ECOTOXICOLOGY

A ubiquitous tire rubber–derived chemical induces acute mortality in coho salmon

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In U.S. Pacific Northwest coho salmon (*Oncorhynchus kisutch*), stormwater exposure annually causes unexplained acute mortality when adult salmon migrate to urban creeks to reproduce. By investigating this phenomenon, we identified a highly toxic quinone transformation product of *N*-(1,3-dimethylbutyl)-*N*′-phenyl-p-phenylenediamine (6PPD), a globally ubiquitous tire rubber antioxidant. Retrospective analysis of representative roadway runoff and stormwater-affected creeks of the U.S. West Coast indicated widespread occurrence of 6PPD-quinone (<0.3 to 19 micrograms per liter) at toxic concentrations (median lethal concentration of 0.8 ± 0.16 micrograms per liter). These results reveal unanticipated risks of 6PPD antioxidants to an aquatic species and imply toxicological relevance for dissipated tire rubber residues.

Humans discharge tens of thousands of chemicals and related transformation products to water (*1*), most of which remain unidentified and lack rigorous toxicity information (*2*). Efforts to identify and mitigate high-risk chemical toxicants are typically reactionary, occur long after their use becomes habitual (*3*), and are frequently stymied by mixture complexity. Societal management of inadvertent, yet widespread, chemical pollution is therefore costly, challenging, and often ineffective.

The pervasive biological degradation of contaminated waters near urban areas ("urban stream syndrome") (*4*) is exemplified by an acute mortality phenomenon that has affected Pacific Northwest coho salmon (*Oncorhynchus kisutch*) for decades (*5*–*9*). "Urban runoff mortality syndrome" (URMS) occurs annually among adult coho salmon returning to spawn in freshwaters where concurrent stormwater exposure causes rapid mortality. In the most urbanized watersheds with extensive impervious surfaces, 40 to 90% of returning salmon may die before spawning (*9*). This mortality threatens salmonid species conservation across ~40% of the Puget Sound land area despite costly societal investments in physical habitat restoration that may have inadvertently created ecological traps through episodic toxic water pollution (*9*). Although URMS has been linked to degraded water quality, urbanization, and high traffic intensity (*9*), one or more causal toxicants have remained unidentified. Spurred by these compelling observations and mindful of the many other insidious sublethal stormwater impacts, we have worked to characterize URMS water quality (*10*, *11*).

Previously, we reported that URMS-associated waters had similar chemical compositions relative to roadway runoff and tire tread wear particle (TWP) leachates, providing an opening clue in our toxicant search (*10*). In this work, we applied hybrid toxicity identification evaluation and effect-directed analysis to screen TWP leachate for its potential to induce mortality (a phenotypic anchor) in juvenile coho salmon as an experimental proxy for adult coho (*6*). Using structural identification by means of ultrahigh-performance liquid chromatography–high-resolution tandem mass spectrometry (UPLC-HRMS/MS) and nuclear magnetic resonance (NMR), we discovered that an antioxidant-derived chemical was the primary causal toxicant. Retrospective analysis of runoff and receiving waters indicated that detected environmental concentrations of this toxicant often exceeded acute mortality thresholds for coho during URMS events in the field and across the U.S. West Coast.

Aqueous TWP leachate stock (1000 mg/liter) was generated from an equal-weight mix of tread particles (0.2 ± 0.3 mm^2 average surface area) (fig. S1) from nine used and new tires (table S1). TWP leachate (250 mg/liter positive controls) was acutely and rapidly (~2 to 6 hours) lethal to juvenile coho (24 hours exposures, 98.5% mortality, n = 135 fish from 27 exposures) (data file S1), even after heating (80°C, 72 hours; 100% mortality, n = 10 fish from two exposures), indicating stability during handling. Behavioral symptomology (circling, surface gaping, and equilibrium loss) (fig. S2 and movie S1) of TWP leachate exposures mirrored laboratory and field observations of symptomatic coho (*5*, *6*). No mortality occurred in negative controls, including solvent- and process-matched method blanks subjected to identical separations (0 of 80 fish, 16 exposures) or exposure water blanks (0 of 45 fish, nine exposures).

Mixture complexity [measured here as number of UPLC-HRMS electrospray ionization (ESI+) chemical features] was a substantial barrier to causal toxicant identification because 250 mg/liter TWP leachate typically contained more than 2000 ESI+ detections. Our fractionation studies, optimized over 2-plus years through iterative exploration of toxicant chemical properties, focused on reducing these detection numbers to attain a simple, yet toxic, fraction amenable to individual compound identifications. Throughout this fractionation procedure, observed toxicity remained confined to one narrow fraction, which is consistent with a single compound or a small, structurally related family of causal toxicants. In initial studies, TWP leachate toxicity was unaffected by silica sand filtration, cation and anion exchange, and ethylenediaminetetraacetic acid (EDTA) (114 μM) addition (*12*), indicating that toxicant(s) were not particle-associated, strongly ionic, or metals, respectively, and validating prior studies that eliminated candidate pollutants (*13*, *14*) as primary causal toxicants.

Mixture complexity was reduced by using cation exchange, two polarity-based separations (XAD-2 resin and silica gel), and reverse-phase high-performance liquid chromatography (HPLC) on a semipreparative C18 column (250 by 4.2 mm ID, 5 μm particle size). After C18-HPLC generated 10 fractions, only C18-F6 (10 to 11 min) was toxic; it contained ~225 ESI+ and ~70 ESI– features (Fig. 1). Having removed ~90% of features, we began to prioritize and identify candidate toxicants by abundance (peak area), followed by fish exposures with commercial standards at fivefold higher concentrations (mixtures at 1 to 25 μg/liter) than those estimated in C18-F6. We identified 11 plasticizers, antioxidants, emulsifiers, and various transformation products, including some well-known environmental contaminants [such as tris(2-butoxyethyl) phosphate] and some that are rarely reported [such as di(propylene glycol) dibenzoate and 2-(1-phenylethyl)phenol] (table S2). We also detected several bioactive, structurally related phenolic antioxidants and their transformation products (2,6-di-*t*-

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butyl-4-hydroxy-4-methyl-2,5-cyclohexadienone, 3,5-di-*t*-butyl-4-hydroxybenzaldehyde, and 7,9-di-*tert*-butyl-1-oxaspiro[4,5]deca-6,9-diene-2,8-dione) (*15*). However, over many rounds of identification and subsequent exposure to juvenile coho, none of these identified chemical exposures reproduced URMS symptoms or induced mortality. Because these identifications used exhaustive environmental scientific literature searches (*10*, *16*, *17*), we suspected a previously unreported toxicant.

To sharpen our search, we used multidimensional semipreparative HPLC using two additional structurally distinct column phases [pentafluorophenyl (PFP) and phenyl]. Parallel fractionations (same column dimensions, mobile phase, and gradient as for C18-HPLC) (*18*) of the toxic silica gel fraction generated toxic fractions of PFP-F6 (10 to 11 min; ~204 ESI+, 60 ESI– features) and phenyl-F4 (8 to 9 min; ~237 ESI+, 75 ESI– features); all other fractions were nontoxic. Across these separations (C18, PFP, phenyl), only four ESI+ and three ESI– HRMS features co-occurred in all three toxic fractions (fig. S3). Of these, one unknown compound [mass/charge ratio (*m*/*z*) 299.1752, $C_{18}H_{22}N_2O_2$, RT 11.0 min on analytical UPLC-HRMS] dominated the detected peak area (10-fold higher intensity in both ESI+ and ESI–). To further resolve candidate toxicants for synthetic efforts, we converted the three-dimensional chromatography workflow from parallel to serial through sequential C18, PFP, and phenyl columns (C18-F6 to PFP-F6 to phenyl-F4; with solvent removal by means of centrifugal evaporation and toxicity confirmation between separations). The purified final fraction was chemically simple (four ESI+, three ESI– detections), highly lethal (100% mortality in 4 hours; n = 15 coho, three exposures), and was again dominated by $C_{18}H_{22}N_2O_2$. Drying this fraction yielded a pink-magenta precipitate (Fig. 1).

Published characterizations of crumb rubber (*16*) and receiving waters (*10*, *17*) did not mention $C_{18}H_{22}N_2O_2$. UPLC-HRMS/MS spectra indicated C_4H_{10} and C_6H_{12} alkyl losses (M-58 and M-84 fragments) (Fig. 2B), but MS^3 and MS^4 fragmentation yielded no additional structural insights (fig. S4). Additionally, in silico fragmentation (MetFrag, CSI:FingerID) of $C_{18}H_{22}N_2O_2$ compounds in PubChem and ChemSpider (15,624 and 17,105 structures, respectively) failed to match observed fragments. Thus, to the best of our knowledge, $C_{18}H_{22}N_2O_2$ was not described in environmental literature or databases and posed a "true unknown" identification problem (*19*). We then assumed a transformation product; industrial manufacturing (such as high heat or pressure, or catalysis) and diverse reactions in environmental systems generate many undocumented transformation products, most of which lack commercial standards.

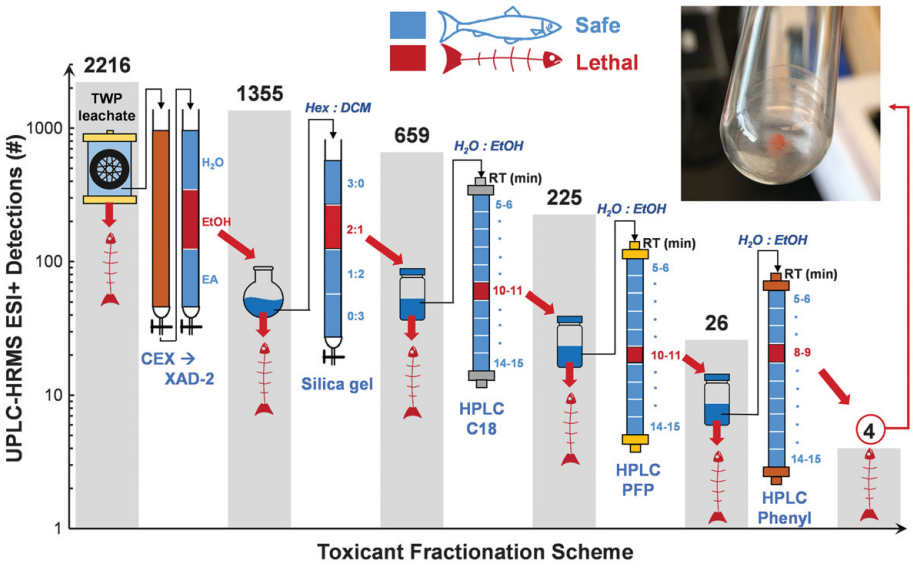

Fig. 1. Tire rubber leachate fractionation scheme. As a metric of mixture complexity and separation efficiency, the numbers above gray bars represent distinct chemical features detected in solid-phase extracted fish exposure water (1 liter) and subsequent fractions by means of UPLC-HRMS. Blue indicates nonlethal fractions; red indicates lethal fractions. All fractionation steps and exposures were replicated at least twice; positive and negative controls were included throughout fractionations. (**Inset**) Purified product (~700 μg from 30 liter of TWP leachate) in the final lethal fraction. TWP, tire tread wear particles; CEX, cation exchange; EA, ethyl acetate; EtOH, ethanol; H_2O, water; Hex, hexane; DCM, dichloromethane; RT, retention time.

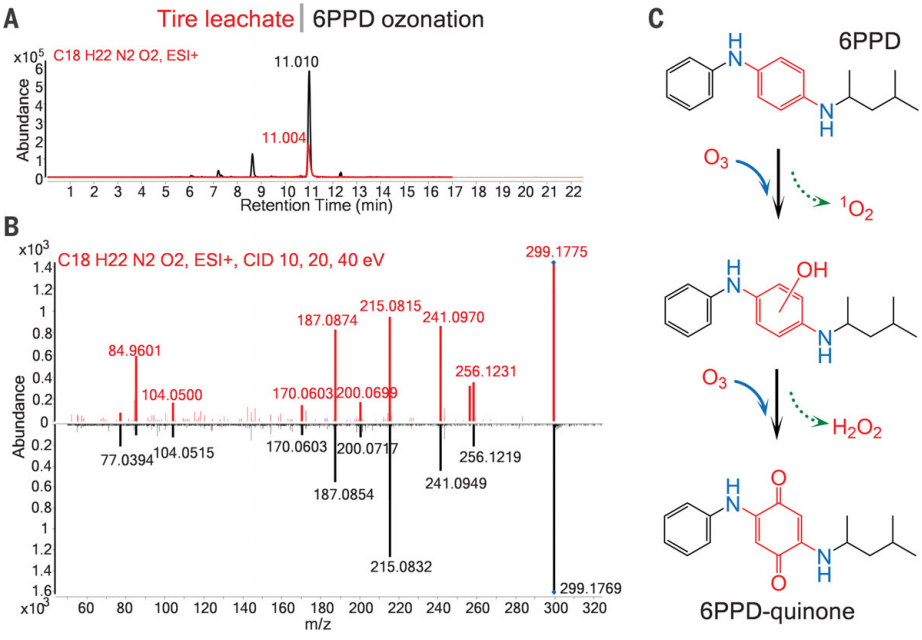

Fig. 2. 6PPD-quinone identification and a proposed formation pathway. (**A**) Extracted ion chromatograms of 6PPD-quinone from UPLC-HRMS (ESI+); red data indicate the final fraction from TWP leachate, and black data indicate the purified 6PPD ozonation mixture. (**B**) Observed MS/MS fragmentation (integrated from 10, 20, and 40 eV) of 6PPD-quinone in the final toxic fraction from TWP leachate (red spectra) and 6PPD ozonation (black spectra). (**C**) One proposed reaction pathway from 6PPD to 6PPD-quinone (alternate proposed formation pathways are provided in fig. S13). Red highlights indicate key changes in the diphenylamine structure during ozonation.

Our breakthrough came by assuming that abiotic environmental transformations commonly modify active functional groups by preferentially altering the numbers of hydrogen and oxygen atoms relative to carbon and nitrogen. By searching a recent U.S. Environmental Protection Agency (EPA) crumb rubber report (*16*) for related formulas ($C_{18}H_{0-x}N_{2-4}O_{0-y}$), several characteristics of the $C_{18}H_{24}N_2$ anti-ozonant "6PPD" [*N*-(1,3-dimethylbutyl)-*N*′-phenyl-p-phenylenediamine] matched necessary attributes. First, 6PPD is globally ubiquitous (0.4 to 2% by mass) in passenger and commercial vehicle tire formulations (*20*), indicating sufficient production to explain mortality observations within large and geographically distinct receiving water volumes. 6PPD was present in TWP leachate but was completely removed during fractionation through cation exchange. 6PPD crystals are purple, similar to the pink-magenta precipitate obtained after fractionation. Most compellingly, neutral losses in 6PPD gas chromatography (GC)–MS spectra matched the $C_{18}H_{22}N_2O_2$ GC-HRMS spectra (fig. S5), and the predicted logK_{ow} of 6PPD (5.6) (K_{ow}, *n*-octanol-water partition coefficient) was close to that for $C_{18}H_{22}N_2O_2$ (5 to 5.5) (*11*). Last, literature detailing the industrial chemistry of 6PPD reactions with ozone [7 days, 500 parts per billion volume (ppbv)] described a $C_{18}H_{22}N_2O_2$ product (*21*), leading us to hypothesize that 6PPD was the likely protoxicant (Fig. 2C).

We tested this hypothesis with gas-phase ozonation (500 ppbv O_3) of industrial grade 6PPD (96% purity) (*21*). A $C_{18}H_{22}N_2O_2$ product formed; UPLC-HRMS analysis demonstrated exact matches of retention time (11.0 min) and MS/MS spectra between this synthetic $C_{18}H_{22}N_2O_2$ and the TWP leachate fractionation-derived $C_{18}H_{22}N_2O_2$ (Fig. 2, A and B). When purified, the ozone-synthesized $C_{18}H_{22}N_2O_2$ formed a reddish-purple precipitate. One-dimensional ^{1}H NMR structural analysis confirmed identical TWP leachate–derived and ozone-synthesized $C_{18}H_{22}N_2O_2$ structures (figs. S6 to S7). Two-dimensional NMR spectra and related simulations revealed isolated tertiary carbons and carbonyl groups (figs. S8 to S12), clearly indicating a quinone structure for $C_{18}H_{22}N_2O_2$ rather than the dinitrone structure reported in the past 40 years of literature describing 6PPD ozonation products (*21*). Therefore, the $C_{18}H_{22}N_2O_2$ candidate toxicant was unequivocally "6PPD-quinone" {2-anilino-5-[(4-methylpentan-2-yl)amino]cyclohexa-2,5-diene-1,4-dione}. Consistent with environmental 6PPD ozonation, reported 6PPD ozonation products $C_{18}H_{22}N_2O$ (formula-matched) and 4-nitrosodiphenylamine ($C_{12}H_{10}N_2O$, standard-confirmed) (*21*) also were detected in ozonation mixtures and nontoxic TWP leachate fractions.

Exposures to ozone-synthesized and tire leachate–derived 6PPD-quinone (~20 μg/liter nominal concentrations) both induced rapid (<5 hours, with initial symptoms evident within 90 min) mortality (*n* = 15 fish, three exposures) (fig. S2 and movie S2), which matched the 2 to 6 hours mortality observed for positive controls. Behavioral symptomology in response to synthetic 6PPD-quinone exposures matched that from field observations, roadway runoff, bulk TWP leachate, and final toxic TWP fraction exposures, confirming the phenotypic anchor (*5*–*9*). Using synthetic 6PPD-quinone (purity ~98%), we performed controlled dosing experiments (10 concentrations, *n* = 160 fish in two independent exposures). 6PPD-quinone was highly toxic [median lethal concentration (LC_{50}) 0.79 ± 0.16 μg/liter] to juvenile coho salmon (Fig. 3B). Estimates of LC_{50} through controlled exposures closely matched estimates derived from bulk roadway runoff and TWP leachate exposures (LC_{50} 0.82 ± 0.27 μg/liter), indicating the primary contribution of 6PPD-quinone to observed mixture toxicity (Fig. 3A). Direct comparisons with 6PPD were performed (LC_{50} 250 ± 60 μg/liter through nominal concentrations) (fig. S14), but confident assessment of 6PPD toxicity was precluded by its poor solubility, high instability, and formation of products during exposure.

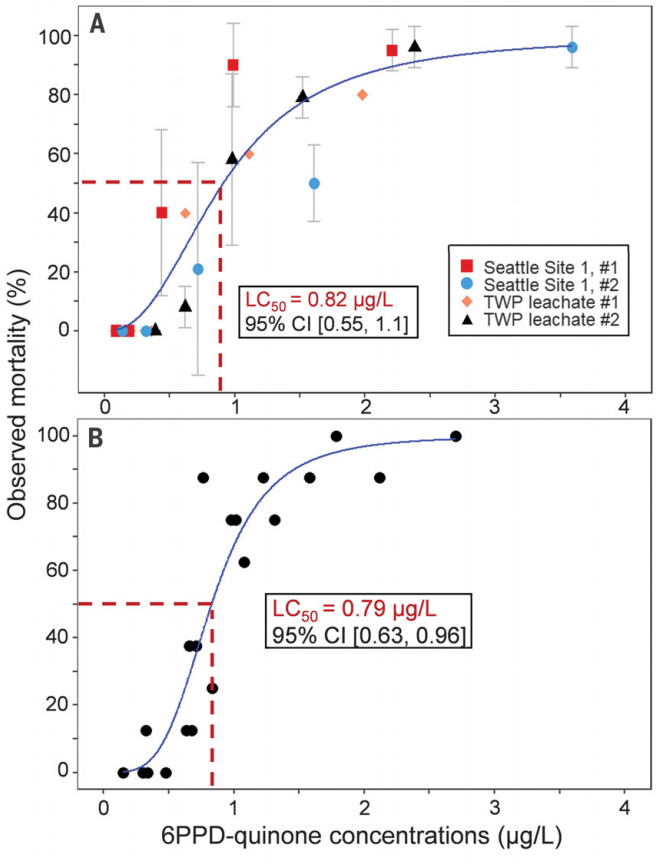

Fig. 3. Dose-response curves. (**A**) Dose-response curve for 24-hour juvenile coho exposures to roadway runoff and TWP leachate (*n* = 365 fish). Error bars represent three replicates of eight fish (except TWP leachate 2, *n* = 5 fish; Seattle site 1, duplicate of *n* = 10 fish). 6PPD-quinone concentrations were from retrospective quantification. (**B**) Dose-response curves for 24-hour juvenile coho exposures to ozone-synthesized 6PPD-quinone (10 concentrations, two replicates, *n* = 160 fish). Curves were fitted to a four-parameter logistic model. CI, confidence interval.

To assess environmental relevance, we used UPLC-HRMS to retrospectively quantify 6PPD-quinone in archived extracts from roadway runoff and receiving water sampling (fig. S15 and table S4) (*10*). In Seattle-region roadway runoff (*n* = 16 of 16 samples), 0.8 to 19 μg/liter 6PPD-quinone was detected (Fig. 4A). During seven storm events in three Seattle-region watersheds highly affected by URMS, 6PPD-quinone occurred at <0.3 to 3.2 μg/liter (*n* = 6 of 7 discrete storm events; *n* = 6 of 21 samples when including samples collected across the full hydrograph). These samples included three storms with documented URMS mortality in adult coho salmon; 6PPD-quinone was not detected in pre- and poststorm samples, but concentrations were near or above LC_{50} values during storms. We also detected 6PPD-quinone in Los Angeles region roadway runoff (*n* = 2 of 2 samples, 4.1 to 6.1 μg/liter) and San Francisco region creeks affected by urban runoff (*n* = 4 of 10 samples, 1.0 to 3.5 μg/liter).

These data implicate 6PPD-quinone as the primary causal toxicant for decades of stormwater-linked coho salmon acute mortality observations. Although minor contributions from other constituents in these complex mixtures are possible, 6PPD-quinone was both necessary (consistently present in and absent from toxic and nontoxic fractions, respectively) and, when purified or synthesized as a pure chemical exposure, sufficient to produce URMS at environmental concentrations. Over the product

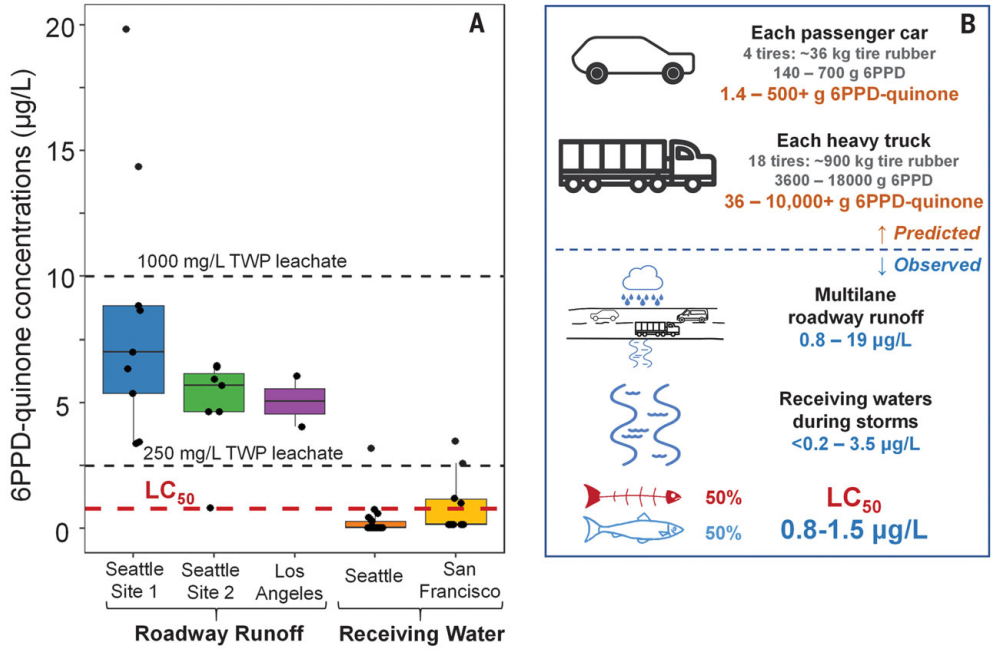

Fig. 4. Environmental relevance of 6PPD-quinone. (**A**) Using retrospective UPLC-HRMS analysis of archived sample extracts, 6PPD-quinone was quantified in roadway runoff and runoff-affected receiving waters. Each symbol corresponds to duplicate or triplicate samples, and boxes indicate first and third quartiles. For comparison, the 0.8 µg/liter LC_{50} value for juvenile coho salmon and detected 6PPD-quinone levels in 250 and 1000 mg/liter TWP leachate are included. (**B**) Predicted ranges of potential 6PPD-quinone mass formation in passenger cars (for example, four tires, ~36 kg tire rubber mass) and heavy trucks (for example, 18 tires, ~900 kg of tire rubber) (represented in orange) and measured 6PPD-quinone concentrations in affected environmental compartments (represented in blue, with experimental data italicized). Predicted ranges reflect calculations applying 0.4 to 2% 6PPD per total vehicle tire rubber mass followed by various yield scenarios (1 to 75% ultimate yields) for 6PPD reaction with ground-level ozone to form 6PPD-quinone.

life cycle, antioxidants [such as PPDs, TMQs (2,2,4-trimethyl-1,2-dihydroquinoline), and phenolics] are designed to diffuse to tire rubber surfaces, rapidly scavenge ground-level atmospheric ozone and other reactive oxidant species, and form protective films to prevent ozone-mediated oxidation of structurally important rubber elastomers (*21*, *22*). Accordingly, all 6PPD added to tire rubbers is designed to react, intentionally forming 6PPD-quinone and related transformation products that are subsequently transported through the environment. This anti-ozonant application of 6PPD inadvertently, yet drastically, increases roadway runoff toxicity and environmental risk by forming the more toxic and mobile 6PPD-quinone transformation product. On the basis of the ubiquitous use and substantial mass fraction (0.4 to 2%) of 6PPD in tire rubbers and the representative detections across the U.S. West Coast (table S4), which include many detections near or above LC_{50} values, we believe that 6PPD-quinone may be present broadly in peri-urban stormwater and roadway run-off at toxicologically relevant concentrations for sensitive species, such as coho salmon.

Globally, ~3.1 billion tires are produced annually for our more than 1.4 billion vehicles, resulting in an average 0.81 kg per capita annual emission of tire rubber particles (*23*). TWPs are one of the most substantial microplastics sources to freshwaters (*24*); 2 to 45% of total tire particle loads enter receiving waters (*25*, *26*), and freshwater sediment contains up to 5800 mg/kg TWP (*23*, *24*, *27*). Supporting recent concerns about microplastics (*24*, *28*), 6PPD-quinone provides a compelling mechanistic link between environmental microplastic pollution and associated chemical toxicity risk. Although numerous uncertainties exist regarding the occurrence, fate, and transport of 6PPD-quinone, these data indicate that aqueous and sediment environmental TWP residues can be toxicologically relevant and that existing TWP loading, leaching, and toxicity assessments in environmental systems are clearly incomplete (*25*). Tire rubber disposal also represents a major global materials problem and potential potent source of 6PPD-quinone and other tire-derived transformation products. In particular, scrap tires repurposed as crumb rubber in artificial turf fields (17) suggest both human and ecological exposures to these chemicals. Accordingly, the human health effects of such exposures merit evaluation.

Environmental discharge of 6PPD-quinone is particularly relevant for the many receiving waters proximate to busy roadways (Fig. 4B). It is unlikely that coho salmon are uniquely sensitive, and the toxicology of 6PPD transformation products in other aquatic species should be assessed. For example, used tires were more toxic to rainbow trout (75% lower 96 hours LC_{50}) relative to new tires (*29*), an observation that is consistent with adverse outcomes mediated by transformation products. If management of 6PPD-quinone discharges is needed to protect coho salmon or other aquatic organisms, adaptive regulatory and treatment strategies (*17*, *30*, *31*) along with source control and "green chemistry" substitutions [identifying demonstrably nontoxic and environmentally benign replacement antioxidants (*22*, *32*)] can be considered. More broadly, we recommend more careful toxicological assessment for transformation products of all high-production-volume commercial chemicals subject to pervasive environmental discharge.

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ACKNOWLEDGMENTS

We thank D. Whittington; S. Edgar (University of Washington Medicine Mass Spectrometry); M. Bozlee (City of Tacoma); J. Protasio; A. Rue (Washington State Department of Ecology); M. Goehring (King County); D. E. Latch (Seattle University); J. E. Baker; C. A. James; A. D. Gipe (University of Washington Tacoma); M. Yu (Mount Sinai); S. D. Richardson (University of South Carolina); J. R. Cameron [National Oceanic and Atmospheric Administration (NOAA) NWFSC]; K. King (U.S. Fish and Wildlife Service); Washington State Department of Transportation; and dedicated citizen scientists from the Miller Walker Community Salmon Investigation, Puget Soundkeeper, and Thornton Creek Alliance. We gratefully thank the Puyallup Tribe and NOAA NWFSC for providing juvenile coho and Agilent Technologies (T.A. and D.C.) for technical support. **Funding:** This work was supported by NSF grants 1608464 and 1803240, EPA grant 01J18101 (E.P.K.), DW-014-92437301 (N.L.S., J.K.M., and J.W.D.), Washington State Governors Funds (J.K.M. and E.P.K.), the Burges Fellowship (H.Z.), the Regional Monitoring Program for Water Quality in San Francisco Bay (A.G. and R.S.), Brazilian foundation agency FAPESP (2018/16040-5 and 2019/14770-9) (F.V.C.K.), NSERC Alliance (ALLRP 549399) and Discovery (RGPIN-2019-04165) Programs, the Canada Foundation for Innovation (CFI), the Ontario Ministry of Research and Innovation, and the Krembil Foundation (A.S.). Disclaimer: Findings and conclusions herein are those of the authors and do not necessarily represent the views of the sponsoring organizations. **Author contributions:** Z.T., H.Z., K.T.P., J.K.M., M.C.D., and E.P.K. designed research; Z.T., H.Z., M.G., K.T.P., C.W., R.H., and A.E.C. performed fractionation experiments; Z.T., K.T.P., R.L., and M.G. performed HRMS and data analysis; Z.T., H.Z., M.G., J.W., K.T.P., C.W., R.H., E.P.K., J.K.M., and A.E.C. conducted fish exposures; J.P., C.W., and J.W. generated TWP particles; J.W., J.P., E.M., and J.K.M. maintained the fish facility and enabled exposure studies; R.G.B., F.V.C.K., R.S., A.J., and A.S. elucidated structures by means of NMR; K.T.P., C.W., F.H., Z.T., M.G., B.D., A.G., and R.S. provided water samples; X.H., Z.T., H.Z., H.H., and M.C.D. performed ozonation experiments; N.L.S. and J.W.D. provided perspectives and context; and Z.T., H.Z., K.T.P., and E.P.K. wrote the manuscript. **Competing interests:** None declared. **Data and materials availability:** Data file S1 includes the record of the juvenile coho salmon exposure experiments. Number of tanks and coho salmon used, mortality results, and treatment information are included in the table. All other data needed to evaluate the conclusions in the paper are present in the paper or the supplementary materials.

SUPPLEMENTARY MATERIALS

science.sciencemag.org/content/371/6525/185/suppl/DC1
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8 July 2020; accepted 5 November 2020
Published online 3 December 2020
10.1126/science.abd6951

A ubiquitous tire rubber–derived chemical induces acute mortality in coho salmon

Zhenyu Tian, Haoqi Zhao, Katherine T. Peter, Melissa Gonzalez, Jill Wetzel, Christopher Wu, Ximin Hu, Jasmine Prat, Emma Mudrock, Rachel Hettinger, Allan E. Cortina, Rajshree Ghosh Biswas, Flávio Vinicius Crizóstomo Kock, Ronald Soong, Amy Jenne, Bowen Du, Fan Hou, Huan He, Rachel Lundeen, Alicia Gilbreath, Rebecca Sutton, Nathaniel L. Scholz, Jay W. Davis, Michael C. Dodd, Andre Simpson, Jenifer K. McIntyre and Edward P. Kolodziej

Science **371** (6525), 185-189.
DOI: 10.1126/science.abd6951originally published online December 3, 2020

Tire tread particles turn streams toxic

For coho salmon in the U.S. Pacific Northwest, returning to spawn in urban and suburban streams can be deadly. Regular acute mortality events are tied, in particular, to stormwater runoff, but the identity of the causative toxicant(s) has not been known. Starting from leachate from new and aged tire tread wear particles, Tian *et al.* followed toxic fractions through chromatography steps, eventually isolating a single molecule that could induce acute toxicity at threshold concentrations of ~1 microgram per liter. The compound, called 6PPD-quinone, is an oxidation product of an additive intended to prevent damage to tire rubber from ozone. Measurements from road runoff and immediate receiving waters show concentrations of 6PPD-quinone high enough to account for the acute toxicity events.

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Science (print ISSN 0036-8075; online ISSN 1095-9203) is published by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005. The title *Science* is a registered trademark of AAAS.