Environmental Science & Technology Letters

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Letter

Acute Toxicity of the Tire Rubber-Derived Chemical 6PPD-quinone to Four Fishes of Commercial, Cultural, and Ecological Importance

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Cite This: *Environ. Sci. Technol. Lett.* 2022, 9, 333−338

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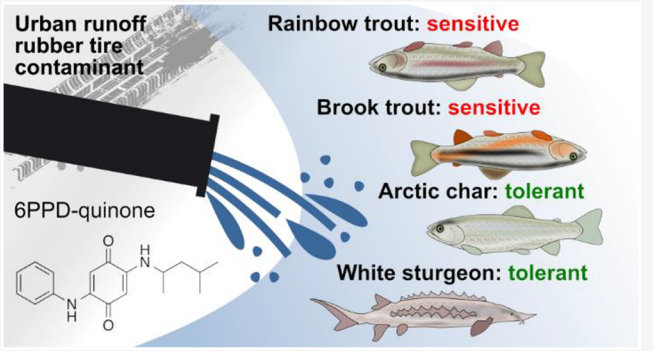
ABSTRACT: *N*-(1,3-Dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine-quinone (6PPD-quinone), a transformation product of the rubber tire antioxidant 6PPD, has recently been identified as the chemical responsible for urban runoff mortality syndrome in coho salmon, with a median lethal concentration (LC_{50}) of <0.1 μg/L. Subsequent studies have failed to confirm comparable sensitivity in other fish species. Here, we investigated the acute toxicity of 6PPD-quinone to rainbow trout, brook trout, Arctic char, and white sturgeon. Fish were exposed under static renewal conditions, and exposure concentrations were verified analytically. Mortalities in brook trout occurred between 1.2 and 20 h, while mortalities began after 7 h and spanned 60 h in rainbow trout. The LC_{50}s in brook trout (24 h) and rainbow trout (72 h) were 0.59 and 1.00 μg/L, respectively. Both species showed characteristic symptoms (increased ventilation, gasping, spiraling, and loss of equilibrium) shortly before death. No mortalities were observed after exposure of either char or sturgeon for 96 h at measured concentrations as high as 14.2 μg/L. This is the first study to demonstrate the acute toxicity of 6PPD-quinone to other fishes of commercial, cultural, and ecological importance at environmentally relevant concentrations and provides urgently needed information for environmental risk assessments of this contaminant of emerging concern.



INTRODUCTION

Stormwater runoff from urban landscapes has long been a cause for environmental concern due to its chemical complexity, toxicity to aquatic organisms, and temporal and spatial dynamics.[1] In addition to road salt, organic contaminants from vehicle emissions and leakage, and toxic metals from brake pad abrasion,[2] tire wear particles (TWPs) have recently become the focus of scientific and public interest.[3] Earlier research into the causes of fish kills following rainfall events along the west coast of the United States, termed coho salmon (*Oncorhynchus kisutch*) urban runoff mortality syndrome (URMS), suggested that rubber tire-derived chemicals might be responsible for this effect because they co-occurred with these mortality events.[4] In a landmark study, Tian et al.[5] applied a combination of fractionation, chemical analysis, and biological testing to pinpoint the causative chemical. The authors found that *N*-(1,3-dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine-quinone (6PPD-quinone) was generated through the environmental oxidation of the common rubber tire antidegradant 6PPD and can cause lethality in coho salmon at a median lethal concentration (LC_{50}) of <0.8 μg/L. Using a commercial standard, a revised LC_{50} in coho salmon of <0.10 μg/L was reported in a follow-up study.[6] Tian et al.[5,6] and subsequent studies have demonstrated the widespread occurrence of 6PPD-quinone in stormwater runoff and surface waters at concentrations of ≤19 μg/L,[7,8] indicating that 6PPD-quinone exposure poses an immediate risk to coho salmon populations. However, it was unknown whether exposure to this pollutant would also affect other aquatic species.

Two follow-up studies have determined the acute toxicity of 6PPD-quinone to a variety of species, including fish (zebrafish, *Danio rerio*; Japanese medaka, *Oryzias latipes*) and invertebrates (*Daphnia magna* and *Hyalella azteca*).[9,10] All tested species were significantly less sensitive than coho salmon: exposure to 6PPD-quinone did not cause lethality in any of the four species studied by Hiki et al.[10] up to concentrations as high as the maximum water solubility, which the authors estimated to range between 34 and 54 μg/L. Varshney et al.[9] observed an LC_{50} of 309 μg/L for zebrafish larvae when ethanol was used as the solvent vehicle. Because of the

Received: January 19, 2022
Revised: February 11, 2022
Accepted: February 14, 2022
Published: March 2, 2022

alarmingly high sensitivity of coho salmon to 6PPD-quinone, environmental risk assessors urgently require data on the acute toxicity of 6PPD-quinone across a greater diversity of fish species, with an emphasis on additional salmonid species.

This study investigated the acute toxicity of 6PPD-quinone across four species of commercial, cultural, and ecological importance to North America: rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*), Arctic char (*Salvelinus alpinus*), and white sturgeon (*Acipenser transmontanus*). Additionally, rainbow trout are an important model fish species used in chemical risk assessment across many jurisdictions.[11,12] This research provides important information for the environmental risk assessment of urban runoff and has the potential to inform regulatory controls of the use of 6PPD in rubber tires.

MATERIALS AND METHODS

Chemicals and Reagents. Native and mass-labeled (d_5) 6PPD-quinone were purchased from Toronto Research Chemicals (Toronto, ON). Stock solutions for exposure of fish to 6PPD-quinone were prepared using dimethyl sulfoxide (DMSO) to achieve a final solvent concentration of 0.01% (v/v) during exposures. Analytical standard solutions of native and mass-labeled 6PPD-quinone were prepared in HPLC-grade methanol.

Fish Source and Culture. Brook trout were from Allison Creek Trout Hatchery (Coleman, AB), were ∼1 year old, were 17.1 ± 1.1 cm in length, and weighed 52.8 ± 7.6 g. Fish were housed in the Aquatic Research Facility (ARF) at the University of Lethbridge and acclimated in 150 L inert glass-fiber Krescel tanks (four fish per tank, 30% daily water renewal) for 2 weeks prior to exposures. Fish were fed a commercial salmonid feed at a daily rate of 1% of body weight during acclimation. Studies were approved by the University of Lethbridge Animal Welfare Committee (Protocol 2111).

Rainbow trout (from Lyndon Hatcheries, New Dundee, ON), Arctic char (from Miracle Springs Inc., North Vancouver, BC), and white sturgeon (wild fish spawned at the Nechako White Sturgeon Conservation Centre, Vanderhoof, BC) were from in-house cultures raised from embryos in the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan. Fish were cultured under flow-through conditions in facility water until they reached the juvenile stage (rainbow trout, ∼2 years, 19.6 ± 1.9 cm, 97.5 ± 28.9 g; Arctic char, ∼3 years, 13.8 ± 1.7 cm, 28.3 ± 9.8 g; white sturgeon, ∼4.5 years, 42.4 ± 4.5 cm, 462.3 ± 159.3 g) and fed with a commercial fish feed at a daily rate of 1% of body weight during acclimation. Even though fish were somewhat larger than recommended according to various guidelines for acute toxicity tests, all fish were sub-adult and the larger size was selected due to availability considerations and to provide sufficient tissues for downstream analyses. Experiments were approved by the University of Saskatchewan Animal Care Committee (Protocol 20070049). A Species at Risk Act (SARA) permit for culture of and experimentation with white sturgeon was obtained from the Department of Fisheries and Oceans Canada (Permit 20-PPAC-00026).

Exposure Experiments. Pilot studies were conducted for each species to establish upper concentration bounds for acute lethality studies. For brook trout, fish were fasted for 24 h, moved to aerated 45 L rectangular glass tanks (two fish per tank) at 10 °C, and exposed for 24 h to nominal concentrations of 0, 0.02, 0.2, 2, or 20 μg/L 6PPD-quinone under static conditions (10 fish total). For the other species, two fish per species were each exposed under static conditions in individual tanks at either 6 or 20 μg/L (two fish total per species). Brook trout and rainbow trout became moribund at 2 and 6 μg/L, respectively, within 4 h of the onset of exposure. Arctic char and white sturgeon did not show any response to concentrations as high as 20 μg/L within 96 h.

Accordingly, in the main experiment, brook trout and rainbow trout were exposed to nominal concentrations of 6PPD-quinone ranging from 0.1 to 6 μg/L (see Table S1 for details). Tanks were cleaned with a series of detergents, disinfectants, and/or ethanol, carefully rinsed, and left to dry before experiments. Due to their lower sensitivity, Arctic char and white sturgeon were exposed to only one nominal concentration (20 μg/L) that could be achieved using the limited amount of chemical available and that was nearing water solubility, while still being environmentally relevant.[5,7,8] Exposures of brook trout were performed in 150 L inert glass-fiber Krescel tanks at 10 ± 1 °C for 24 h (two replicate tanks with four fish each; two controls at five concentrations, 56 fish total). A shorter exposure period was chosen for brook trout due to a much faster onset of symptoms compared to rainbow trout. Test solutions were continuously aerated, recirculated, and temperature controlled. Rainbow trout, white sturgeon, and Arctic char were exposed in 700 L glass-fiber Min-o-Cool tanks containing 500, 500, and 300 L of test solution, respectively, at 12 ± 1 °C for 96 h under static renewal conditions. Water was exchanged at 40−60% (white sturgeon) or 75% (rainbow trout and Arctic char) daily (two replicate tanks and one extra control replicate with five fish each for rainbow trout, 65 fish total; two replicate tanks with five fish each for Arctic char, 20 fish total; three replicate tanks with two fish each for white sturgeon, 12 fish total). Control tanks were dosed with the DMSO solvent vehicle at the same level as all other tanks [0.01% (v/v)]. Average (±SD) water quality parameters were as follows for brook trout: temperature, 10.3 ± 0.7 °C; pH, 6.74 ± 0.13; DO, 99.8 ± 11.5%; ammonia, 0.13 ± 0.11 mg/L; hardness, 131 ± 2.33 mg/L. Average (±SD) water quality parameters were as follows for other species: temperature, 12.8 ± 0.8 °C; pH, 8.35 ± 0.45; DO, 92.8 ± 13.2%; ammonia, 0.14 ± 0.15 mg/L; hardness, 132 ± 6.80 mg/L. Water samples were collected for analytical confirmation of concentrations of 6PPD-quinone ∼1 h after the initial dosing of tanks, which occurred after acclimation of fish for 48−96 h. For rainbow trout, Arctic char, and white sturgeon, a water sample was also taken every 24 h prior to water changes or after most fish in a tank became moribund. Samples were immediately spiked at 50 μg/L with 6PPD-quinone-d_5 and stored at −20 °C until they were analyzed. Fish were observed during most of the exposure duration, immediately removed once they became moribund, and humanely euthanized using >250 mg/L buffered MS-222. Characteristic signs of 6PPD-quinone exposure leading to brook trout and rainbow trout becoming moribund (increased ventilation rate, gasping on the water surface, permanent loss of equilibrium, and spiraling motion) were observed during regular tank inspections and noted and would have resulted in death within 0.5 h if fish were not euthanized.

Biological Sampling. The fork length (millimeters) and weight (grams) of each fish were determined after euthanasia. Blood samples were obtained from the caudal vein using heparinized syringes, and blood glucose concentrations determined using hand-held meters (brook trout, OneTouch

https://doi.org/10.1021/acs.estlett.2c00050
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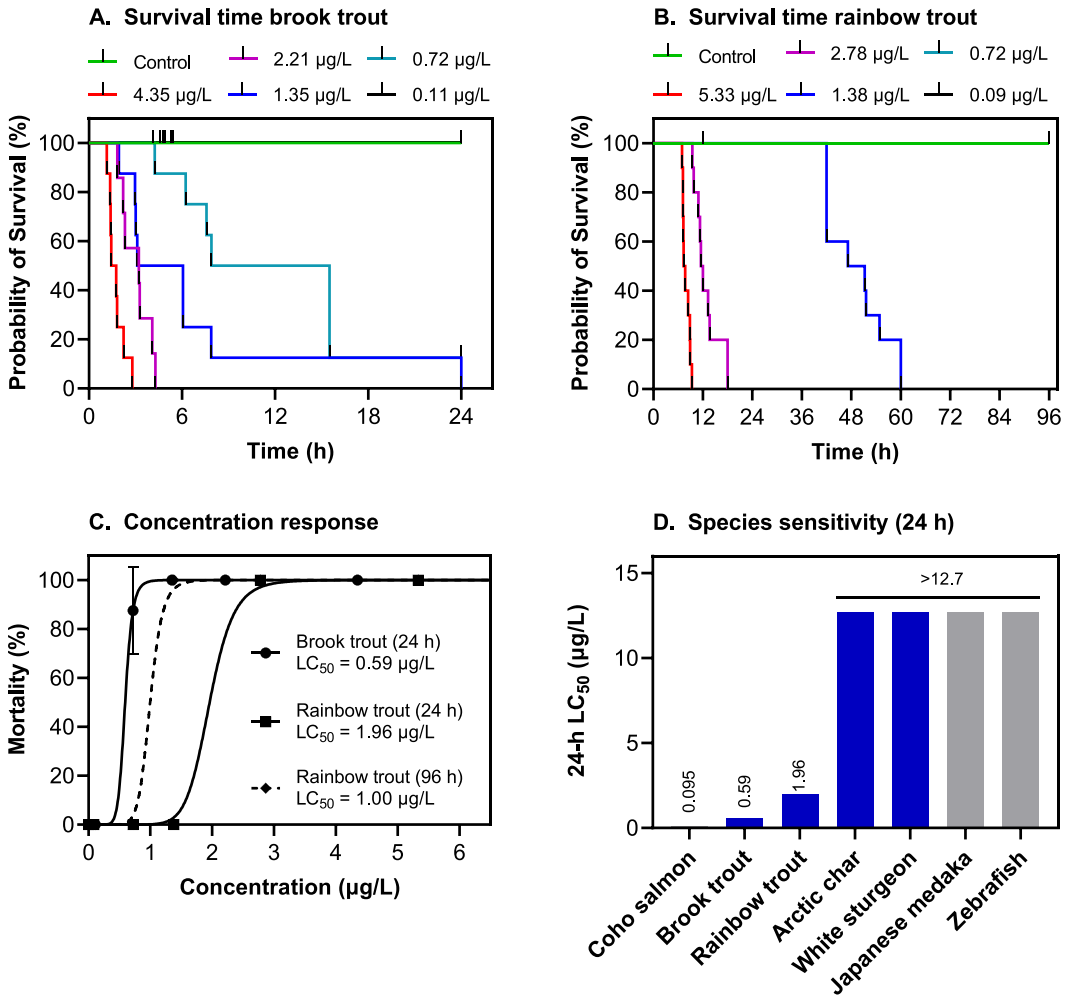

Figure 1. Relationships among exposure time, exposure concentration, and survival in (A) brook trout and (B) rainbow trout over exposure durations of 24 and 96 h, respectively. Median lethal concentrations at 24 and 96 h of exposure were interpolated for both species using (C) two-parameter logistic regression and (D) compared with those of other previously studied species. All concentrations are based on measured concentrations. Blue bars in panel D are from this study, while values for coho salmon, Japanese medaka, and zebrafish have been previously published.[6,9,10]

Ultra 2 m, LifeScan, Malvern, PA; all other species, Contour Next meter, Ascensia, Basel, Switzerland). The percent hematocrit was determined in brook trout using a StatSpin CritSpin microhematocrit centrifuge (StatSpin, Norwood, MA).

Analytical Chemistry. Instrumental verification of exposure concentrations of 6PPD-quinone followed the method outlined by Challis et al.[7] with modifications. Briefly, samples were analyzed on a Vanquish UHPLC instrument coupled with a Q-Exactive HF Quadrupole-Orbitrap hybrid mass spectrometer (Thermo-Fisher). An isotope dilution strategy using 6PPD-quinone-d_5 was applied for quantification. Average measured exposure concentrations were calculated and used for subsequent data analysis instead of nominal exposure concentrations. A detailed description of the analytical methods is provided in the Supporting Information.

Data Analysis and Statistics. The percent mortality for each concentration and replicate was calculated at 24 h for brook trout and at 24 and 96 h for rainbow trout to account for differences in time to death between both species. LC_{50}s were interpolated for each time point using logistic regression of the percent mortality versus average measured exposure concentrations. Blood glucose measurements were analyzed for normality and heteroscedasticity using Kolmogorov−Smirnov's test and Levene's test, respectively. Because the data sets violated the assumptions for one-way analysis of variance (ANOVA), a nonparametric Kruskal−Wallis's test with Dunn's post hoc test was performed. A p value of ≤ 0.05 was considered indicative of statistically significant differences. All plots were created and statistically analyzed using Prism 9 (GraphPad, La Jolla, CA).

RESULTS AND DISCUSSION

Analytical Verification of Exposure Concentrations. Average concentrations of 6PPD-quinone measured over the exposure periods deviated <16% from nominal values across all species with the exception of the low-treatment groups for brook trout and rainbow trout (Table S1). There was an average loss of 14% (1.7% and 32% in the high- and low-treatment groups, respectively) of the test chemical over the 24 h window between water changes, suggesting exposure levels were stable throughout the experiments. Losses were slightly greater at the higher exposure concentrations used for Arctic char and white sturgeon. Hiki et al.[10] reported a 17−73% decrease in 6PPD-quinone concentrations over 48 h between water changes for zebrafish and medaka, confirming the

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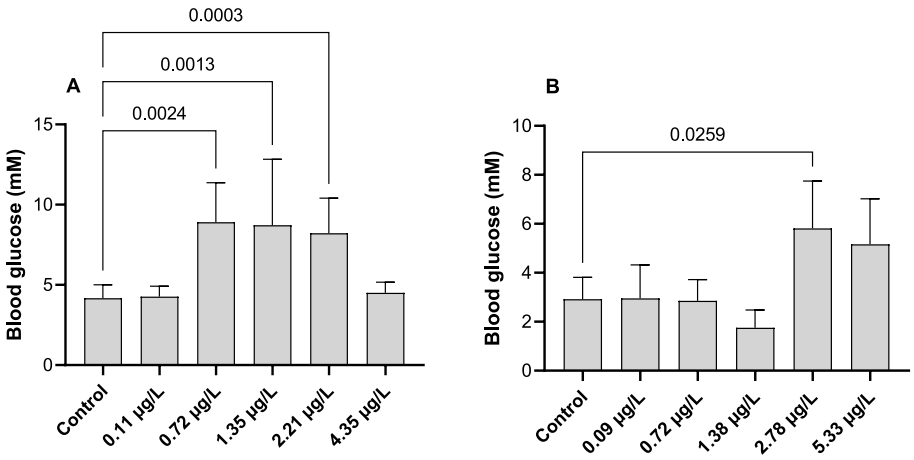

Figure 2. Blood glucose concentrations in (A) brook trout and (B) rainbow trout in moribund animals and those that survived until termination of the experiment following exposure to graded nominal concentrations of 6PPD-quinone. Bars depict the mean, and error bars the standard deviation of 4−15 fish per treatment and species, rather than tank replicates. Because control fish taken down at different sampling times did not differ significantly, individuals were pooled in a single control group for this analysis. Concentrations are based on measured exposure concentrations. Numbers above the brackets indicate the *p* value of statistical comparisons of blood glucose concentrations in 6PPD-quinone-exposed fish with those of the control group (Kruskal−Wallis ANOVA on ranks with Dunn's post hoc test).

relative stability of this chemical under static renewal conditions.

Acute Toxicity of 6PPD-quinone in Fish. Exposure to 6PPD-quinone resulted in significant acute effects in two of the four tested species, which varied as a function of chemical concentration, exposure time, and species (Figure 1). Brook trout were most sensitive with 100% of mortalities in the high-treatment group occurring within 3 h of exposure and a 24 h LC_{50} of 0.59 μg/L [95% confidence interval (CI) of 0.48−0.63 μg/L], which is similar to previous observations in coho salmon.[5] A slightly greater LC_{50} of 1.00 μg/L (95% CI of 0.95−1.05 μg/L) was recorded for rainbow trout after 72−96 h (1.96 μg/L after 24 h, 95% CI of 1.86−2.06 μg/L), while no mortalities were observed for either Arctic char or white sturgeon at measured concentrations as high as 14.2 and 12.7 μg/L after 96 h. Interestingly, in rainbow trout, the first signs of morbidity did not manifest until 7 h after commencing exposures and maximum mortalities occurred at 60 h, which was significantly longer than the times for brook trout and coho salmon.[5] The LC_{50} values reported here for brook trout (0.59 μg/L) and rainbow trout (1.00 μg/L) were ∼6-10-fold greater than that of coho salmon (0.10 μg/L) and are well within ranges of environmental concentrations of 6PPD-quinone previously reported in Canadian and U.S. surface waters after stormwater runoff events.[5−8] While no mortality of endangered white sturgeon or Arctic char was observed after exposure to 6PPD-quinone, potential subchronic or chronic impacts have not been fully studied and cannot be excluded at this time.

These results support earlier reports that identified marked differences in the sensitivity of fishes to exposure with 6PPD-quinone and TWP leachates.[3,5,10] Previous studies have hypothesized that sensitivity to 6PPD-quinone may be unique to salmonids.[9] These authors, who assessed the acute toxicity of this chemical to Japanese medaka and zebrafish, did not observe any significant mortalities up to the limit of the water solubility of 6PPD-quinone, which was estimated to range between 34 and 54 μg/L. While this is in accordance with the lack of effects reported in white sturgeon in this study, our results for Arctic char as well as those reported for TWP leachates by McIntyre et al.[3,5] for chum salmon (*Oncorhynchus keta*) clearly demonstrate the tolerance of these two salmonid species. Thus, we can conclude that sensitivity to acute exposure with 6PPD-quinone is highly variable among fishes in general, and salmonids specifically, even among species from the same genus such as brook trout and Arctic char representing the genus *Salvelinus*, and rainbow trout, chum salmon, and coho salmon representing the genus *Oncorhynchus*.

In cases in which mortalities occurred, both brook trout and rainbow trout exhibited behaviors consistent with those observed in coho salmon,[3,5] including hovering close to the water surface, accelerated opercular movements, gasping, and spiraling motion. This is in accordance with the hypothesis by McIntyre et al.[3] and Varshney et al.[9] that these types of behavior are suggestive of 6PPD-quinone causing cardio-respiratory distress. A significant increase in blood glucose concentrations observed at 0.72−2.21 μg/L in brook trout and 2.78 μg/L in rainbow trout (Figure 2) indicates that 6PPD-quinone impacted energy metabolism, although the underlying mechanisms for this increase are currently unclear. Additionally, hematocrit of brook trout exposed to 0.72−4.35 μg/L 6PPD-quinone significantly increased from an average of 42% in the control group to 68% at 4.35 μg/L (Figure S4). This agrees with observations by Blair et al.,[13] who found even more pronounced increases in hematocrit in coho salmon following exposure to urban runoff. The authors also provided evidence of the disruption of the blood−brain barrier in exposed fish, which might be one of the reasons for the observed increases in hematocrit. However, it is currently unclear if this is the key event ultimately responsible for causing death or if other processes are involved.

Environmental Implications and Risk Assessment. Salmonids are of significant ecological, commercial, and recreational importance in many countries around the world, and this study highlights that the acute toxicity of 6PPD-quinone previously reported for coho salmon[3,5] is also of significant concern for other key receptors, including rainbow trout and brook trout. While there have only been a limited number of studies that characterized the presence of 6PPD-

https://doi.org/10.1021/acs.estlett.2c00050
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quinone in surface waters and urban runoff,[7,8] available reports clearly highlight that commonly found concentrations of this emerging contaminant exceed toxicity thresholds reported here and by Tian et al.[5] Hence, 6PPD-quinone appears to pose a significant and widespread ecological risk to these species, and potentially other salmonids, especially downstream of urban areas and in smaller water bodies receiving roadway runoff.[5,7,8] However, other ecologically relevant genera and families of fishes have not been studied to date, which represents an important uncertainty at this point.

The observed differences in the temporal dynamics of time to death among the three species for which acute effects of 6PPD-quinone have been observed to date are interesting. While in coho salmon[5] and brook trout morbidities at the greatest concentrations were observed as early as 1−2 h after initiation of exposure, in rainbow trout the first mortalities did not occur until ∼7 h at comparable concentrations. As exposure conditions were comparable among experiments in terms of temperature (10−13 °C), pH (6.7−8.3), and DO (>90% saturation), it is unlikely that these parameters would have been a driving factor. Despite the similar LC_{50} values observed for all three species, these differences may have significant implications for ecological risk assessment of urban runoff events. The shorter time to death for coho salmon and brook trout may increase their risk of mortality prior to dilution of stormwater in receiving water bodies over time after rain events.

Future Research Needs. For more comprehensive future risk assessments of 6PPD-quinone in aquatic ecosystems, it is imperative to study its acute and sublethal effects in a broad range of fish species. More research into the potential respiratory or cardiovascular mechanisms of action is needed to conclusively and comprehensively elucidate the specific mechanism by which 6PPD-quinone triggers URMS in select salmonids and possibly other fishes. Most importantly, drivers of species differences in sensitivity need to be studied; i.e., why are some salmonids more sensitive than others? Several native salmonid fish species (e.g., cutthroat trout, *Oncorhynchus clarkii*; bull trout, *Salvelinus confluentus*)[14−16] are at risk of extinction in parts of their native range, and the contribution of 6PPD-quinone to their stock status needs to be urgently investigated.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.2c00050.

Additional details of the chemical analytical methods (Text S1) and results (Table S1 and Figures S1 and S2) and results of hematocrit measurements in brook trout (Figure S3) (PDF)

Video of characteristic symptoms of brook trout exposed to 6PPD-quinone, here loss of equilibrium and spiraling (MOV)

Video of characteristic symptoms of rainbow trout exposed to 6PPD-quinone, here gasping (MOV)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This project was supported partially by a financial contribution from Fisheries and Oceans Canada. Additional funding was provided to M.B., L.W., D.J., M.H., and S.W. through the Discovery Grants program of the Natural Sciences and Engineering Research Council of Canada (NSERC). Instrumentation used for chemical analyses within this research was obtained with funding from Western Economic Diversification Canada (WED) and the Canadian Foundation for Innovation (CFI). J.K.C. was supported through the Banting Postdoctoral Fellowship program of NSERC. M.B. is currently a faculty member of the Global Water Futures (GWF) program, which received funds from the Canada First Research Excellence Funds (CFREF). S.W. and M.H. were supported by the Canada Research Chairs Program. The authors acknowledge the animal care support of Zoë Henrikson and Dale Jefferson (ATRF) and Holly Shepherd and Mamun Shamsuddin (ARF).

Analytical support was provided by Shakya Kurukulasuriya, Jenna Cantin, and Xiaowen Ji.

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https://doi.org/10.1021/acs.estlett.2c00050
Environ. Sci. Technol. Lett. 2022, 9, 333−338