutants like PAHs, BTs, and Zn. However, further studies on TWP's transport, fate, and environmental concentrations are needed to better evaluate the associated mass flows of constituent elements and chemicals [99]. This study and others demonstrated that these leached constituents could induce toxicity in various biological systems, although more studies using different model species exposed to environmentally realistic concentrations are still needed. Furthermore, understanding the interplay between particle weathering, chemical additives, and environmental impacts is essential for a comprehensive evaluation of the risks posed by TWP. Additionally, certain compounds leaching from TWP, such as benzothiazole, are suspected to be highly bioaccumulative and were found to accumulate in marine organisms [43]. Therefore, the accumulation of the most concerning compounds in the environment and the food chain must also be considered.

4.7.2. Implications for human health

As mentioned earlier, TWP are widespread, and inhaled PM10, smaller particles, and other sources like food and water contamination contribute to human exposure. While the health risk from inhaling TWP is considered low [52], the implications of airborne contaminants for human health are not fully understood [45], with notable gaps in existing literature, including the impact of exposure via the food [6]. Although human exposure evaluation is beyond the scope of this article, it's crucial to recognize that, besides particle contamination, TWP could be a source of chemical contaminants in products intended for human consumption, as seen with BTs and PAHs detected in drinking water and food products [100]. Therefore, identifying and monitoring the presence of toxic molecules in TWP leachate capable of inducing biological responses, such as signaling disruption, as shown in the present study, is essential for managing human health risks.

4.8. Conclusion

Abundant in the environment, TWP have the potential to elevate the chemical burden on living organisms. However, their impacts within the complex environmental context, where organisms face numerous biotic and abiotic stressors, remain unclear. This study highlights observed adverse effects across various biological models, potentially linked to one or more leachable organic chemicals in seawater. The demonstrated toxicity spans multiple biological levels, from intracellular to whole organisms, suggesting mechanisms such as oxidative stress generation and membrane disruption and specific actions like receptor binding that disrupt cellular signals. Therefore, increased efforts are needed to understand how additives in car tires affect the health of both wild animals and humans, ultimately impacting well-being. Such insights will contribute to refining regulatory practices concerning tire manufacturing additives globally. Strict control of most toxic additives and minimizing their quantity to essential levels for tire function is necessary. Additionally, exploring safer, well-established alternatives is imperative.

Environmental implication statement

Tire wear particles (TWP) pose a significant environmental threat, contributing to microplastic pollution and releasing harmful chemical contaminants. This study unveils the hazardous nature of TWP leachates, introducing detrimental compounds to aquatic ecosystems. Identified pollutants, notably polyaromatic hydrocarbons, induce acute toxicity, impede algae growth, induce embryotoxicity, alter behavior, and disrupt signaling pathways. These revelations emphasize TWP as a hazardous material affecting aquatic life at multiple levels, posing risks to the health of both ecosystems and, potentially, human well-being. Enhancing our understanding of TWP leachate mechanisms is crucial

for mitigating these risks and safeguarding aquatic ecosystems from deleterious effects.

Research ethics

This work has received research ethics approval from the regional ethics committee of Umeå regarding zebrafish experimentations, and a certificate of approval is available upon request.

CRediT authorship contribution statement

Clara Kempkens: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Jessy LEDU-CARREE:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Rodrigo Almeda:** Writing – review & editing, Writing – original draft, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Steffen Keiter:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Viktor Sjöberg:** Writing – review & editing, Validation, Methodology, Investigation. **Magnus Engwall:** Resources, Funding acquisition. **Oleksandr Kotlyar:** Visualization, Validation, Software, Formal analysis. **Andi Alijagic:** Visualization, Validation, Software, Methodology, Investigation, Formal analysis. **Maria Larsson:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Anna Rotander:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author(s) used ChatGPT, a language model developed by OpenAI, and Grammarly's AI writing assistant in order to improve language fluency. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Chemical analysis

Table A1
Organic compounds detected in the SPE-TWP extract (at 1 g peq L^{-1}, $n = 1$) analyzed by gas chromatography coupled with Orbitrap mass spectrometry using the High-Resolution MS/MS Spectral Library (HRMS) and the National Institute of Standards and Technology Library (NIST). Retention Time (RT), Retention Index (RI), Quantification mass (Qu mass), Non-available (N/A).

RT	RI	Qu mass	Compound	Formula	Match Factor	R. Match Factor	Library	RI Library	Mass Error
7.50	1230	135.0136	Benzothiazole	C7H5NS	0.87	0.87	In-house HRMS	1232	− 1.5
9.41	1347	84.0808	o-Nicotine	C10H14N2	0.80	0.87	In-house HRMS	1349	0.4
10.90	1440	158.0962	2,2,4-trimethyl-1 H-quinoline	C12H15N	0.93	0.85	In-house HRMS	1449	− 0.6
13.29	1599	135.0804	4-tert-Octylphenol	C14H22O	0.86	0.87	In-house HRMS	1602	0.7
13.39	1607	181.0013	2-(Methylthio)benzothiazole	C8H7NS2	0.99	0.98	In-house HRMS	1610	0.6
15.91	1788	178.0777	Phenanthrene	C14H10	0.81	0.96	External HRMS	1833	1.1
19.43	2047	204.0569	4 H-Cyclopenta[def]phenanthren-4-one	C15H8O	0.77	0.95	In-house HRMS	2043	1.2
20.50	2119	202.0776	Fluoranthene/Pyrene	C16H10	0.86	0.92	External HRMS	2103	0.7
25.87	2445	177.0883	Hexamethoxy methyl melamine	C15H30N6O6	0.96	0.94	In-house HRMS	2448	0.0
28.01	2569	177.0884	Hexamethoxy methyl melamine isomer	C15H30N6O6	0.96	0.98	In-house HRMS	2448	0.6
7.58	1235	88.0518	Neodecanoic acid	C10H20O2	715	727	NIST14	N/A	N/A
8.02	1262	88.0519	Acetic acid, diethyl-	C6H12O2	751	772	NIST14	N/A	N/A
8.90	1316	93.0574	Formamide, N-phenyl-/ 2,4,6-Cycloheptatrien-1-one, 2-amino-	C7H7NO	805	821	NIST14	1221	N/A
9.87	1376	120.0807	Benzenamine, N-(2,2-dimethylpropyl)-N-methyl-	C12H19N	771	781	NIST14	N/A	N/A
9.93	1379	132.0808	2-Heptanamine, N-(phenylmethylene)-	C14H21N	743	757	NIST14	N/A	N/A
10.58	1420	138.1278	Cyclohexanamine, N-cyclohexyl-	C12H23N	661	793	NIST14	1442	N/A

(*continued on next page*)

Table A1 (*continued*)

RT	RI	Qu mass	Compound	Formula	Match Factor	R. Match Factor	Library	RI Library	Mass Error
11.31	1466	103.0417	Phthalimide/o-Cyanobenzoic acid	C8H5NO2	765	805	NIST14	1450	N/A
12.76	1563	132.0808	N-Phenylcyclohexylamine	C12H17N	787	861	NIST14	N/A	N/A
12.87	1570	158.0963	Cyclohexanamine, N-(phenylmethylene)-	C13H17N	782	782	NIST14	1658	N/A
13.61	1621	169.0883	Diphenylamine/Pyridine, 2-(4-methylphenyl)-	C12H11N	794	844	NIST14	1621	N/A
14.41	1677	154.0652	2,5-Cyclohexadien-1-one, 4-(phenylimino)-	C12H9NO	802	804	NIST14	N/A	N/A
15.80	1778	194.0837	1 H-Benzimidazole, 2-phenyl-/ 1 H-Pyrrolo[2,3-*b*] pyridine, 2-phenyl-	C13H10N2	785	826	NIST14	N/A	N/A
17.77	1928	185.0833	3-Hydroxydiphenylamine	C12H11NO	875	877	NIST14	N/A	N/A
18.22	1961	181.9967	2-Benzothiazolesulfenamide, N-(1,1-dimethylethyl)-	C11H14N2S2	700	715	NIST14	N/A	N/A
20.31	2106	211.1230	1,4-Benzenediamine, N-(1-methylethyl)-N'-phenyl-	C15H18N2	787	812	NIST14	N/A	N/A
20.96	2148	135.0138	Methyl dithio-3-methylbenzoate	C9H10S2	710	896	NIST14	N/A	N/A
24.14	2342	268.1935	1,4-Benzenediamine, N-(1,3-dimethylbutyl)-N'-phenyl-	C18H24N2	698	704	NIST14	N/A	N/A
33.27	2881	180.1747	Thiohydroxylamine, S-benzothiazole-2-yl-N,N-dicyclohexyl-	C19H26N2S2	801	806	NIST14	N/A	N/A

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