Increased temperature and turbulence alter the effects of leachates from tire particles on fathead minnow (*Pimephales promelas*)

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14 KEYWORDS: microplastics, tire wear particles, environmental stressors, chemical leachate,
15 climate change

19

20 ABSTRACT. Tire particles are of concern as a stressor due to the combination of their chemical
21 constituents, high emission rates, and global distribution. Once in the environment, they will
22 interact with physical parameters (e.g., UV, temperature). The interaction of chemical pollution
23 with changing physical environmental parameters is often underestimated in ecotoxicology.
24 Here, we investigate the role of temperature, mechanical stress (i.e., turbulence), UV and CO₂ on
25 the effects of tire leachates on fish. Two samples of tire particles were exposed to four different
26 levels of each physical stressor. A toxicological assessment was performed with fathead minnow
27 embryos assessing five end points (hatching success, time to hatch, length, deformities, and heart
28 rate). Results showed that variations of temperature and mechanical stress affect the
29 toxicological impact of tire leachates. Zn and/or PAHs (pyrene, phenanthrene, chrysene,
30 benzo[a]pyrene, anthracene, naphthalene, fluoranthene, and benzo[ghi]perylene) were identified
31 in the leachate and tire samples by Raman/SERS and GC/MS respectively.

32 (abstract art)

33

34 INTRODUCTION

35 In a world undergoing global change, complex interactions between anthropogenic pollutants
36 and environmental parameters related to climate change (e.g., temperature, UV, CO₂) are of great

interest. Climate change is not only responsible for the overall warming of the planet [1], but also influences weather severely by causing longer droughts and higher temperatures in some regions and more intense deluges in others [2]. Environmental parameters such as UV, changing CO₂, turbulence, temperature, and pH may impact the fate of chemical pollutants and how they affect ecosystems. For plastic pollution, mechanical erosion [3], temperature [3], [4], oxygen [5], and UV light [4], [6], [7] affect the rate of plastic degradation. As local conditions change, they may impact how plastics break down into microplastics (plastic debris <5mm in size [6]) and the extent to which they leach chemicals into the environment.

Tire - particles (herein after referred to as T-P), are a common form of microplastic pollution and are of particular concern as a potential stressor due to the combination of high emission rates (millions of tons annually) [8], [9], [10], [11] and widespread global distribution [12]. It is estimated that 1 kg of tire particles is released per driver, per year in Europe [13] and the global mean is 0.8 kg/driver/yr (range 0.23 to 4.7 kg/person/yr) [11]. Non-airborne T-Ps on roads have a size distribution ranging from nanometers to several hundred micrometers [8]. Road runoff and subsequent transport in watersheds can lead to particles in aquatic ecosystems [11]. Kole et al. [14] suggest that 12% of total T-P emissions will end up in surface waters.

Once in the environment, T-P are available to various species of wildlife [10]. Exposure may be by direct ingestion of the particles, or by ingestion of constituent chemical leachate.

Car tires are known to contain toxic chemicals added during manufacturing [15], [16], [8], [12]. The chemical additives or byproducts in tires vary by manufacturer, however, zinc (Zn) and polycyclic aromatic hydrocarbons (PAHs) are common constituents in tires and are of concern [17]. Previous studies detected Zn [9], [18], [15], [10], [19], pyrene [20], [21], phenanthrene

[18], [20], naphthalene [18], [22], fluoranthene [18], [15], [20], benzo[ghi]perylene [16], [20], coronene [16], [22], indeno[1,2,3-cd]pyrene [16], [22], benzo[e]pyrene [16], benzo[a]pyrene [16], [22], and dibenzo[a,h]anthracene [22] in tire particles. Additionally, other tire-related compounds, including nitrogen-containing compounds, polyethylene glycols, octylphenol ethoxylates, and polypropylene glycols, have been reported in urban runoff [12]. In fish, chemicals associated with tires have been suggested as a source of toxicity. Analysis of the urban road runoff suggested that elevated mortality of adult coho salmon is correlated with chemical leaching from TWP (since the chemical signature of the leachates were most chemically similar to the waters with observed toxicity, compared to other vehicle-related sources) [12].

Several exposure studies have investigated the influence of TP on aquatic organisms, and the outcomes have varied widely. Some authors report no toxicity from TP leachates [23], while others report toxicity [24], [17], [12]. These discrepancies may be due to differences in tire brand, tire type, sample preparation or leaching conditions, which are inconsistent across studies [12], [9],[24],[17],[23],[25]. Environmental parameters, such as differences in temperature, UV light exposure, pH, and mechanical stress, might also affect TP leachate concentrations and properties.

The aim of the current study is to investigate whether chemicals that leach from TPs affect fish embryos, and how this varies under the influence of different levels of environmental parameters (i.e., UV light, temperature, mechanical stress and CO₂). The environmental parameters of interest are related to those that are affected by our changing climate [4], e.g changing UV light due to stratospheric ozone depletion [26], temperature rise, resulting from high CO₂ emissions [27] and mechanical stress, representing increased storm events [28]. We leached TPs in water under varying levels of UV, mechanical stress, temperature and CO₂. Fish embryos were then

exposed to the leachate created from TPs under these different conditions. The following hypotheses were tested: 1) the toxicity of leachate from TPs will vary among tire types (e.g., brands), and 2) the toxicity of leachate will vary among leaching condition parameters (i.e., environmental factors related to climate change). Leaching experiments were performed using Lake Ontario water and toxicity tests were conducted using a freshwater fish that is common across North America (Fathead minnow, *Pimephales promelas*). Fathead minnow are extensively used for toxicological tests due to their ecological importance, broad distribution and tolerance to a wide range of water types [29]. We measured five endpoints: hatching success, time to hatch, body length at hatch, deformities (presence and severity), and embryonic heart rate.

MATERIALS AND METHODS

Experimental Design.

Four independent full factorial tests were conducted with 12 treatments each. Each test had two factors; Factor 1 TP with three levels (Tire 1, Tire 2, and a no tire Control) and Factor 2 was one of four individual environmental parameters (i.e., temperature, mechanical stress, UV, or CO₂) with four levels consisting of different concentration or intensity levels. The different levels for the test with temperature included 4±0.5 °C, 14±0.5 °C, 24±0.5 °C, and 34±0.5 °C, for mechanical stress 0 rpm, 50 rpm, 100 rpm, and 200 rpm, for UV 0 µW/cm², 500±30 µW/cm², 1000±30 µW/cm², and 1500±30 µW/cm², and for CO₂ 400±50 ppm, 600 ±50 ppm, 800±50 ppm, and 1000±50 ppm. We chose a CO₂ gradient in accordance with four near-future climate change scenarios, where 1000 ppm in 2100 is a worst-case scenario [30]. The temperature represents the lowest, average minimum and average maximum recorded temperatures in Ontario, in addition to the hottest recorded temperature in Toronto in 2017 [31], [32], [33]. UVA gradient was chosen

based on technical capabilities of lamp, incorporating a gradient from indirect to no-sunlight. For mechanical stress, turbulence data was not available, and thus we chose a range of numbers based on what seemed reasonable. For leaching experiments, we included three replicates of each treatment (n=3). These replicates were then carried into toxicity tests with fathead minnow. Chemical characterization of the TP leachate and the tires were conducted using Surface Enhanced Raman Spectroscopy (SERS) and Gas Chromatography with Mass Spectroscopy (GC/MS), respectively. SERS was chosen based upon its high sensitivity of detection for a broad range of PAHs for the small volume of the samples, while GC/MS was used for quantitative assessment of chemicals associated with the TP.

Sample preparation

Two new tires were purchased from Amazon in April 2018: Tire 1 (Bridgestone, Ecopia EP422, all season, sidewall markings 205/60R16 92H) and Tire 2 (Michelin energy saver a/s, all season, sidewall markings 205/60R16 91V). The outside ring, or top of both tires were manually cut into small TPs in the shape of squares with an area of $3 \pm 2 \text{ mm}^2$ (measured from a sample of 738 particles) and dried in the oven (at 45 °C) for three days to eliminate moisture. Fifty samples of 2.3 g of TPs from each tire were weighed using a microbalance scale METTLER TOLEDO, XP6 and stored in the freezer (at -20 °C) to avoid uneven sample aging. Forty liters of carbon-filtered, UV sterilized, City of Burlington water (sourced from Lake Ontario) were collected from Environment Canada in April 2018 and stored in the fridge (at 4 °C). One day before leaching experiments, all tire samples and Lake Ontario water were transferred into an environmental chamber to reach the experimental temperature of 18.8 °C (except for temperature experiments, which had different values). On the day of each experiment, 2.3 g of T-Ps were mixed with 230 ml of Lake Ontario water to reach a concentration of 10 g/l (between 600 and

750 TPs per sample), the concentration was chosen based on the estimation to observe negative effect [15]. All leaching experiments took place inside a 250 ml amber soda-lime glass bottles from VWR International LLC, except for the UV experiment where 250 ml clear borosilicate glass bottles (VWR) were used for better UV light transmission.

Leaching Trials to Prepare Solutions for Toxicity Tests

Leaching trials lasted ten days and were staggered to meet space requirements (Figure 1). All experiments except CO₂ were conducted inside environmental chambers (from Percival Scientific F30B, Series 101) at a temperature of 18.8±0.5 °C (except for when temperature was a changing parameter) to match the beginning of the spawning season for fathead minnow [34]. CO₂ experiments were performed inside a NewAir AW-121E 12 Bottle Thermoelectric Wine Cooler at 18.8±0.1 °C because of its ability to keep constant CO₂ levels. See the supporting information for more details on how physical stressors were introduced for each leaching experiment. After 10 days of leaching, TPs were separated from the lake water using individual stainless steel sieves with a mesh size of 1mm. Next, 220 ml of the leachate (inside the 250 ml amber soda-lime glass bottles from VWR International LLC) was transferred to Environment Canada for toxicological experiments on the same day, 10 ml (in amber soda-lime glass bottles from McMaster Carr) was left for Raman/SERS analysis (also for same-day measurements). See the supplementary information for more details about quality assurance and quality control.

Figure 1. Scheme of each of the four individual tests with twelve treatments each (n=3). For each environmental stressor there are four intensities. For each intensity, each of three material types were run (Tire 1, Tire 2, and a no tire Control). Dates are included to show the timing of all tests across all four experiments.

Fathead minnow embryo exposures

Toxicity tests with TP leachates and fathead minnow (*Pimephales promelas*) embryos were conducted at Environment and Climate Change Canada's Aquatic Life Research Facility (Burlington, ON) under the Standard Operating Procedure GWACC-119 "Fish embryo toxicity (FET) tests using tissue culture plates", approved by the Animal Care Committee (operated under the approval of the Canadian Council of Animal Care). Newly fertilized fathead minnow embryos were purchased from Aquatox Laboratories (Guelph, ON). The eggs used in testing had been fertilized < 18 h before the start of the exposure and were selected based on criteria in Marentette et al [35]. The embryo exposures were performed in environmental chambers with controlled temperature, light, and humidity (25 °C, 16 h light: 8 h dark, 60 % humidity). Embryos were exposed using daily static renewal methods in 24-well cell culture plates (Falcon,

Becton, Dickenson and Co., New Jersey, USA), following the method of Marentette et al [36]. For more information on water quality data as well as preparation of exposure solutions see Supporting Information Table S1. Since each treatment (control, Tire 1, Tire 2) was experimentally replicated three times, we used one replicate plate per replicate extract. In each replicate plate, there were two control wells to test for any difference between plates. In addition, there were two replicate plates for controls, and this was to test whether there were any differences between days in which experiments were performed. Note, these controls are different from the “no tire Control” treatments, which were exposed to different stressors. Thus, for each treatment there were three replicate plates per treatment, six control wells across the three plates per treatment to account for between-plate variability, and two additional control plates to account for between-day variability. Plates contained 12 embryos (10 exposed embryos and 2 control embryos). Eggs from ≥ 4 breeding groups were used for each sample (per plate). All embryo checking and solution changes were performed in a 25° C environmental chamber to ensure consistent temperatures throughout the manipulations. Embryos were assessed and moved to new plates containing fresh tire leachate daily.

Any dead embryos were removed from plates and euthanized. At two days post fertilization, five embryos per plate were videotaped in their exposure well for counting heart rates. At 4-5 days post-fertilization embryos began to hatch, and time-to-hatch was noted for each. Hatched embryos were assessed for deformities, hatching success, and total length (measured on a dissecting microscope) before being euthanized. Deformities that were assessed at hatch included severe, moderate, and mild scorings of edemas and changes to circulation (necrotic but alive, cardiac edema, yolk edema, bubbles under skin, hemorrhage, or other), craniofacial deformities (small face, eye edema, or other), and spinal deformities (helix coil, lordosis (belly

out), kyphosis (belly in), scoliosis (to the side), bent tail fin, or other). In these experiments we also assessed tube heart, fused jaw, and several changes to the eyes (small eyes, less eye pigment = “glassy eyes”, or total lack of eye pigment).

Using Raman to assess the leachate

Each leachate sample was qualitatively assessed for the presence or absence of 16 EPA priority PAHs (acenaphthene, acenaphthylene, anthracene, benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[ghi]perylene, benzo[k]fluoranthene, chrysene, dibenz[ah]anthracene, fluoranthene, fluorene, indeno[1,2,3-cd]pyrene, naphthalene, phenanthrene, pyrene) and Zn using Surface Enhanced Raman Spectroscopy (SERS) [37]. Clustered gold nanoparticle suspensions (CNP-Au) were used as SERS substrates and prepared by Ocean Optics according to the method by Lee and Meisel [38], see supporting information in the attachment for details. Before each measurement, 100 μ l of CNP-Au was mixed with 100 μ l of the sample on top of a glass slide or glass petri dish and placed in the oven under 45 °C for 1h to allow liquid evaporation and crystal formation. The full description of this method as well as PAH reference measurements are presented elsewhere [39]. All SERS measurements were recorded at room temperature with a Raman microscope (Xplora Plus from HORIBA France SAS). The laser beam was focused through a standard 50x objective with a 1.9 μ m laser spot size. All Raman/SERS spectra were recorded with an excitation wavelength of 785 nm, beam filter of 10 %, and 10 mW laser power. The laser beam exposure time was 10 s, and accumulation of 10. The intensity of the Raman peak at 520 cm^{-1} from silicon was normalized before each data acquisition. Data was analyzed using LabSpec 6 software. According to previous studies [39],

206 CNP-Au substrate is capable of detecting 16 EPA priority PAHs in concentrations between 1 ppb
207 and 10 ppm.

208 **Extraction and Analysis of PAHs in Tire Samples**

209 The tire samples were extracted and analyzed for 52 PAHs (unsubstituted, alkylated and
210 dibenzothiopenes) by the Environment and Climate Change Canada (ECCC) Pacific and Yukon
211 Laboratory for Environmental Testing (PYLET; Vancouver, BC), (Table S2 in SI) [40], [41].
212 The method has been validated and accredited by ECCC and the Canadian Association for
213 Laboratory Accreditation, Inc., and further details are provided in the Supporting Information.
214 Briefly, the tire samples were extracted by shaker and centrifuge with dichloromethane. The
215 extracts were analyzed by gas chromatography mass spectrometry using an Agilent 7890A gas
216 chromatograph coupled with an Agilent 7000C triple quadrupole mass spectrometer (GC-
217 MS/MS). The analytical method has been described previously [40], [41]. Concentrations in $\mu\text{g/g}$
218 of PAHs analyzed in Tire 1 and Tire 2 are presented in Table S3 in SI.

219 **Statistical Analyses**

220 Four individual two-factor ANOVAs ($\alpha = 0.05$, factor 1 = material type, factor 2 = level of
221 stressor) were run to measure whether there were differences in hatching success, time to hatch,
222 total length, deformities or heartbeat in Fathead minnows across treatments for each individual
223 environmental parameter. Because some experiments were run on different dates, we also ran
224 Kruskal-Wallis tests to determine whether there were differences amongst controls run on
225 different days. We took a p value of >0.05 to mean that there were no differences among
226 individual experiments and thus we proceeded to run two-factor ANOVAs individually for each
227 environmental factor. To test for homogeneity of variance, we ran a Cochran's C-test. Student-

Newman-Keuls (SNK) tests distinguished significantly different treatment means. Statistical analyses were performed using RStudio.

RESULTS AND DISCUSSION

Response of fathead minnow to TWP leachates

The effects of TP leachates on fathead minnow embryos were assessed under four different environmental parameters (temperature, mechanical stress, UV, CO₂). Basic water quality measurements of the various TP leachates suggested no undue stress to the embryos from changes in conductivity, pH, dissolved oxygen, and ammonia. Conductivity of the leachate solutions ranged from 346 to 431 $\mu\text{S}/\text{cm}$, and pH ranged from 8.2 to 8.6. Dissolved oxygen (7.5 to 8.5 mg/l) and free ammonia (0.0 to 0.1 mg/l) were also within tight ranges and would not be expected to cause adverse effects to embryos. All water quality data are provided in SI Table S1. Overall, there were no statistical differences between control samples run on different days for any endpoint except for hatching success. However, because the hatching success for the control samples was always 80% or higher, we continued with our analyses for each of the four independent experiments. A summary of results for the entire leaching experiment can be found in SI, Table S4.

Temperature

Leachates were used in exposure experiments with fathead minnow embryos to measure the effects of TP leachates under four temperatures: 4, 14, 24 and 34 °C. There were no significant effects of time to hatch across all treatments (Figure 2a). For hatching success, there was a pattern for leachates from Tire 2 where hatching success decreased with increasing

temperature. Statistically, there was a significant interaction term for material and temperature (Figure 2b), with Tire 2 at the highest temperature being significantly different from all other treatments. In addition, for Tire 2, hatching success was also significantly different under 24 °C from some treatments in the Control and Tire 1, and only under some temperatures. For length, there was a significant interaction term for material and temperature, but with no apparent trend (Figure 2c). For heart rate, there was a significant difference among materials, with both tire treatments having slower heart rates than the controls (Figure 2d). There was also a significant difference among temperatures, with higher temperatures of leaching generally leading to a faster heart rate. For deformities, there was a significant interaction term for material and temperature (Figure 2e), with high levels of deformities in all tire leachates compared to the controls. For Tire 1 at 14 °C, 75% of the fish had deformities, and 100% of the fish had some level of deformity for all remaining tire treatments. The severity of the deformities also differed among treatments, with the most severe deformities observed in fish exposed to leachates from Tire 2. Additionally, a trend showing increasing deformity severity with temperature was evident (Figure 2e).

Overall, the effects of temperature were consistent between tires, although the tire type and composition did affect the severity of effects. Tire 2 had lower hatching success, shorter lengths, slower heart rate and more severe deformations compared to those of Tire 1. The severity of deformities observed increased with increasing temperature regardless of tire type. These results suggest that the toxicological impact of the leachate will vary greatly with geography, via temperature, with warm places seeing more impact than cold places. Additionally, changes to seasonal temperatures expected with climate change – and warming in particular – will increase the leaching rate of chemicals from TP, with downstream effects on freshwater fish.

Figure 2. Toxicological test results of tire leachates (Tire 1, Tire 2 and Control) under different temperatures, five endpoints: a) time to hatch (days); b) hatching success (percentage); c) total length (mm); d) heart beats per minute; e) deformity (percentage); The color grading of the bars represents different temperatures; f) deformity severity. The color grading represents deformation severity.

Mechanical Stress

Leachates were used in exposure experiments with fathead minnow embryos to measure the effects of TP leachates under four levels of mechanical stress: 0, 50, 100, and 200 rpm. There were no significant effects of time to hatch across all treatments (Figure 3a). Similarly as with temperature, the hatching success of Tire 2 decreased with increasing mechanical stress, and was significantly different from both Tire 1 and the Control (Figure 3b). Although, there appears to be a pattern whereby embryos exposed to tire leachates are shorter in length – and in a stepwise fashion for Tire 2 (Figure 3c) – there is no significant difference among treatments. For heart rate there was a significant difference among materials, with Tire 1 being different from Tire 2 and both tire treatments having slower heart rates than the controls (Figure 3d).

For deformities, fish exposed to leachate from both tires had significantly more deformities than the control (Figure 3e), with leachates from Tire 1 and Tire 2 leading to 100% deformities for all levels of mechanical stress. The severity of the deformities also differed among treatments, with the most severe deformities observed in fish exposed to leachates from Tire 2. Tire 2, specifically, also showed increasing deformity severity with increasing mechanical stress (Figure 3f).

Overall, this suggests that rainfall events, which may vary with a changing climate, may also influence the leaching rate of chemicals from TP, which may affect freshwater organisms.

296 Additionally, extreme events, like hurricanes, tsunamis, earthquakes, and floods represent
297 additional mechanical stress-related pressure on TP pollution. Similar to temperature treatments,
298 the mechanical stress experiments suggest that the toxicity of TPs on organisms likely depends
299 on the tire type. Tire 2 again demonstrated lower hatching success, slower hatch rate and more
300 severe deformities compared to Tire 1.

Figure 3. Toxicological tests results of tire leachates (Tire 1, Tire 2 and Control) exposed to different mechanical stress; five endpoints: a) time to hatch (days); b) hatching success (percentage); c) total length (mm); d) heart beats per minute; e) deformity (percentage); Plot color represents different mechanical stress; f) deformity severity. Plot color represents deformation severity.

UV

Several studies report that the toxicity from tire leachates increased after exposure to UV light [15]. We used leachates from Tire 1 and Tire 2 in exposure experiments with fathead minnow under four UVA treatments: 0, 500, 1000, and 1500 $\mu\text{W}/\text{cm}^2$. Similar to the previously described experiments, there were no significant effects for time to hatch across all treatments (Figure S1a in SI). For hatching success, fish exposed to Tire 2 with UV exposure of 500 $\mu\text{W}/\text{cm}^2$ had significantly lower hatching success than all other treatments (Figure S1b). Similarly, fish exposed to Tire 2 at 1000 $\mu\text{W}/\text{cm}^2$ had significantly lower hatching success compared to the Control and Tire 1, but only under some UV intensities. For length, fish exposed to Tire 2 were significantly smaller than fish exposed to the Control and Tire 1 (Figure S1c). For heart rate (Figure S1d), there was a significant difference among materials, with Tire 2 producing slowest heart rates.

For deformities, fish exposed to Tire 1 and Tire 2 had significantly more deformities than fish exposed to the Control (Figure S1e). With the exception of fish exposed to Tire 2 at 500 $\mu\text{W}/\text{cm}^2$, all tire treatments had 100% deformities. The severity of the deformities also differed among treatments, with the most severe deformities observed in fish exposed to leachates from Tire 2 (Figure S1f). Overall, we observed material-related differences, but without an obvious

increasing trend with increasing UV. This suggests that variations of UVA light are less important for influencing toxicity of the chemicals in TP than UV exposure in general.

CO₂

Elevated levels of carbon dioxide could change water pH and promote chemical leaching from the TP, therefore exposure experiments with fathead minnow embryos were conducted to measure the effects of TP leachates under four CO₂ concentrations: 400, 600, 800 and 1000 ppm. There were no significant effects of time to hatch across all treatments (Figure S2a in SI). For hatching success, only fish exposed to Tire 2 under the 800 ppm treatment had significantly lower hatching success compared to all other treatments (Figure S2b). For length, there was a significant interaction term for material and CO₂, with fish exposed to Tire 2 at 400, 800 and 1000 ppm being significantly smaller than all Control fish (Figure S2c). For heart rate (Figure S2d), Control leachates at 400ppm and 600ppm are different the rest of the samples, suggesting that water pH change may result in a slower heartbeat.

For deformities, fish exposed to Tire 1 and Tire 2 had significantly more deformities than fish exposed to the Control (Figure S2e). All fish exposed to Tire 1 and fish exposed to Tire 2 at 400, 600 and 1000ppm had 100% deformities. The severity of the deformities did not follow any pattern, and fish exposed to Tire 2 at 800 ppm had the most (Figure S2f). Overall, CO₂ exposure results suggests that slight pH variations in the water will unlikely have a big influence on TP leaching and its effect on freshwater organisms, and in particular fathead minnow.

Loss of pigmentation

The exposure of fish embryos to aromatic hydrocarbons has been found to decrease pigmentation [42], [43]. In this study, all fish embryos across all TP treatments had a noticeable lack of pigmentation in their eyes (Figure 4). Figure 4 shows an example of three fish embryos from the CO₂ experiment (similar for all stressors). Figure 4a shows an embryo exposed to the leachate from a control sample (i.e. water sample exposed to the CO₂ = 600 ppm stressor but containing no tire material). Figures 4b and 4c show embryos exposed to the leachate from Tire 1 (after 600 ppm) and Tire 2 (after 600 ppm), respectively. The lack of eye pigmentation is clear. Other researchers have noted the lack of eye pigmentation when fathead minnow embryos were exposed to chlorinated phenols [44]. In addition, benzo(a)pyrene can affect mouse retinal epithelial cells [45]. Zn may also be a factor, as this metal is important in retinal epithelium and photoreceptors [46]. The lack of eye pigment in the current study may be due to the exposure to PAHs, Zn and/or other components of the tire leachate; further investigation is needed to determine what caused this effect, and whether it continues into later life stages, and what that ultimately means for the health of the fish.

Figure 4. One example of Fathead minnows exposed to the leachates from the CO₂ experiments at 600 ppm a) control; b) Tire 1 ; c) Tire 2. The black bar represents 1 mm.

PAHs and metals associated with TPs leachate

SERS

To qualitatively characterize some of chemicals in TP leachate, we used Raman/SERS measurements. Since PAHs and Zn has been classically attributed as environmental tire markers [16], [15], [47], [8] we chose to target the 16 EPA priority PAHs and Zn, although other studies demonstrate that tire particles may leach other polar compounds which may be the cause of some of the observed effects in this study [12], [48]. Despite differences in toxicological effects, according to Raman/SERS measurements, both types of car tires under all treatments produce a similar spectrum (see figure S3 in SI). This is an indicator of a similar basic tire production recipe for the chemicals targeted. However, the Raman technique is not quantitative or exhaustive, and the toxicological effects of the tire leachates to the fish embryos will likely be chemical specific and depend on the amount of the leached chemicals.

To detect chemicals of interest in TP leachates, it is necessary to measure “reference” spectra of these chemicals in different concentrations to identify whether there is a dose-response. An example of a reference measurement for Zn is in Figure 5a (shown for concentrations of 0.1, 1 and 100 ppm in RO (reverse osmosis) water). Here, there is a dose-intensity response in the areas of 600 cm^{-1} , 640 cm^{-1} and 1015 cm^{-1} , compared with the blank spectrum of RO water. Raman signals in the similar spectral range appear in almost all samples (see SI, Table S5), for example, we can see similar peaks in Tire 1 and Tire 2 leachates from the 800 ppm CO_2 treatment (Figure 5a). Note, that SERS/Raman signals are slightly shifted in different samples. This is expected due to chemisorption processes between sample molecules and the substrate, [49].

Figure 5. Raman measurements of target pollutants (Zn, and several PAHs) and tire leachate samples, a) Raman/SERS spectra of dose-intensity response spectra of Zn in three different concentrations in RO water, as well as two tire leachate samples (Tire 1 and Tire 2 from tire leachates after exposure to $\text{CO}_2=800\text{ppm}$); b) Normalized Raman/SERS spectra of the naphthalene (Nap), anthracene (Ant) and pyrene (Pyr), as well as Tire 1 (T1_+24°C) sample from temperature treatment of +24°C; c) Normalized Raman/SERS spectra of the chrysene (Cry), benzo[a]pyrene (BaP) and phenanthrene (Phe) as well as Tire 2 (T2_+24°C) sample from temperature treatment of +24°C. CNP-Au substrate, $\Lambda=785\text{ nm}$, laser power at the sample 10 mW, 10 s integration time.

After comparing the SERS spectral shifts of 16 EPA priority reference PAHs (presented in Table S6 in SI) and all tire leachate samples, the highest spectral matches are attributed to five PAHs: pyrene, phenanthrene, chrysene, benzo[a]pyrene, anthracene and naphthalene, (see Figure 5b and 5c), which is in agreement with other studies [18], [16], [20], [21], [22]. For more detail,

see Tables S5 (Raman shift from all samples) and S6 (Raman shift from 16 EPA PAHs) in SI. A follow up study is necessary to determine whether these compounds were related to any observed toxicological effect.

GC/MS

Tire 1 and Tire 2 were also extracted to measure PAHs and alkylated PAHs in the tire material. The concentrations of individual compounds are given in Table S2 in SI. The Σ PAHs in Tire 1 and Tire 2 was 141.2 and 90.4 $\mu\text{g/g}$ respectively; total concentrations of the 16 EPA PAHs was 24.0 and 11.1 $\mu\text{g/g}$, respectively. The differences in PAH concentrations could be attributed to the differences in the formulations of tires. These were also evident in the differences in composition profiles (concentration of analyte/total concentration) of PAHs (Figure S4 in SI). Previous studies have reported total concentrations in the range 31-200 $\mu\text{g/g}$ [50], [51], [52]. Of the 16 EPA PAHs, the order of dominance was pyrene>fluoranthene>benzo[ghi]perylene in Tire 1 and pyrene>fluoranthene>phenanthrene in Tire 2. Pyrene, fluoranthene and benzo[ghi]perylene have been observed to be similarly dominant in other tires for the priority PAHs [50], [52], [53]. There is limited information on alkylated PAHs (APAHs) in TWP, however, these were the most dominant compounds in both tires, accounting for over 70 % composition. In Tire 1, c3-fluoranthene; c3-phenanthrene/anthracene, c2-c3-fluoranthene/pyrene were the dominant APAHs; in Tire 2, c4-fluoranthene/pyrene and c2-c3-benzo[a]anthracene/chrysene were dominant.

In regards to toxicity tests, the severity of deformities was more pronounced in Tire 2. These deformities increased with increasing temperature and mechanical stress. Although total concentrations of parent EPA priority PAHs in Tire 1 were greater than Tire 2, Tire 2 had a

higher % composition of alkylated PAHs (81 vs 71.4 %) and a smaller composition of the more volatile-semi-volatile components (i.e. naphthalene, c1-c4 naphthalene, acenaphthene, acenaphthylene, fluorene, c1-c2 fluorene, etc.) (31.1 vs 47.2 %). Tire 2 also had higher concentrations of indeno[1,2,3-cd]pyrene (9x), benzo(b)chrysene (7x), and C2-benzo[a]anthracene/chrysene (5x) compared to Tire 1. The emissions of PAHs from TPs would be affected by their physical-chemical properties and interactions with the tire materials, which would in turn dictate volatilization and the generation of tire wear particles in the presence of (mechanical) stress. The results of this study suggest that the less volatile and alkylated PAHs associated with Tire 2 could be contributing to the increased toxicity observed. Still, further work would be needed to determine which compounds caused toxicity, and there may be other compounds associated with toxicity that were not targeted in our chemical analysis.

Implications of the study

Researchers have demonstrated that tire particles are present in nature [8]-[12], and their sources are predicted to increase in the future. Moreover, studies have demonstrated that leachate from car tires can be toxic to animals [12], [24], [17]. Here, we investigate how tire particles leachate may impact fish, including under various physical stressors associated with climate change. Our investigation of the effects of individual environmental stressors on chemical leaching from car tires is a first step towards a study on how multiple stressors in parallel may affect tire leachates and their toxicity in nature. Here, we found that T-P toxicity varies by Tire type and among physical parameters (particularly temperature and mechanical stress). The impact of TP will be non-uniform globally, being strongly influenced by local weather conditions. With climate change, this suggests that increasing temperatures and changes in

weather patterns may affect how tire r particles affect wildlife. The leachates of both tire samples produced toxic effects (mostly affecting hatching success and deformities) as well as caused a lack of eye pigmentation in fish embryos. Through SERS and GCMS, we show that PAHs and Zn were present in the leachates, and may have contributed to toxic effects. Via GCMS, we show that Tire 1 had a greater concentrations of parent PAHs, and Tire 2 had a greater proportion of alkylated PAHs – which may explain the differences in effects between the two. Future studies should look at the full suite of compounds in tire particle leachate and use effect directed analysis to determine which chemicals are causing the effects that we observed. Such data may inform chemical formulas for tires that are more benign by design.

ASSOCIATED CONTENT

Supporting Information:

- Description of experimental setup
- Quality assurance and quality control (QA/QC) of the experiment.
- Table S1. Summary of basic water quality data for controls and tire leachates
- Preparation of exposure solutions
- Description of CNP-AU substrate preparation procedure
- Table S2: PAHs analyzed, abbreviations used, internal standards and PAHs used for quantitation, as well as method detection limits (MDLs) by GC/MS.
- Description of PAH tire extraction and quantitation
- Table S3: Concentrations in $\mu\text{g/g}$ of PAHs analyzed in Tire 1 and Tire 2.

- Table S4: Summary of toxicological test results for the entire experiment
- Figure S1: Toxicological tests results of tire leachates (Tire 1, Tire 2 and Control), exposed to different UV light.
- Figure S2: Toxicological tests results of tire leachates (Tire 1, Tire 2 and Control), exposed to different CO₂ amount
- Figure S3 Raman/SERS measurements of T1 and T2 leachates
- Table S5. Raman/SERS measurements of the tire leachates on CNP-Au substrate
- Table S6. Raman/SERS measurements of 16 EPA PAHs on CNP-Au substrate.
- Figure S4: Concentrations in µg/g of PAHs analyzed in Tire 1 and Tire 2.

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Anna Kolomijeca – planned, carried out and evaluated multistressor experiments

Joanne Parrott – helped planning and supervised toxicological tests

485 Hufsa Khan, Kallie Shires, Stacey Clarence, Cheryl Sullivan – contributed equally by
486 conducting toxicological experiments and helping to evaluate data

487 Leah Chibwe – Conducted GC/MS analysis

488 David Sinton – helped planning and supervising work process

489 Chelsea Rochman – planned, helped prepare experiments, conducted statistical analyses as well
490 as overall supervision of the work process.

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