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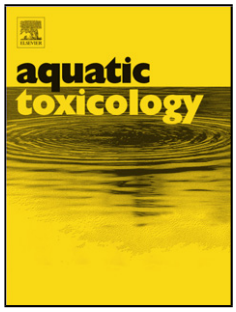

Tire wear particle and leachate exposures from a pristine and road-worn tire to *Hyalella azteca*: comparison of chemical content and biological effects

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Tire wear particle and leachate exposures from a pristine and road-worn tire to *Hyalella azteca*: comparison of chemical content and biological effects

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Highlights

- Particles abraded from pristine tires exhibit greater toxicity than particles from a worn tire.
- Acute toxicity profiles differ depending on tire type and fraction (particle or leachate exposure).
- Pristine tire wear particle exposure diminished growth of *Hyalella azteca*.
- 1-octanethiol, present in pristine tire particles, could be a possible causative agent.

Abstract

Tire emissions have emerged as an environmental contaminant of concern. To fully understand their effects to biota, research is needed from different stages of a tire's lifecycle. In this study we exposed freshwater *Hyalella azteca* to tire wear particles (TWPs) as particle suspensions or their respective chemical leachates (the chemicals released from tire particles into water) from a pristine (P-TWP) and worn (W-TWP) tire of same make and model. Acute and long-term toxicity experiments on *H. azteca* showed that P-TWP suspensions were more toxic than W-TWP suspensions with estimated LC_{50} values of 364 ± 64 particles (0.19 ± 0.03 g L^{-1}) and 3073 ± 211 particles (0.91 ± 0.06 g L^{-1}), respectively. However, leachates from W- and P-TWPs appeared equally toxic, but did not conform to a sigmoidal dose-response pattern and LC_{50} values could not be derived. In long-term tests (21 d) P-TWP suspensions showed no significant effects on *H. azteca* mortality (p=0.970) or reproduction (p=0.123), but growth was significantly reduced (p=0.003) at the highest concentration tested (250 particles mL^{-1} or 0.127 g L^{-1}). Chemical analysis of both particle types and their leachates showed that four compounds, benzothiazole, 1-indanone, aluminium and zinc, consistently leached from TWPs into water. Analysis of the two TWPs showed

a difference in the concentration of the various compounds. Specifically, P-TWPs contained significantly more 1-octanethiol, phenanthrene, anthracene and aluminium than W-TWPs, suggesting that they are possible candidates for the increased toxicity observed following P-TWP exposure.

Keywords: tyre/tire wear; TWP; TRWP; polymer; microplastic.

1. Introduction

Micronized rubber particles ('microrubber', tire wear particles (TWP) and tire and road wear particles (TRWP)) abraded from car tires on road surfaces have emerged as a pollutant of interest within the broad scope of microplastic (MP) pollution (Rochman et al., 2019; Halle et al., 2020). An estimated global per capita release of 0.81 kg of tire emissions enter the environment each year (Kole et al., 2017). Micronized rubber particles have been found in all environmental compartments and most likely contribute a significant proportion of the observed polymer debris in the environment (Wik & Dave, 2009; Boucher & Friot, 2017; Evangeliou et al., 2020). However, efforts to determine the potential ecotoxicological impacts of TWPs are still in their infancy. These knowledge gaps needs to be urgently addressed in order to understand the potential environmental risk (Wagner et al., 2018; Halle et al., 2020).

Previous research on the biological effects of TWP exposure, spanning the past 30 years, has primarily been based on the toxicity of the leached fraction ('leachate'). Tire leachate is a complex

suite of toxic substances predominantly trace metals, polycyclic aromatic hydrocarbons and assorted volatile organics. These organics are mainly used in vulcanization and as antioxidants, and may be released into the aqueous environment (Turner & Rice, 2010; Marwood et al., 2011). The effects of tire leachate exposure range from none to significant on the molecular, cellular and organismal levels (Halle et al., 2020). Acute mortality has been the most often tested endpoint for tire leachates, and toxicity varies depending on test species and also the methods used for generating TWPs and leachate. Generally though, a 48h EC_{50} in the range of 0.1-10 g/L has been observed (Wik & Dave, 2006; Mantecca et al., 2007; Wik et al., 2009; Turner & Rice, 2010; Marwood et al., 2011; Villena et al., 2017). Other less investigated endpoints includes embryogenetic effects (Gualtieri et al., 2005; Mantecca et al., 2007) and biomarker responses related to, for instance, the detoxification of accumulated chemicals and/or their metabolites (Stephensen et al., 2003, 2005). Most recently, leachates from a variety of polymer materials were tested on the algae, *Raphidocelis subcapitata*, and the mussel, *Mytilus galloprovincialis*. This study showed differential toxicity between leached additives from MPs and TWP leachate, and found that TWP leachate was the most toxic of the tested materials (Capolupo et al., 2020). Compounds such as zinc and benzothiazole were detected in the TWP leachate and overall the higher toxicity could be attributed to the higher chemical content of organics and metals in TWP leachate (Capolupo et al., 2020). Another recent study examined the influence of four physical stressors on TWP leachate toxicity. Temperature and mechanical stress were shown to affect the toxicological impact of TWP toxicity, underlining the variable hazard of TWPs and its leachate that is likely to occur in different environments (Kolomijeca et al., 2020).

While research on tire-related toxicity has focused mainly on the effects of the leached fraction, growing attention is being placed on the role of TWPs as being potentially capable of eliciting

negative impacts following ingestion. Ingestion of TWPs was first observed in *Daphnia magna* (Wik & Dave, 2009) and later documented in *Gammarus pulex* (Redondo-Hasselerharm et al., 2018), *Hyalella azteca* (Khan et al., 2019) and opossum shrimps from the mysidae family (Halle et al., 2020). In the study by Khan et al. (2019), the freshwater crustacean *H. azteca* was shown to ingest TWPs indiscriminately with a gut retention time of 24-48 h. Although TWP-induced mortality followed an expected concentration-response curve, from which an LC_{50} of 3426±172 particles mL^{-1} (0.99 g L^{-1}) was determined, leachate-induced mortality did not conform to a sigmoidal pattern and therefore an LC_{50} was not derivable. Toxicity profiles of TWP suspension and leachate was sufficiently different to suggest a dissimilar mechanism of toxicity between particle and leachate exposures in the acute phase. Mortality, reproductive output (neonate production) and net growth were all significantly impacted at the higher exposure concentrations (500-2000 particles mL^{-1}, 0.15 to 0.59 g L^{-1}) following 21 d exposure to TWP suspensions. In contrast to this, exposure of freshwater benthic invertebrates to TWPs within sediment matrices showed overall negligible effects on the tested species (Panko et al., 2013; Redondo-Hasselerharm et al., 2018).

In the present study we determined the toxicity of pristine TWPs (P-TWPs) and its leachate to *H. azteca* in comparison to the toxicity of road worn TWPs (W-TWPs) and its leachate as previously detailed in Khan et al. (2019). TWPs were produced from the same make and model of tire. Acute and chronic toxicity endpoints were assessed in accordance with, and simultaneously with, our previous research (Khan et al., 2019). A suite of contaminants in P- and W-TWP suspensions and leachates was additionally analyzed to investigate whether the chemical content of the different tire types and fractions could explain any toxicological differences between treatments. Whilst recognizing that the vast majority of tire debris released into the environment will consist of W-

TWPs and that P-TWPs represent a miniscule proportion, the comparison is warranted considering the emerging investigations into the differential impacts caused by aged and weathered MPs compared to their pristine counterparts. For instance, Jaikumar et al. (2019) recently found that pristine primary MPs caused a greater impact on Cladoceran reproduction than weathered secondary MPs. Conversely, MPs taken from the marine environment are perceived as being more toxic than pristine MPs owing to their greater sorbed chemical load (Rochman et al., 2013). The only study to date to investigate the influence of tire aging on aquatic toxicity was conducted with only leachates and found that the road-worn tire leachate exhibited greater toxicity to rainbow trout (*Oncorhynchus mykiss*) compared to pristine and breakwater tire leachate (Day et al., 1993).

2. Materials and methods

2.1. Production and physical characterization of tire wear particles

Both worn-TWP (W-TWPs) and pristine TWP (P-TWPs) were produced as previously described in Khan et al. (2019). Briefly, TWPs were produced by grinding the surface of the respective pristine or road worn tire of the same make and model (Continental Premium Contact 5 50175/65 R14 82) using a course grind stone (size: 200mm x 25 mm) rotating at 4000 rpm. TWPs were then dry sieved through a 500 µm metal sieve prior to use. The worn tire had been used under Danish driving conditions for approximately 10,000 km. The two tires, although controlled for by make and model, were of different production batches and thus may have had minor variations in composition.

To accurately add TWP to the exposures, TWPs were dispersed in water at a concentration of 0.1 g mL^{-1} with use of a surfactant (0. 1% TWEEN80, Cospheric LLC). A ratio of TWP weight to particle number was determined by counting the number of particles in a set volume of the dispersion (10 µL) (Khan et al., 2015). Previously, the average W-TWP particle weight was

determined to be 0.29 µg/particle (Khan et al., 2019). The average particle weight for P-TWPs in the present study was determined as 0.51 µg/particle. The TWPs were imaged and photographed (NIKON SM218) to determine the average diameter of the TWPs using ImageJ software.

2.2. Preparation of leachates from tire wear particles

TWP leachate was prepared from 1 g of TWPs immersed in 10 mL deionized water and shaken vigorously for 60 s by hand. To mimic leaching during the acute biological experiments samples were submerged in a temperature regulated water bath at 25℃on a shaking platform (Thermo Scientific Precision SWB 27). After 48 h the leachate-TWP mixture was filtered through a 0.45µm SimplePure nylon syringe filter (Henke-Sass Wolf, United States), and the leachate collected. The particles in the filter were subsequently re-suspended and rinsed with an additional 5 mL of deionized water. The two leachate fractions were pooled, resulting in a total leachate volume of 15 mL.

2.3. Extraction and digestion procedures of particle and leachate fractions

Extracts of TWPs and leachates were analyzed with gas chromatography mass spectrometry (GC-MS, Agilent 7890B GC with a 5977A MS and a 7693A ALS) to quantify organics. The analyzed organics were the US-EPA's 16 priority PAHs (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, ideno(1.2.3-cd)pyrene and benzo[ghi]perylene) and three non-PAH organic compounds (1-octanthiol, benzothiazole and 1-indanone) selected based on a non-target GC-MS screening of the extracts

combined with a literature search on ecotoxicologically relevant compounds. To quantify metals, inductively coupled plasma mass spectrometry was used (ICP-MS, Agilent 7700x equipped with an ASX-500 auto-sampler in collision mode with Helium). The investigated metals were aluminum (Al), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), lead (Pb), mercury (Hg), nickel (Ni) and zinc (Zn). All analyses were performed in triplicates.

2.3.1. Extraction of organic compounds from particles and leachates

PAHs were extracted from 1 g of TWPs in 7.6 mL of ultrapure cyclohexane (Rathburn chemicals Ltd., Walkerburn, United Kingdom). PhenanthreneD10, fluoranthreneD10, benzo(a)pyreneD12 and dibenzo(a,h)anthraceneD14 were added as internal standards at a concentration of 100 ppb (Chem Service, West Chester, Pennsylvania, United States). The samples were ultrasonicated for 2 h after which 1 mL of supernatant was transferred to a GC vial for analysis (section 2.4.1). The three selected non-PAH organic compounds, 1-octanethiol. benzothiazole and 1-indanone, were extracted using the same procedure as the PAHs, except the internal and calibration standards differed. An internal standard of 2.5 ppm dibromooctafluorobiphenyl (Aldrich Chemistry, United States) was added to each sample and pure analytical reference standards for each compound (>97%, Merck) were obtained for quantification by calibration curves (0-1000 ppb in cyclohexane).

To extract organics from the prepared leachate solution (section 2.2.) 1 mL NaOH (pure grade diluted to 5M, VWR, Radnor, Pennsylvania, United States) was added to prevent protonation of cyclic compounds before 5 mL cyclohexane was added. This mixture was shaken and left for phase separation to occur. Once the phases had separated, two organic phase samples were analyzed. To analyze PAHs 0.5 mL of the leachate extract was diluted in 0.5 mL cyclohexane (1:1), and to

examine 1-octanethiol, benzothiazole and 1-indanone 1 mL of leachate extract was transferred undiluted to a GC vial prior to analysis (section 2.4.2).

2.3.2. Digestion of metals from particles and leachates

Metal analysis was conducted by digesting 0.50 g of TWPs in 10 mL aqua regia (5:3:1 mixture of $HCl:H_2O:HNO_3$ containing a Rh procedural standard). This was followed by 40 min heating (up to 120℃) in a microwave (Multiwave ECO, Anton Paar GbmH) to ensure complete digestion. Samples were diluted 1:51 with a rinsing solution (9:0.3:0.15 ratio of $H_2O:HNO_3$:HCl containing an Au procedural standard) prior to ICP-MS analysis with three internal standards added.

To examine metals from the leachate, 5 mL of leachate solution (section 2.2.) was digested with 10 mL aqua regia solution. Rh procedural standard was added before the samples were digested and samples were diluted prior to ICP-MS analysis as described for particles.

2.4. Chemical analysis of particle and leachate fractions

2.4.1. Analysis of organic compounds

Appropriate columns, standards and GC-MS procedures were used for the 16 PAHs and three non-PAH organics, respectively. To quantify PAHs a standard calibration mix, 'PAH Calibration Mix' (Supelco, Bellefonte, Pennsylvania, USA), was used and a standardized PAH detection procedure was applied using an Agilent J&W Select PAH capillary column (30 m x 0.25 mm, df=0.15µm). A selected ion monitoring (SIM) method was optimized to distinguish and quantify the three non-PAH organic compounds using an adjusted GC program. The selected organic constituents were: 1-octanethiol (CAS: 111-88-6), benzothiazole (CAS: 95-16-9) and 1-indanone (CAS: 83-33-0). The

ions detected for the SIM-method was 146 for 1-octanethiol, 135 for benzothiazole and 132 for 1-indanone. The analysis of the three compounds was carried out using an Agilent J&W DB-XLB 122-1232 capillary column (30 m x 250 µm, df=0.25 µm) with the following GC-parameters. Flow: 1.7 mL min^{-1}, inlet temperature: 300°C, carrier gas: He, injection volume: 1 µL, and the temperature program was as follows: 45°C in 2.25 minutes, 40°C increase per minute from 45°C to 250°C then 3 minutes hold.

2.4.2. Analysis of metals

All acids used during analysis of metal content in TWPs and associated leachates were PlasmaPURE grade (SCP Science, Quebec, Canada). Two procedural standards were added to monitor any loss during digestion and analysis. These were 1000 ppm Rhodium (Rh) for the digestion procedure and 1000 ppm gold (Au) for the analysis (Sigma-Aldrich, Saint Louis, Missouri, United States and SCP Science, Quebec, Canada, respectively). Three internal standards were added to each sample in a concentration of 1 ppm (scandium (Sc), indium (In) and lutetium (Lu), SCP Science, Quebec, Canada) before the samples were analyzed for a suite of nine elements. The calibration standards used to quantify the investigated metals was PlasmaCAL custom standard (SCP Science, Quebec, Canada).

2.5. Toxicity experiments

2.5.1. Maintenance conditions of Hyalella azteca culture

Hyalella azteca were retrieved from an established laboratory culture at Roskilde University and maintained in artificial freshwater medium in two 10 L aquariums. Freshwater medium was prepared from stock solutions of $NaHCO_3$ (84.05 mg L^{-1}), NaBr (1.032 mg L^{-1}), KCl (3.73 mg L^{-1}),

$CaCl \cdot 2H_2O$ (147.04 mg L^{-1}) and $MgSO_4 \cdot 7H_2O$ (61.68 mg L^{-1}) dissolved in demineralized water and diluted 1:300 in tap water and demineralized water at a 1:3 ratio (Pedersen et al., 2013). The culture was maintained at 25℃ with a 16:8 light:dark photoperiod, fed with rabbit chow (Chrisco, Køge, Denmark) twice a week.

2.5.2. Preparation of TWP and leachate exposures

TWPs were coated with a surfactant (0.1% Tween80) and shaken at 300 rpm on an orbital shaker (Janke & Kunkel, KS 250) 48 h prior to use in particle exposure treatments. Particles were then rinsed three times in demineralized water to eliminate excess surfactant before *H. azteca* exposures. Similarly, leachate exposures were prepared by first coating particles in surfactant and rinsing them three times in demineralized water before dispersion of the particles in freshwater media. Particles were added to 50 mL media and left under the same conditions (25℃) as the acute toxicity particle exposure for 48 h, to mimic the leaching that would occur in the particle exposures. Lastly, the particles were sieved from the solution using a 1 µm nylon mesh and the leachate was distributed among the exposures for the leachate exposures to begin.

2.5.3. Acute toxicity exposures

Acute mortality from P- and W-TWP suspensions and leachates to juvenile (7-9 d) *H. azteca* was determined according to Khan et al. (2019). Experiments were performed in '*Daphnia magna* multi-well plates' (Dapthox kit, MicroBioTests Inc.) with five individuals per well, four wells per concentration on each plate, and three replicate plates (i.e. 20 organisms per concentration x 3 replicates, n=3). The concentration range for W-TWP suspension exposure was 0-15000 particles mL^{-1} (4.45 g L^{-1}) and the range for W-TWP leachate exposure corresponded to leached chemicals from 0-125000 particles mL^{-1} (37.01 g L^{-1}), as previously reported by Khan et al. (2019). Here, the

concentration range for acute P-TWP suspension exposure was 0-5000 particles mL^{-1} (2.55 g L^{-1}) and P-TWP leachate exposure range corresponded to the leached chemicals from 0-50000 particles mL^{-1} (25.52 g L^{-1}).

2.5.4. Long-term toxicity exposures

Long-term exposures of *H. azteca* to particles were conducted as 21-d static renewal tests with 21-23 d old organisms, using mortality, reproduction and growth as endpoints (Khan et al., 2019). The concentration range for long-term experiments using W-TWP suspensions were previously 0-2000 particles mL^{-1} (0.59 g rubber L^{-1}) (Khan et al., 2019), whereas in the present study concentrations of 0-250 particles mL^{-1} of P-TWPs (0.13 g rubber L^{-1}) were used. Exposures were conducted in 250 mL glass beakers filled with 150 mL freshwater media, where media and particles were changed weekly. Exposure beakers had individual constant air-supplies, and temperature and photoperiod were the same as laboratory culture. Mortality and reproduction was recorded weekly from test beakers containing 10 randomly assigned organisms per beaker (n=5). Growth was recorded separately under the same set-up conditions as above, containing one *H. azteca* per beaker in order to track growth of the individual organisms (n=10). Each individual's length was determined from eye to uropod ('tail') as an average of three measurements at each time point using Image J software.

2.6. Statistical analysis

Assumptions of normality were tested using Kolmogorov-Smirnov and equal variance was assessed based on visual inspection of homoscedasticity and residual plots using GraphPad Prism 8. Chemical data of particles and leachate were tested compound by compound in a two-way ANOVA

with tire type (worn and pristine) and fraction (particle and leachate) as variables. The concentrations of organic compounds and metals in particles and leachate of the two tire types are presented in Table 1. All concentrations are expressed in ppm (µg g^{-1} tire rubber) ± 95% CL.

In acute toxicity experiments calculation of LC_{50} values were only applicable to the particle exposures, as leachate exposure did not conform to a sigmoid-shaped concentration-response curve. For acute particle data LC_{50} values were derived with 95% confidence limits using R 'drc' package (Ritz & Streibig, 2005). For all acute exposures no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values were determined, for each tire type and fraction with concentration as variable, by a one-way ANOVA followed by a post-hoc Dunnett test. Potential differences in long-term mortality were tested using a two-way ANOVA for each tire type with time and concentration as variables. The biological endpoints, reproduction and growth, were tested using two-way ANOVAs with tire type and concentration as variables. When the ANOVA showed significant differences on the interaction a Tukey post-hoc test was used to establish pair-wise differences between treatment groups. A significance level of 0.05 was used throughout. ANOVAs were performed on GraphPad Prism v8 (GraphPad Software, CA, United States).

3. Results

3.1. Physico-chemical characteristics

3.1.1. Size distribution of particles

P-TWPs and W-TWPs had an average diameter of 210 ± 116 µm and 176 ± 120 µm, respectively (n=100, Figure 1A). The median diameter of P-TWPs was 199 µm whereas the median diameter of W-TWPs was 146 µm, skewing the distribution of the majority of W-TWPs towards a slightly

smaller size fraction than the majority of P-TWPs. This is also reflected from the particle weights (section 2.1) where W-TWPs on average weigh less than P-TWPs.

3.1.2. Chemical content of particles

In W-TWPs the three non-PAH organics were all present, ordered by decreasing concentration as benzothiazole > 1-indanone > 1-octanethiol. The content of these were summed to 31.61 ± 5.73 µg g^{-1}, which corresponded to ~74%, ~20% and ~6% of the total non-PAH organic content, respectively. Of the 16 US-EPA priority PAHs were pyrene > fluoranthene > phenanthrene > benzo[ghi]perylene > anthracene had the highest concentration. These five PAHs summed to 60.1 ± 0.12 µg g^{-1} out of a total of 65.40 ± 0.14 µg PAH g^{-1}, which is ~92% of the total PAH load in W-TWPs. The three metals of highest concentration in W-TWPs were Zn >Fe > Al equating to 87%, 8.5% and 3.4% of the total metal content, respectively. Zn accounted for ~8300 µg g^{-1} of a total combined metal concentration of 9500 µg g^{-1}.

In P-TWPs the three non-PAH organics were also present but the concentration of 1-octanethiol was significantly increased by a factor of 8.2 compared to W-TWPs ($p<0.01$), changing the order to benzothiazole > 1-octanethiol > 1-indanone, for a sum of 40.44 ± 3.42 µg g^{-1}. This corresponded to ~50%, ~37% and ~13% of the total non-PAH organics content, respectively. P-TWPs also differed from W-TWPs in their ranked concentration order of the 16 US-EPA priority PAHs as follows: pyrene > phenanthrene > fluoranthene > anthracene > benzo[ghi]perylene, for a sum of 70.1 ± 0.21 µg g^{-1} out of a total PAH concentration in P-TWPs of 74.18 ± 0.23 µg g^{-1} (~95% of the total PAH load). The metals of highest concentration in P-TWPs were Zn>Fe>Al as with W-TWPs. Again, Zn contributed most (85%). Fe and Al contributed 9.3% and 5.1% of the total metal load, respectively. Many of the measured compounds were only present in the TWP (particle) and not in leachate.

Three compounds, benzo(b)fluoranthene, benzo(k)fluoranthene, and benzo(a)pyrene, were only measured in W-TWPs, albeit in low concentrations.

3.1.3. Chemical content of leachates

The chemical composition and load of TWP leachates were similar between tire types. The compounds tested that readily leached from W- and P-TWPs included two tire-associated organics, benzothiazole and 1-indanone, and two metals, Al and Zn. Independent of tire type, PAHs did not generally leach from the particle to freshwater media, and only few PAHs could be measured above detection limits in the leachate fractions.

Generally, the measured group of metals did not tend to leach to the surrounding media, with an average of 0.4‰ of the total particle metal content being released. Only two metals, Al and Zn, were detected in the leachate fraction. Of the leached metal fraction, averaged across both tire types, Zn and Al represented ~86 % and ~13 %, respectively.

The chemical compounds that leached most readily were the 'non-PAH organics' group, although 1-octanethiol did not leach out into the water. On average, 14% of the total non-PAH organic particle load leached from particles into the water, independent of tire type, with benzothiazole and 1-indanone contributing ~75 % and ~25 %, respectively. When comparing the specific compounds' leachability directly from particle concentration to leachate ~16% of benzothiazole leached from W-TWPs to W-TWP leachate and ~19% leached from P-TWPs to P-TWP leachate. Likewise, 1-indanone showed a trend of increased percentage leaching from P-TWPs with ~16% leaching from W-TWPs to leachate and ~28% from P-TWPs to leachate.

3.1.4. Comparison of particle and leachate content (tire type and fraction)

The chemical composition of the different tire types' particle fractions were similar, as were the summed chemical loads. However the individual contributions of single compounds did fluctuate between tire types. Most noticeably of the total organics, 1-octanethiol, phenanthrene and anthracene were all more abundant in P-TWPs than W-TWPs ($p<0.001$ for all), whereas pyrene was more abundant in W-TWPs than in P-TWPs ($p<0.001$). Hg, Pb, Zn, Fe and Cu showed no significant differences in particle concentrations between tire types ($p=0.275$ for Cu and $p>0.99$ for the rest). Al, Cr, Cd and Ni concentrations were significantly higher in P-TWPs compared to W-TWPs ($p\leq0.05$ for all), albeit most were in low concentrations. Four out of 28 compounds leached out independently of tire type, these were benzothiazole, 1-indanone, aluminum and zinc.

3.2. Effects of TWP and leachate exposure to *Hyalella azteca*

3.2.1. Acute toxicity

A dissimilar concentration-response pattern was observed when comparing the acute toxicities of tire types (W-TWP and P-TWP) and their respective fractions (particle suspensions and leachate) (Figure 2). Mortality in the 48 h controls averaged close to 10% per replicate with a maximum of three out of twenty *H. azteca* deceased.

A 48 h LC_{50} of 0.91 ± 0.06 g L^{-1} (equivalent to 3073 ± 211 particles) and 0.19 ± 0.03 g L^{-1} (equivalent to 364 ± 64 particles), was determined for W-TWP and P-TWP suspensions, respectively. Thus, acute exposure to P-TWP suspensions results in greater toxicity to *H. azteca* compared to exposure to W-TWP suspensions (Figure 2A), both in relation to TWP weight and particle number. This is also the tendency when comparing NOEC and LOEC values for W-TWP

suspensions and P-TWP suspensions. The NOEC and LOEC values for W-TWP suspensions were determined to be 3000 and 4000 particles mL^{-1}, respectively (0.87 and 1.16 g L^{-1}), and 500 and 1000 particles mL^{-1} (0.26 and 0.51 g L^{-1}) for P-TWP suspensions. This corroborates the result that exposure to P-TWP suspensions leads to greater toxicity to *H. azteca* than exposure to W-TWP suspensions.

Toxicities from exposure to P-TWP and W-TWP leachate were similar, with data points overlapping throughout the tested concentration range (Figure 2B). The NOEC and LOEC values for W-TWP leachate were 500 and 1000 particles mL^{-1} (0.14 and 0.29 g L^{-1}), respectively. The LOEC value for P-TWP leachate was estimated to be 500 particles mL^{-1} (0.26 g L^{-1}), whereas a NOEC value for P-TWP leachate could not be estimated, as 500 particles mL^{-1} was the lowest particle density tested for P-TWP in the leachate test. Considering the lower particle density required to elicit a toxic response, this suggests that P-TWP leachate toxicity was higher than W-TWP leachate toxicity, although the graphical representation does not show this. When expressed on a weight basis, the LOEC particles concentrations of 1000 W-TWPs (0.29 g L^{-1}) and 500 P-TWPs (0.26 g L^{-1}) for W-TWP and P-TWP leachate, respectively, are similar.

When comparing fraction exposures (particle suspension or leachate) of the same tire type, we observe dissimilar acute toxicities depending on tire type. In the case of W-TWP suspension versus W-TWP leachate, the leachate fraction is more toxic at low concentrations, whereas particles become more toxic at the higher concentrations (Figure 2C). This difference in toxicity at lower exposure concentrations is corroborated by the NOEC and LOEC values with W-TWP leachate values (500 and 1000 particles mL^{-1}, respectively) being lower than W-TWP suspension values

(3000 and 4000 particles mL^{-1}, respectively). Conversely, the exposure of *H. azteca* to P-TWP suspensions and corresponding leachates showed a pattern of the particles being equally or more toxic than the leachate throughout the tested concentration range (Figure 2D). However, this is not supported by the LOEC values of P-TWP suspension and leachate exposures indicating that leachate toxicity (LOEC of 500 particles mL^{-1}) is higher than particle toxicity (LOEC of 1000 particles mL^{-1}). Note, however, the pronounced differences in variability around means between the two exposure types at the LOEC concentrations, which diminishes statistical power to discriminate between the treatments in the comparison of estimated LOEC values.

3.2.2. Long-term toxicity

The survival of *H. azteca* over a 21 d exposure was not significantly affected by the tested concentration range (0-250 particles mL^{-1}) of W-TWP ($p=0.238$) or P-TWP ($p=0.970$) (Figure 3A). Mortality in the control groups did not exceed 4 %. When conducting a two-way ANOVA, significant interaction was found between tire type and concentration for *H. azteca* growth ($p=0.003$). To further examine the relationships a post-hoc multiple comparison Tukey-test was conducted on this interaction, revealing a significant difference between the highest exposure concentration, of 250 particles mL^{-1} (0.13 g L^{-1}) of P-TWPs, to all other treatments (all p-values ≤ 0.033, Figure 3B). Net growth for P-TWP exposure thus diminished from 20.8 ± 5.5 % in control treatments to 5.7 ± 3.2 % in the highest concentration of 0.13 g L^{-1}. Conversely, no effects were seen for reproduction. Although the total reproductive output for the highest test concentration of P-TWPs was very low (Figure 3C), there were no statistically significant differences on either interaction or main effects of tire type and concentration when analyzing reproduction by a two-way ANOVA (Interaction term, $p=0.123$; tire type, $p=0.454$; concentration, $p=0.218$).

4. Discussion

The present study demonstrated that TWPs in the aquatic environment may affect the acute mortality and long-term growth in *H. azteca*. Particle suspensions were generally more toxic than leachate, with a lower test range (up to ~1000 particles mL^{-1}) of W-TWP leachate being the exception, a pattern that can likely be ascribed to the higher bioavailability of soluble compounds. P-TWP suspensions were more toxic than W-TWP suspensions, most likely due to a number of chemical compounds being more abundant in P-TWPs. Based on previous research (Day et al., 1993) our expectation was, that the fractions of the worn tire (TWP suspension and leachate) would be of higher toxicity than the fractions of the pristine tire, since road-worn TWPs might accumulate pollutants from the road surface during their lifespan. This would be similar to what has been described with the sorption of exogenous chemicals as seen for weathered MPs compared to virgin MPs (Rochman et al., 2013; Velez et al., 2018). However, our expectations were not corroborated. The differing chemical profiles of the worn- and pristine- TWP and leachate fractions is the most plausible explanation of the differential toxicities.

Specifically, we found that the summed chemical concentrations were higher in particles compared to leachate independent of tire type. In W-TWP fractions, the summed difference in concentration from W-TWP suspensions to W-TWP leachate was by a factor of ~1400, and similarly the summed difference was by a factor of ~900 from P-TWP suspensions to P-TWP leachate. The chemical composition of tire particles was similar between the two tire types, but the concentrations of the present chemicals differed along with the leachable portion of compounds, resulting in unique characteristics for the two different fractions. The main differences in the chemical content of the particle fractions were higher concentrations of 1-octanethiol, phenanthrene, anthracene and

aluminum in P-TWPs compared to W-TWPs. Each of these are plausible causative agents explaining the higher toxicity observed in the biological exposures of P-TWP suspensions. The organic compounds did not readily leach out from TWPs to the aqueous media. This suggests that the lower acute toxicity of P-TWP leachate compared to P-TWP suspensions is explained by these chemicals not being released by the P-TWPs into water. However, the measured chemical loads could not explain the differences in toxicity between W-TWP suspensions and its leachate. The summed chemical concentration in W-TWP particles was higher than in the leachate (derived from the same mass of particles), but exposure to W-TWP leachate was more toxic at lower concentrations compared to particle exposure. This could be explained by the presence of distinct chemical compounds with a higher propensity to leach that are found only or more abundantly in W-TWPs. In this study we can most likely exclude benzothiazole, 1-indanone, Al and Zn as reasons for increased W-TWP leachate toxicity, as the concentration of these chemicals in the leached fractions were relatively similar between the two tire types. Considering the total number of compounds used in tire manufacturing, many other eligible candidate compounds could presumably be found.

The observed difference in biological responses, possibly caused by differences in chemical content, is also seen for TWPs when comparing weathered and virgin crumb rubber (CR) in soil. Here, the virgin CR affected earthworms by impacting their growth (Pochron et al., 2017), whereas weathered CR reduced their survival time (Pochron et al., 2018). In this study, the P-TWPs also caused decreased growth and at a lower concentration than W-TWPs. Another study on aquatic toxicity found that W-TWP leachate exposure to *O. mykiss* exhibited greater toxicity than P-TWP leachate (Day et al., 1993). We, however, did not observe this in the current study when comparing the acute toxicity of W-TWP leachate with P-TWP leachate. Nevertheless, Day et al. (1993) found

differences in the toxicity to different species exposed to pristine or road-worn tire leachates. Not all tested organisms were equally sensitive to the different tire leachates, but some reacted to all treatments. Inhibition of enzymatic activity in screening assays furthermore indicated that some metabolic processes were sensitive to these compounds. Interestingly, two of these assays showed that pristine tire leachate were more toxic than road worn tire leachate (Day et al., 1993), highlighting the complexity of cause-effect relationships within tire wear toxicology. Differences in toxicity across treatments was similarly found in this study, underlining the continued need to better understand the bioavailability of single compounds and chemical mixtures, both when in suspension and bound in or to particles that can be ingested.

Follow-up studies were conducted by Day et al. (1993) to assess if the toxic substances affecting *O. mykiss* were volatile and it was suggested that the compounds of interest were water-soluble, non-volatile and slow to degrade. Wik and Dave (2006) also found that leachate toxicity to *D. magna* was reduced after running the leachate solution through a C18 SPE column, indicating that toxicity was caused by non-polar organic compounds. Bartlett et al. (2012) furthermore investigated the toxicity of storm water runoff (a mixture of PAHs and metals) on *H. azteca*. Here several metals were measured at concentrations exceeding the Canadian water guidelines and effect concentrations, but they concluded that the bioavailability, measured as bioaccumulation, to *H. azteca* remained low. One of the contributing factors to this was the decrease in metal concentration from particulate to dissolved phase (0.7–40,000 $\mu g\ g^{-1}$ in sediment to 0.04–300 $\mu g\ L^{-1}$ in filtered pond water) (Bartlett et al., 2012). This indicated that most metals were bound to the particulate fraction in water. The low propensity for metals to leach into the surrounding media is corroborated by the findings in this study, where only a small fraction of the metal load in particles was found to

leach out, and predominantly Zn and Al. The number of "compounds of interest" in relation to TWP suspensions and TWP leachate could thus be reduced to Zn, Al and maybe a few PAHs.

Some PAHs used in tire manufacturing and extender oils are already regulated in the European Union at a maximum concentration of either 1mg kg^{-1} of benzo(a)pyrene or 10 mg kg^{-1} as a sum of 8 PAHs (European Commision, 2006). Hence, future investigations could focus on investigating the numerous other chemicals used in tire manufacturing that we do not yet fully understand in relation to TWP suspensions and TWP leachate. For example, the use and possible ecotoxicological effects of 2-mercapto-benzothiazole as an antioxidant and curing agent in tires is important to elucidate. Partly because its primary degradation product, benzothiazole, has been located 100 km downstream of a tire production plant at a concentration of 20 mg/L (Puig et al., 1996). As shown in this and other studies benzothiazole compounds readily leach from TWPs (He et al., 2011; Llompart et al., 2013; Liao et al., 2018; Asheim et al., 2019; Halsband et al., 2020) and can thus be expected to be found anywhere that TWPs are located. In this study we also found benzothiazole and other non-PAH organics, in both particulate and leachate fractions, making these compounds suitable candidates for further investigation. Benzothiazoles have previously been described as a metal chelating agent (De Wever & Verachtert, 1997), and is used as a curing agent interacting with Zn. A recent review describes the interest in reducing the use of Zn-based curing activators (e.g. benzothiazoles) in the rubber vulcanization process. As the use of ZnO is high and the product is not fully consumed in the process, chemicals are thus available for leaching to the environment (Mostoni et al., 2019).

Considering that benzothiazole and Zn represent two of four chemicals found in the leachate in this study, it underlines the importance of studying the toxicity effects of these further. While both bioavailable metals and non-PAH organics are highly relevant in the field of TWP toxicity, benzothiazoles, other non-PAH organics and their metabolites are particularly interesting as they are usually found through non-target screenings of TWPs (Marwood et al., 2011; Capolupo et al., 2020; Tian et al., 2021). As the overall concentration of PAHs present in the two tested tires was low and the literature generally describes bioavailability of particle bound metals as low (Deaver & Rodgers, 1996; Warren et al., 1998; Bartlett et al., 2012), the need to fully uncover the remaining chemicals used in tire manufacturing is emphasized. The group of 'non-PAH organics' could hold a special interest as it is not regulated nor tested on a regular basis. Ensuring the development of continuous knowledge and expertise in this area of particle and leachate pollution will provide for a good scientific foundation in this currently under-investigated field of research.

Our focus on the chemical differences between treatments is in keeping with the current trend of research related to TWP and leachate toxicity. However, it would be remiss to not recognize that toxicities caused by TWP suspension exposure could also result from the ingestion of the tire rubber particle. A variety of organisms have been shown to indiscriminately consume TWPs (Wik & Dave, 2009; Redondo-Hasselerharm et al., 2018; Khan et al., 2019), which raises the possibility that the tire wear particle may act as a 'Trojan horse' delivering the chemical load into the organism to leach *in vivo* (Khan et al., 2019). But also, that the particle may cause a physical irritation in the *H. azteca* digestive tract. The observed reduction in growth rate (Figure 3) may suggest an effect resulting from impaired feeding behavior or altered nutritional intake. Similarly, the congruence between W-TWP and P-TWP suspension toxicity curves at higher particle concentrations (Figure 2A) may also suggest a common mechanism related to the physical nature of the particle, again potentially indicating feeding impairment or even respiratory distress. Such explanations, of course,

require further investigation, but highlight the need to recognize that TWPs exist simultaneously as waterborne contaminants that leaches a suite of chemicals and as anthropogenic particulates that are consumed.

5. Conclusion

The present study demonstrated that TWPs in the aquatic environment may affect the acute mortality and long-term growth in *H. azteca*. Furthermore, it showed that particle suspensions are generally more toxic than leachate, with a lower test range (up to ~1000 particles mL^{-1}) of W-TWP leachate being the exception, a pattern that can likely be ascribed to the higher bioavailability of soluble compounds. P-TWP suspensions were more toxic than W-TWP suspensions, most likely due to a number of chemical compounds being more abundant in P-TWPs. These include 1-octanethiol, anthracene, phenanthrene and Al. The lower toxicity of leachates from worn and pristine tires, in comparison to suspended TWPs, is probably due to the absence, or very low concentration, of these compounds in leachates, along with a generally lower PAH and metal concentration. Four of the tested compounds were able to leach at 25℃, these were benzothiazole, 1-indanone, Al and Zn. The highest concentrations employed in this study likely exceed environmental realism, however, it should be noted that empirical data on TWPs in the aquatic environment is exceedingly scarce and is analytically challenging (Halle et al., 2020). Nonetheless, our results highlight the importance of understanding road use, weathering and the life cycle of a tire when assessing the potential ecotoxicological impact of TWPs and their leachates in the aquatic environment.

Author Contribution List:

Louise Lynn Halle: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Validation; Visualization; Writing - original draft, Writing - review & editing.

Annemette Palmqvist: Conceptualization, Funding acquisition, Methodology, Project administration, Writing - review & editing, Supervision.

Kristoffer Kampmann: Supervision, Methodology, Funding acquisition.

Anders Jensen: Methodology, Data curation; Formal analysis.

Tobias Hansen: Methodology, Data curation; Formal analysis.

Farhan R. Khan: Conceptualization; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Writing - original draft; Writing - review & editing.

Declaration of interests

The authors declare that they have no known competing interests or personal relationships that could have appeared to influence the work reported in this paper. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Figures and tables

Figure 1. Morphology and size distribution of tire wear particles.

Size distribution (measured as the longest diameter) is shown for worn (W-TWP, dark grey bars) and pristine (P-TWP, light grey bars) tire wear particles, respectively (A). Imaged W-TWPs (B) and P-TWPs (C) were determined to have an average size of 176 ± 120 µm and 210 ± 117 µm respectively (n=100). The median was 146 µm for W-TWPs and 199 µm for P-TWPs. W-TWPs generally weigh less (0.29 µg particle^{-1}) than P-TWPs (0.51 µg particle^{-1}).

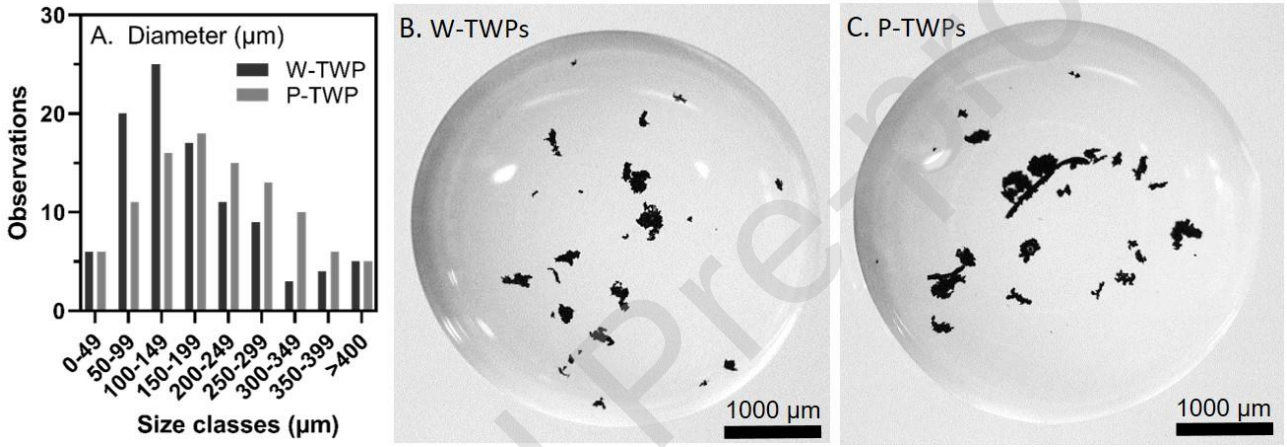

Figure 2. Acute (48 h) mortality of tire wear particles and leachate exposure to *Hyalella azteca.* Concentration-response exposures of worn (W-TWP, dark grey) and pristine (P-TWP, light grey) tire wear particles (closed circles) and their respective leachates (closed triangles) after 48 hours to *Hyalella azteca*. Data points in the four graphs represent means with error bars denoting SD. W-TWP data is that of Khan et al. (2019). Comparison of TWP fractions from the two tires show that P-TWPs have a higher overall toxicity compared to W-TWPs (A) whereas the toxicity of the leachate fractions is similar between tire types (B). When comparing the two fractions of each tire, dissimilar patterns are observed. Here, W-TWPs have a lower toxicity than W-TWP leachate at lower exposure concentrations, but higher toxicity than W-TWP leachate at higher exposure concentrations (C). Conversely, P-TWP suspensions have a higher toxicity compared to P-TWP leachate across the entire exposure range (D).

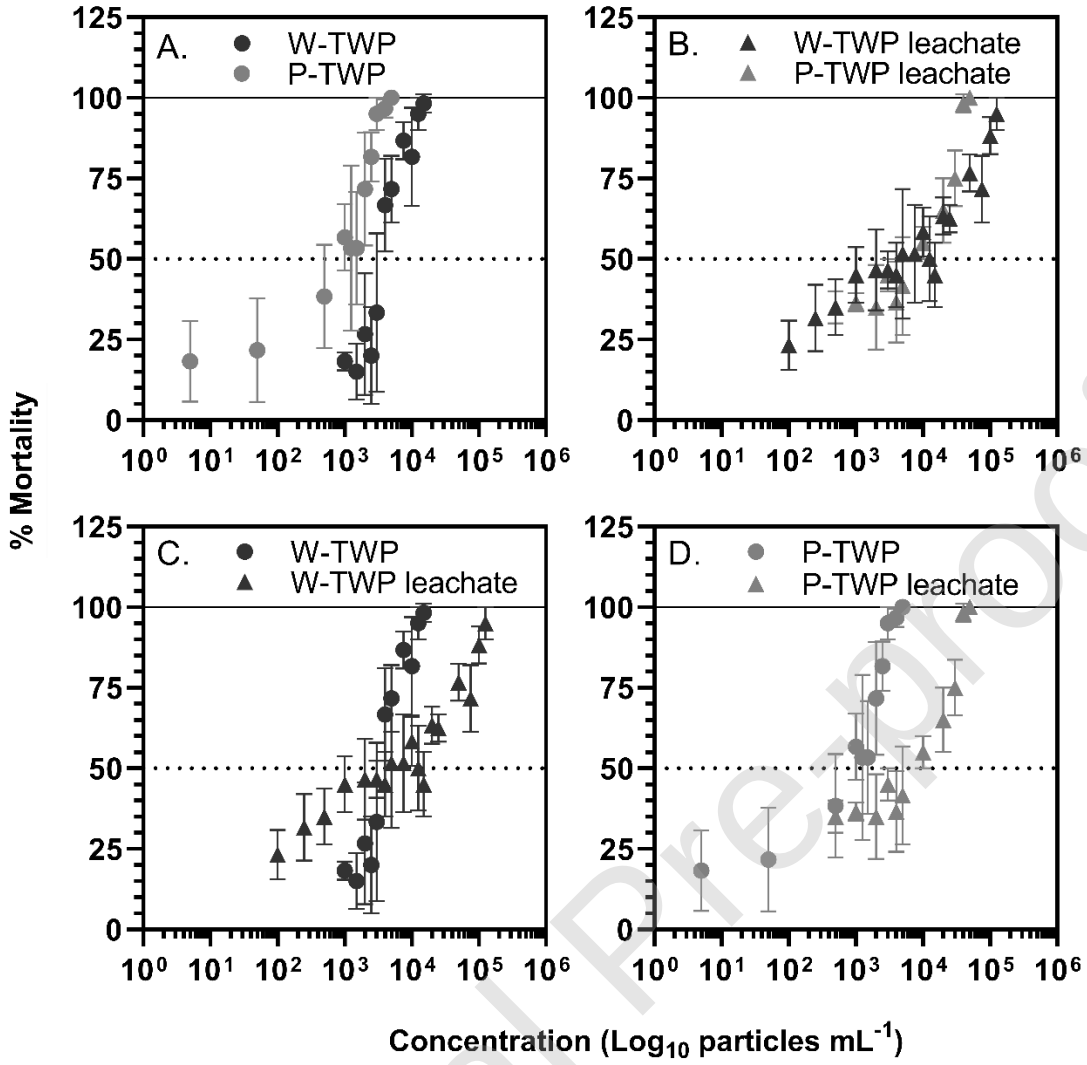

Figure 3. Long-term effects of exposure to worn (W-TWP) and pristine (P-TWP) tire wear particles. Survival, growth and reproduction effects measured after a 21 d static renewal test exposing *Hyalella azteca* to tire particles from W-TWPs (dark grey) or P-TWPs (light grey). W-TWP data is that of Khan et al. (2019). Survival (A, n=5 each with 10 organisms) was scored by observing a heartbeat under stereomicroscope, the bars show mean survival ± 95% CL. Growth (B, n=10 individual organisms) was measured as net growth in percentage from day 0-21 of the experiment. Reproduction (C, n=5 each with 10 organisms) was measured as total reproductive output over the entire exposure period, The boxplots illustrates 25th, 50th and 75th percentiles with the dots representing each individual datapoint, the bars show minimum and maximum values.

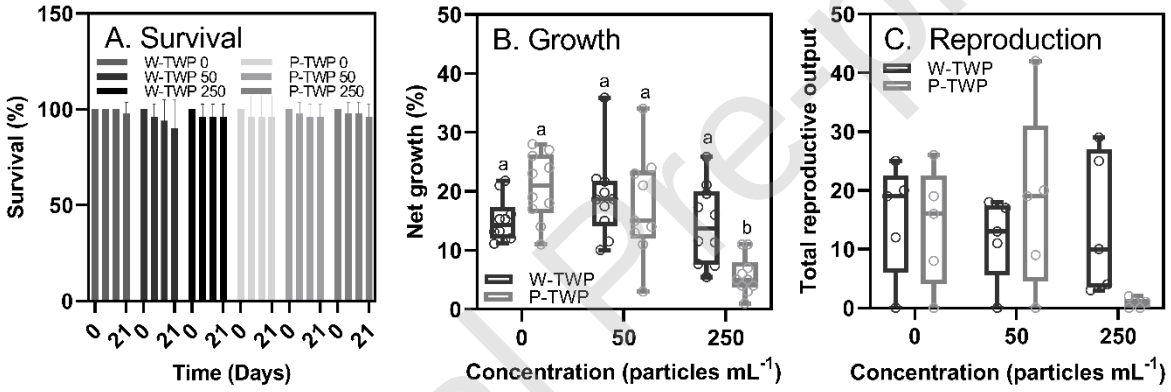

Table 1. Chemical analysis of worn and pristine tire particles and leachate.

Chemical content of particles and leachate from worn- (W-TWP) and pristine (P-TWPs) tire wear particles, all units expressed as µg/g ± 95% CL (n=3 for all samples).

GROUP	LOD	LOQ	COMPOUND	WORN TIRE		PRISTINE TIRE	
				Particle suspensions [µg/g]	Leachate [µg/g]	Particle suspensions [µg/g]	Leachate [µg/g]
Organics	0.002	0.01	1-octanethiol	1.82 ± 0.89	<0.01	14.96 ± 2.34	<0.01
	0.24	0.79	Benzothiazole	23.33 ± 2.77	3.81 ± 0.73	20.22 ± 1.27	3.90 ± 0.91
	0.001	0.005	1-indanone	6.46 ± 2.07	1.09 ± 0.22	5.26 ± 0.26	1.46 ± 0.32
	∑ Non-PAH organics			31.61 ± 5.73	4.90 ± 0.95	40.44 ± 3.86	5.36 ± 1.23
	0.002	0.01	Naphtalene	0.42 ± <0.01	<0.01	0.45 ± <0.01	0.01 ± <0.01
	0.02	0.08	Acenaphthylene	0.82 ± <0.01	<0.01	1.26 ± <0.01	<0.01
	0.02	0.08	Acenaphthene	0.38 ± <0.01	<0.01	0.38 ± <0.01	<0.01
	0.02	0.08	Fluorene	0.47 ± <0.01	<0.01	0.51 ± <0.01	<0.01
	0.02	0.08	Phenanthrene	5.32 ± 0.04	0.02 ± <0.01	17.06 ± 0.17	0.01 ± <0.01
	0.02	0.08	Anthracene	2.27 ± <0.01	<0.01	6.91 ± 0.07	<0.01
	0.02	0.07	Fluoranthene	10.15 ± 0.07	<0.01	12.68 ± 0.13	<0.01
	0.02	0.08	Pyrene	37.99 ± 0.25	<0.01	29.99 ± 0.31	<0.01
	0.02	0.08	Benzo[a]anthracene	0.41 ± <0.01	<0.01	0.87 ± <0.01	<0.01
	0.02	0.08	Chrysene	0.61 ± <0.01	<0.01	0.16 ± <0.01	<0.01
	0.01	0.03	Benzo(b)fluoranthene	0.70 ± <0.01	<0.01	<0.01	<0.01
	0.01	0.02	Benzo(k)fluoranthene	0.14 ± <0.01	<0.01	<0.01	<0.01
	0.01	0.02	Benzo(a)pyrene	0.41 ± <0.01	<0.01	<0.01	<0.01
	0.01	0.02	Dibenzo(a.h)anthracene	<0.01	<0.01	<0.01	<0.01

	0.01	0.02	Indeno(1.2.3-cd)pyrene		0.51 ± <0.01	<0.01	0.48 ± <0.01	<0.01
	0.02	0.08	Benzo[ghi]perylene		4.37 ± 0.03	<0.01	3.43 ± 0.03	<0.01
	∑ PAHs				64.98 ± 0.43	0.05 ± 0.06	74.18 ± 0.77	0.05 ± 0.02
Metals	0.25	0.82	Aluminum	Al	324.97 ± 73.57	0.26 ± 0.07	521.75 ± 126.48	0.75 ± 0.08
	0.01	0.04	Cadmium	Cd	0.82 ± 0.02	<0.01	0.91 ± <0.01	<0.01
	0.04	0.12	Chromium	Cr	23.74 ± 8.41	<0.01	36.45 ± 2.73	<0.01
	0.05	0.17	Copper	Cu	54.82 ± 48.60	<0.01	25.51 ± 6.42	<0.01
	0.82	0.25	Iron	Fe	805.52 ± 299.27	<0.01	953.61 ± 118.35	<0.01
	0.20	0.68	Lead	Pb	7.39 ± 0.13	0.02 ± 0.03	7.92 ± 0.57	<0.01
	0.05	0.17	Mercury	Hg	0.88 ± 0.46	<0.01	0.89 ± 0.02	<0.01
	0.05	0.16	Nickel	Ni	8.25 ± 1.17	<0.01	10.34 ± 0.74	<0.01
	2.34	7.79	Zinc	Zn	8298.70 ± 262.91	1.56 ± 0.23	8644.05 ± 579.18	5.53 ± 0.54
	∑ Metals				9525.09 ± 694.55	1.86 ± 0.32	10201.43 ± 834.52	6.31 ± 0.63
∑ Chemical load					9622.11 ± 699.73	6.81 ± 1.18	10316.05 ± 838.17	11.72 ± 1.71