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6PPD-Quinone: Revised Toxicity Assessment and Quantification with a Commercial Standard

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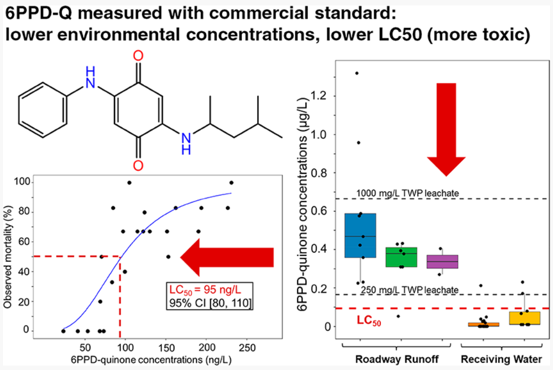
ABSTRACT: Stormwater exposure can cause acute mortality of coho salmon (*Oncorhynchus kisutch*), and 6PPD-quinone (6PPD-Q) was identified as the primary causal toxicant. Commercial standards of 6PPD-Q recently became available; their analysis highlighted a systematic high bias in prior reporting concerning 6PPD-Q. A 6PPD-Q commercial standard was used to re-confirm toxicity estimates in juvenile coho salmon and develop a liquid chromatography-tandem mass spectrometry analytical method for quantification. Peak area responses of the commercial standard were ~15 times higher than those of in-house standards, and the updated LC_{50} value (95 ng/L) was ~8.3-fold lower than that previously reported. These data support prior relative comparisons of the occurrence and toxicity while confirming the substantial lethality of 6PPD-Q. While environmental concentrations are expected to be lower, 6PPD-Q also was more toxic than previously calculated and should be categorized as a "very highly toxic" pollutant for aquatic organisms. Isotope dilution-tandem mass spectrometry methods enabled accurate quantification (limits of quantification of <10 ng/L) within environmental samples.



■ INTRODUCTION

Stormwater runoff is an important contaminant transport pathway in rapidly urbanizing areas,[1] and the complex mixtures of stormwater contaminants often substantially degrade receiving water quality.[2,3] While heavy metals and polycyclic aromatic hydrocarbons have long been regulated pollutants in stormwater, recent studies have reported numerous emerging organic contaminants such as various pesticides, pharmaceuticals, plasticizers, and vehicle and tire rubber-related contaminants.[4−7] Such contaminants can impact aquatic organisms; one compelling example is the stormwater-linked urban runoff mortality syndrome (URMS) of coho salmon (*Oncorhynchus kisutch*) in the Pacific Northwest (USA). Every autumn, recurrent acute mortality occurs when adult coho salmon return to near-urban creeks to spawn.[8] Across sub-basins, mortality rates were most correlated with road density and traffic intensity.[9,10]

While investigating URMS, we previously identified 6PPD-quinone {6PPD-Q; 2-anilino-5-[(4-methylpentan-2-yl)amino]-cyclohexa-2,5-diene-1,4-dione}, an ozonation product of 6PPD, as the primary causal toxicant for long-standing observations of coho mortality.[11] Exposure experiments demonstrated acute toxicity at trace levels (LC_{50} of 0.8 μg/L), and retrospective analysis confirmed detection within roadway runoff and receiving waters, including during URMS events. Because the parent antioxidant compound 6PPD is ubiquitous in tire rubbers, 6PPD-Q would be expected to occur widely in roadway-impacted environments globally, although the hazards of its exposure and toxicity to humans and other organisms remain mostly unknown. Recent studies have confirmed the occurrence of 6PPD-Q in surface waters,[12,13] dusts,[14,15] and fine particulates.[16] Therefore, its potential toxicological effects and ubiquitous occurrence merit monitoring of 6PPD-Q to understand its environmental fate and enable management.

As a newly discovered transformation product, the toxicological experiments and quantification reported by Tian et al.[11] used our own 6PPD-Q standards purified from ozone synthesis and tire wear particle leachate (see the Supporting Information). Recently, a commercial standard and an isotope-labeled standard (D5-6PPD-Q) became available. During analysis, we observed a substantially higher (~15-fold) peak area response of the commercial 6PPD-Q standard versus those of our in-house standards (Figure S1). This observation implied a systematic high bias to the environmental and

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exposure concentrations we reported previously.[11] The lower responses of in-house standards could be caused by the limited solubility or sorption of 6PPD-Q, and we also are investigating possible oxidative polymerization and solid formation within our in-house stocks as potential fate outcomes for quinones.

To correct for potential inaccuracy in previous 6PPD-Q reporting due to the lower purity and/or performance of our in-house standards, we repeated our exposures to juvenile coho salmon with the commercial standard and revised our measured environmental concentrations. We also developed an isotope dilution analytical method based upon liquid chromatography-tandem mass spectrometry (LC-MS/MS). Using these improved quantitative methods, these data represent a timely communication of our current knowledge of the toxicity and expected environmental concentrations of 6PPD-Q.

■ MATERIALS AND METHODS

Chemicals. Commercial standards of 6PPD-Q (10 mg, 98.8% purity, solid) and D5-6PPD-Q (solution in acetonitrile, 100 mg/L) were purchased from HPC (Atlanta, GA). Methanol (LCMS grade), ethanol (absolute, 200 proof), and formic acid (HPLC grade) were purchased from Fisher Scientific. Deionized water (18 MΩ-cm) was generated by a Milli-Q Ultrapure Water System. The 6PPD-Q stock solution (stored at −20 °C) was made by dissolving 5 mg of the HPC standard in 50 mL of ethanol.

Coho Salmon and Exposure Experiments. Juvenile coho salmon used for exposures were obtained courtesy of the Puyallup Tribe of Indians, from the same stock (Diru Creek) and cohort (now age 1+, 30−64 g) as in the previous study.[11] Fish were reared at Washington State University's Puyallup Research and Extension Center on a 12 h:12 h light:dark cycle in a custom recirculating water system and fed commercial food (Biovita, Bio-Oregon, Oregon, WA). Fish system water was dechlorinated municipal water treated by reverse osmosis to Type 3 (>4 MΩ-cm, <0.25 μS/cm) in a RiOs 200 purification system (Millipore Sigma) and then reconstituted with buffered Instant Ocean (Blacksburg, VA) salts to pH ∼7.6 and 1300 μS/cm conductivity at 10−13 °C. Experiments conformed to Experimental Protocol 04860-002, approved by Washington State University's Institutional Animal Care and Use Committee.

For exposures, glass aquaria were randomly placed in recirculating water baths to control temperature. Static aerated exposures were prepared by diluting various volumes of the 6PPD-Q stock solution in 10 mL of ethanol (350−1400 ng/mL), which was then mixed with 70 L of system water; negative controls were pure ethanol (10 mL). Solvent:exposure water ratios matched those of Tian et al.[11] Solutions and negative controls were made within 24 h of exposure. Larger exposure volumes (70 L) and fewer fish per aquarium ($N = 6$) were used here to accommodate the larger fish (30 L with 8−10 fish per aquarium used previously[11]). In two range-finding experiments (0.16−4.0 μg/L and 20−200 ng/L), five concentrations were tested, with five or six fish per concentration. To determine LC_{50}, six concentrations of 6PPD-Q and a negative control were tested. Exposures were repeated in triplicate (batches 1−3, across 3 weeks). Including 30 fish in the second range finding and 108 fish in the three definitive experiments, 138 fish contributed to dose−response exposures (Table S1; controls not included). Four fish jumped out of aquaria during exposures, so 134 fish contributed to the dose−response curve. In all aquaria, safe conditions of temperature (10−13 °C), conductivity (1170−1370 μS/cm), pH (7.6−8.0), and dissolved oxygen (>98% saturation) were verified before fish were transferred. Just prior to the introduction of fish, 1 L of exposure water was sampled from each aquarium, stored on ice or refrigerated, and extracted within 24 h for analysis. For all exposures, mortality rates were recorded at 24 h. Dose−response curves were calculated in R 3.6.2 using a two-parameter log−logistic model in the *drc* package.

Analytical Method. For quantification, duplicates of 200 mL of exposure water from each aquarium (corresponding to one concentration) were spiked with 5 ng of D5-6PPD-Q (100 ng/mL, 0.05 mL) as the internal standard, mixed, and equilibrated (20 min) before extraction. Solid phase extraction (SPE) used Oasis HLB cartridges (6 mL, 200 mg) (see the Supporting Information for details). Eluents were concentrated under a gentle nitrogen flow and volumized to 1 mL for analysis.

Quantification used an Agilent 1290 (Santa Clara, CA) Infinity ultra-high-performance liquid chromatograph (UHPLC) coupled to an Agilent 6460A triple-quadrupole mass spectrometer. Detection used electrospray ionization (ESI+) and multiple-reaction monitoring (MRM) modes. 6PPD-Q concentrations were estimated from a seven-point calibration curve [0.025−50 μg/L (Figure S2)] with D5-6PPD-Q as the isotopic internal standard (25 μg/L, identical to the concentrations of sample extracts). Detailed parameters and method information can be found in Tables S2 and S3 and the text of the Supporting Information.

Quality Assurance/Quality Control. Negative controls were included in batches 1−3 of definitive exposures for LC_{50} determination; all coho salmon ($N = 18$) survived negative controls. Negative controls were processed in a manner identical to that of dosed samples, and additional SPE method blanks were included by extracting and analyzing 200 mL of DI water using identical methods. After laboratory materials (e.g., rubber stoppers) with potential to generate background signals had been screened and removed, 6PPD-Q was not detected above the limit of detection in blanks. Intraday/interday precisions were determined by comparing 2.5 μg/L standard responses multiple ($N \geq 3$) times across one analytical batch or in batches across different dates. All exposure water samples were extracted in duplicate, and then measured and nominal concentrations were compared.

Matrix spikes were performed by spiking 6PPD-Q (5 and 50 ng/L) into Miller Creek (47°27′2.2″N, 122°20′44″W; Burien, WA) baseflow and DI water samples. Absolute recoveries were 58−95%; relative recoveries were 89−116% for the spikes (details in the Supporting Information). The limits of detection (LOD) and quantification (LOQ) were determined as the lowest concentrations giving signal:noise (S:N) ratios of 3 and 10, respectively. The instrumental LOD and LOQ were calculated from low-concentration 6PPD-Q standards (0.025 and 0.1 μg/L), and the method LOD and LOQ (reflecting the mass concentration via SPE) were calculated from spiked creek water samples.

■ RESULTS AND DISCUSSION

Peak Area Response and Toxicity Confirmation. Upon receipt, we first compared the commercial standard (HPC) to our in-house standard[11] across the same nominal concentration range and observed an unexpected difference between

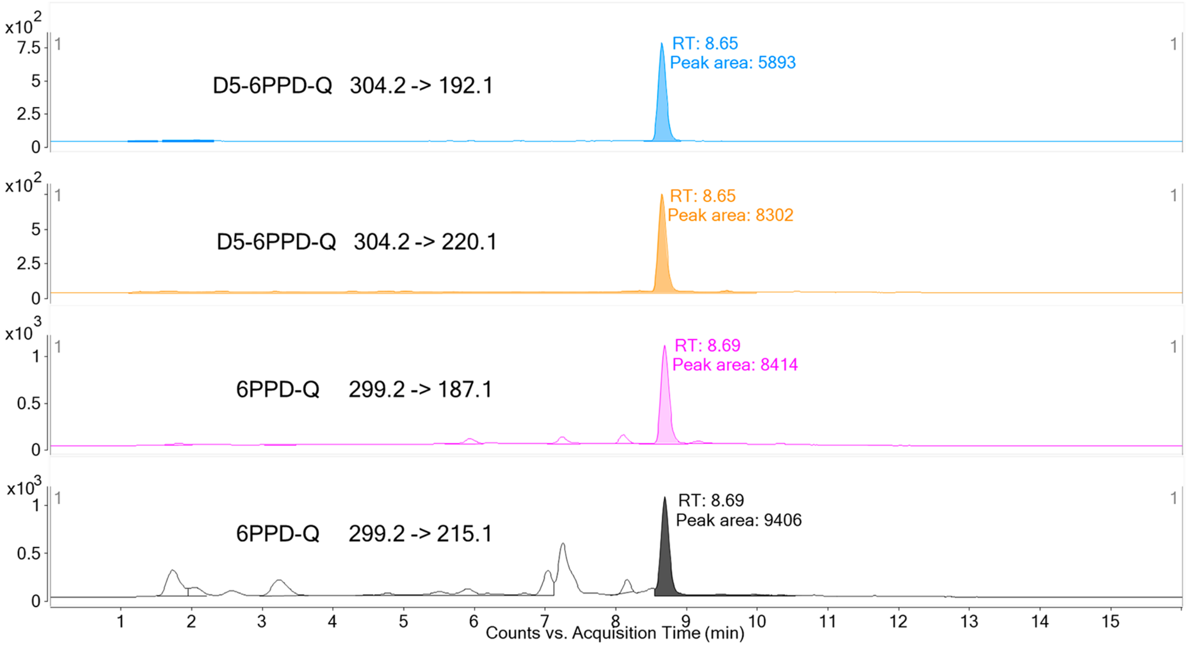

Figure 1. Chromatograms of 6PPD-Q in a stormwater-impacted creek measured by HPLC-MS/MS in MRM mode (measured concentration of 48 ng/L). The top two transitions were the quantitative and qualitative ions of D5-6PPD-Q (internal standard, spiked at 25 ng/L), and the bottom two transitions were the quantitative and qualitative ions of 6PPD-Q. RT, retention time.

the peak area responses of the two calibration curves (Figure S1). The peak area difference was confirmed on both qTOF-HRMS (~15-fold) and MS/MS (~18-fold) instrument platforms, with slight differences caused by the different sensitivities and/or linear ranges of the two instruments. Meanwhile, at identical nominal concentrations, the red/magenta color of the commercial standard solution was visually more intense than that of in-house standards. Ultraviolet−visible spectrophotometry also indicated substantially reduced absorbance for the in-house stock solutions versus the commercial standard stocks at key diagnostic wavelengths [e.g., 360 nm (Supporting Information and Figure S3)].

We then evaluated the toxicity of the commercial 6PPD-Q standard to juvenile coho salmon through two range-finding exposures. Our first exposure utilized a wide nominal concentration range (0.16−4.0 μg/L) that included our previously reported LC_{50} value (0.8 μg/L, derived from exposures using in-house stocks). Observed mortality rates were 100% (five of five) at 0.8, 1.8, and 4.0 μg/L, while four of five coho salmon died at 0.16 and 0.36 μg/L. Notably, coho salmon in the 4.0 μg/L aquarium were symptomatic in ~40 min and all perished in <2 h, which is faster than any of our prior observations. Another exposure series (0.02−0.2 μg/L) demonstrated 100% mortality (six of six) at 0.2 μg/L 6PPD-Q, 67% (four of six) at 0.1 μg/L, and 0% (zero of six) at 0.02, 0.04, and 0.06 μg/L. These results confirmed the substantial toxicity of 6PPD-Q for coho salmon but also indicated that our previous observations with in-house standards had systematically underestimated toxicity.

On the basis of mass spectrometry and these preliminary exposures, we concluded that the 6PPD-Q masses of our in-house standards were lower than we expected. In considering various options for mass loss, we most suspected that we had inadvertently formed a solid precipitate or similar impurity not detected by LC-HRMS or NMR during the end stages of our 6PPD-Q purification or handling processes. The reduced responses of in-house 6PPD-Q might be caused by lower than expected solubility and sorption losses to some filter and system materials; quinones also can undergo many interesting reaction types such as oxidative polymerization and other complexations.[18−20] Although we continue to investigate potential loss mechanisms, we cannot yet conclusively explain this mass loss. On the basis of our experience, while noting the potential for redox-active quinones to exhibit some complex and uncommon fate mechanisms, we caution other investigators to carefully track 6PPD-Q stability and recovery (e.g., peak area response, validated against commercial standards, measured vs nominal concentrations[21]) over time and under experimental conditions. Further research should characterize the fate and stability of 6PPD-Q under a broad range of conditions.

Quantification Method. To improve quantification accuracy, we developed an isotope dilution analytical method using the commercial standard and an isotopic internal standard (D5-6PPD-Q). Using high-performance LC-MS/MS detection, we evaluated transitions of both native and deuterated 6PPD-Q standards (Figure S4), optimized the instrumental parameters to improve sensitivity, and validated performance with environmental samples. These studies indicated that the complex matrix in stormwater and roadway runoff was the primary challenge to analytical performance, with matrix suppression at large extraction volumes (e.g., 1 L) sometimes completely suppressing 6PPD-Q detection.[11] Matrix dilution and reduced extraction (or injection) volumes mitigated matrix suppression and tended to improve performance; thus, we used 200 mL extraction volumes to optimize sensitivity. For location-specific stormwater or roadway runoff sampling, we recommend adjusting extraction volumes in response to observed matrix suppression or sample compositions. Spiking concentrations of the isotopic standard (25 ng/L in samples, 5 μg/L in extracts) were adjusted to reflect expected environmental concentrations while maintaining sufficient sensitivity.

Chromatograms of 6PPD-Q and D5-6PPD-Q in a creek stormwater sample are shown (Figure 1). With optimized parameters (Table S2), the instrument limit of quantification was 0.16 pg on column (31 ng/L in solutions). When applied to analysis of baseflow and stormwater samples in receiving water, method limits of quantification were 2.5 and 5.1 ng/L, respectively. In comparison with our original semiquantification method using LC-qTOF-HRMS,[11] the improved sensitivity came from the more specific MS/MS transitions used for quantification, an optimized processing procedure, the increased detector linear range, and higher standard purity. Spike recovery trials indicated 89−116% relative recovery of 6PPD-Q in creek samples and <3% intraday and interday variation. Given the highly toxic nature of 6PPD-Q and often trace (<100 ng/L) environmental concentrations present in runoff-impacted receiving waters, sensitive, accurate, and robust methods will be needed to maintain performance even at concentrations of ≤10 ng/L.

Updated Dose−Response Curve. To update the dose−response curve and LC_{50} estimate with the commercial standard, we performed additional 6PPD-Q exposures on juvenile coho salmon. Exposure concentrations were quantified using the LC-MS/MS analytical method; Table S1 reports nominal concentrations, measured concentrations, and mortality details. From the updated dose−response curve (Figure 2), an LC_{50} of 95 ng/L was estimated for 6PPD-Q [95% confidence interval (CI [80, 110])]. This result was substantially lower than our previous result (790 ng/L, 95% CI [630, 960]) estimated from our in-house standards and was consistent with more dilute 6PPD-Q stock solutions. This ~8.3-fold difference in the LC_{50} is a combined result of the peak area response (~15-fold) and the 6PPD-Q recovery without internal standard normalization (60−70%).

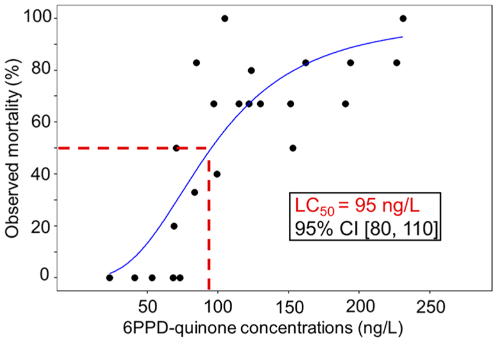

Figure 2. Dose−response curves for 24 h juvenile coho salmon exposures using the commercial HPC 6PPD-Q standard (N = 134 fish). All negative control fish survived and did not show any symptoms (N = 24; six in range finding and 18 in definitive exposure). Curves were fitted to a two-parameter logistic model. CI, confidence interval.

Environmental Significance. The commercial standard analysis exposed a systematic high bias to our previously reported environmental concentrations and toxicity assessment for 6PPD-Q. Therefore, while *relative* comparisons between environmental concentrations and the LC_{50} in our previous work remain valid[11] (all retrospective analysis and dose−response curves were based on the same standard and quantification method), *absolute* concentrations should be revised lower on the basis of our current knowledge of the peak area response for 6PPD-Q. In general, the environmental concentrations and LC_{50} values for coho salmon should be approximately 1 order of magnitude lower than we previously reported.[11,17,22]

To illustrate the updated LC_{50} and observed environmental concentrations, we revised our previous occurrence data[11] while including additional 6PPD-Q data reported recently (Figure 3). Consistent with previous results, 6PPD-Q concentrations in almost all roadway runoff samples exceeded the LC_{50} for coho salmon and concentrations in runoff-impacted receiving waters, including storms where acute mortality occurred, were again near or above the LC_{50}. We note that environmental concentration data in Figure 3B, derived from archived sample extracts,[11] still employ semi-quantification without isotopic standard correction as an inherent aspect of retrospective analysis. Because they are not recovery-corrected, these data likely still underestimate actual concentrations by ~30−40%. With isotope dilution normalization, more stormwater samples would be expected to contain concentrations above the LC_{50}. Overall, these results re-confirm the lethality of 6PPD-Q to coho salmon and the substantial capability for mortality outcomes at environmentally relevant concentrations.

Since the initial identification, additional reports of 6PPD-Q occurrence[13−15,23] have confirmed its ubiquity in roadway-impacted environments at reported concentrations up to the low microgram per liter range. Because rubber products like tires, including end-of-life applications in recycled and repurposed materials, are ubiquitous in modern society, continued documentation of 6PPD-Q in various environmental compartments (e.g., soil, air, and biological tissues) is expected. As demonstrated by our quantitative difficulties, accurately characterizing previously unknown contaminants, especially unintentionally produced transformation products that lack commercial standards, remains a challenging and yet critical task for environmental researchers. The updated LC_{50} (95 ng/L) suggests that 6PPD-Q is among the most toxic chemicals known for aquatic organisms, at least to coho salmon.[21] Compared directly to pollutants categorized as "very highly toxic" to sensitive aquatic organisms (mortality at <100 ppb, Table 1, rationale in the Supporting Information), the LC_{50} for coho salmon (mortality at ~0.1 ppb) places 6PPD-Q among a very small group of pollutants, mostly organophosphate or organochlorine pesticides, with acute toxicity expectations at tens of nanograms per liter.

Many pressing knowledge gaps remain with respect to the environmental fate, transport, and toxicological implications of 6PPD-Q in tire rubber-impacted environments. Aspects of quinone stability and fate, representing contaminant structures less commonly considered and documented in environmental fields, are clearly both analytically and environmentally important to understand. The mechanisms of toxicity of 6PPD-Q to coho salmon need to be characterized, while its toxicity, both acute and sublethal, to additional organisms needs to be broadly evaluated, including insights into mechanisms of species-specific sensitivities.[21,22] Critically, 6PPD-Q reminds us that many synthetic chemicals do not simply disappear upon environmental release; we should be especially careful with chemicals like antioxidants that may have toxic properties[24] and are also designed to react. The lifetime mass balance of 6PPD, 6PPD-Q, and related chemicals in rubber tires that are subject to widespread environmental

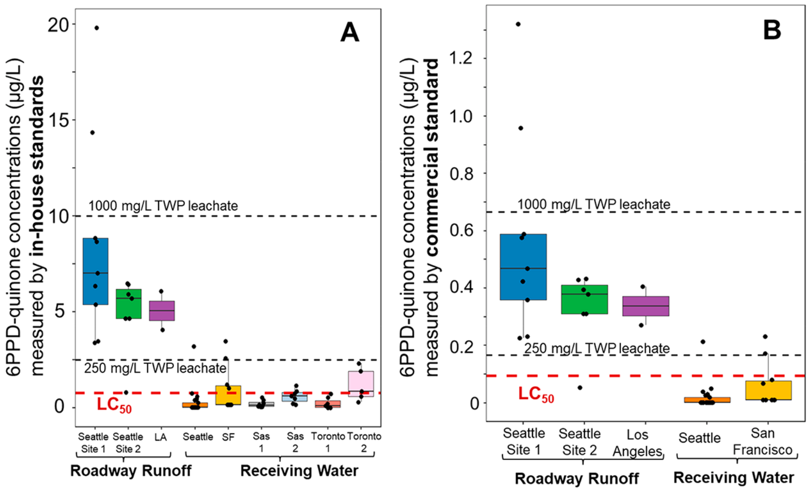

Figure 3. 6PPD-Q environmental concentrations and toxicity estimates derived from different standards. (A) 6PPD-Q concentrations estimated using in-house standards. Data for Seattle, San Francisco, and Los Angeles were taken from ref 11. Sas 1 and Sas 2 were snowmelt and surface water samples from Saskatoon, Canada, reported in ref 12. Toronto 1 and Toronto 2 were surface waters from Toronto, Canada, reported in refs 13 and 23. (B) Updated 6PPD-Q concentrations and toxicity thresholds estimated using a commercial standard (HPC), reflecting data reported in ref 11. The updated concentrations are based on retrospective UPLC-qTOF analysis of archived sample extracts, divided by a factor of 15 (qTOF peak area difference between the in-house and commercial 6PPD-Q standards). 6PPD-Q was quantified in roadway runoff and runoff-impacted receiving waters. Each symbol corresponds to duplicate or triplicate samples; boxes represent first and third quartiles. For comparison, the 95 ng/L LC_{50} value for juvenile coho salmon and detected 6PPD-quinone levels in 250 and 1000 mg/L TWP leachate are included. Modified from ref 11 with permission from AAAS.

Table 1. Comparison of the Toxicity of 6PPD-Q to Coho Salmon with Those of the Most Toxic Chemicals for Which the U.S. Environmental Protection Agency Has Established Aquatic Life Criteria[a]

chemical class	name	most sensitive species	LC_{50} (ppb)	95% CI	ref	CMC (ppb)	EPA document
OP	parathion	*Orconectes nais*	0.04	0.01−0.2	25	0.065	EPA 440/5-86-007
quinone	6PPD-Q	*O. kisutch*	0.10	0.08−0.11	this study	not available	not available
OC	mirex	*Procambaris blandingi*	0.10	not reported	26	0.001	EPA 440/5-86-001
OP	guthion	*Gammarus fasciatus*	0.10	0.073−0.014	25	0.01	EPA 440/5-86-001
OP	chlorpyrifos	*Gammarus lacustris*	0.11	not reported	27	0.083	EPA 440/5-86-005
OC	endrin	*Perca flavescens*	0.15	0.12−0.18	28	0.086	EPA 820-B-96-001
OC	4,4′-DDT	*O. nais*	0.18	0.12−0.30	25	1.1	EPA 440/5-80-038
OP	diazinon	*Ceriodaphia dubia*	0.25	not reported	29	0.17	EPA-822-R-05-006
metal	cadmium	*Oncorhynchus mykiss*	0.35	not reported	30	1.8	EPA-820-R-16-002
OC	methoxychlor	*O. nais*	0.50	0.25−1.8	25	0.03	EPA 440/5-86-001
OC	dieldrin	*Pteronarcella badia*	0.50	0.37−0.67	28	0.24	EPA 820-B-96-001
OP	malathion	*G. fasciatus*	0.76	0.63−0.92	25	0.1	EPA 440/5-86-001
OC	toxaphene	*Ictalurus punctatus*	0.8	0.5−1.2	31	0.73	EPA 440/5-86-006

[a] The rationale for the toxicity comparison can be found in SI text. Abbreviations: OP, organophosphate; OC, organochlorine; CMC, criterion maximum concentration; CI, confidence interval.

dispersal should also be thoroughly characterized to advance the safe use, disposal, and recycling of these materials as we work toward more environmentally benign and sustainable consumer products.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.1c00910.

> Details on the use of in-house standards, water extraction, instrumental methods and parameters, ultraviolet−visible measurement, toxicity comparison rationale, nominal and measured exposure concentrations of 6PPD-Q, chromatograms, mass spectra, and standard calibration curves (PDF)

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Notes

The authors declare no competing financial interest.

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