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Acute Toxicity of 6PPD-Quinone to Early Life Stage Juvenile Chinook (*Oncorhynchus tshawytscha*) and Coho (*Oncorhynchus kisutch*) Salmon

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Abstract: The breakdown product of the rubber tire antioxidant *N*-(1,3-dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine-quinone (6PPD)-6-PPD-quinone has been strongly implicated in toxic injury and death in coho salmon (*Oncorhynchus kisutch*) in urban waterways. Whereas recent studies have reported a wide range of sensitivity to 6PPD-quinone in several fish species, little is known about the risks to Chinook salmon (*Oncorhynchus tshawytscha*), the primary prey of endangered Southern Resident killer whales (*Orcinus orca*) and the subject of much concern. Chinook face numerous conservation threats in Canada and the United States, with many populations assessed as either endangered or threatened. We evaluated the acute toxicity of 6PPD-quinone to newly feeding (~3 weeks post swim-up) juvenile Chinook and coho. Juvenile Chinook and coho were exposed for 24 h under static conditions to five concentrations of 6PPD-quinone. Juvenile coho were 3 orders of magnitude more sensitive to 6PPD-quinone compared with juvenile Chinook, with 24-h median lethal concentration (LC50) estimates of 41.0 and more than 67 307 ng/L, respectively. The coho LC50 was 2.3-fold lower than what was previously reported for 1+-year-old coho (95 ng/L), highlighting the value of evaluating age-related differences in sensitivity to this toxic tire-related chemical. Both fish species exhibited typical 6PPD-quinone symptomology (gasping, increased ventilation, loss of equilibrium, erratic swimming), with fish that were symptomatic generally exhibiting mortality. The LC50 values derived from our study for coho are below concentrations that have been measured in salmon-bearing waterways, suggesting the potential for population-level consequences in urban waters. The higher relative LC50 values for Chinook compared with coho merits further investigation, including for the potential for population-relevant sublethal effects. *Environ Toxicol Chem* 2023;42:815–822. © 2023 His Majesty the King in Right of Canada and The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC. Reproduced with the permission of the Minister of Fisheries and Oceans Canada.

Keywords: Aquatic toxicology; Contaminants of emerging concern; Contaminants; *N*-(1,3-dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine-quinone (6PPD)-6-PPD-quinone; Chinook salmon; Coho salmon

INTRODUCTION

Urban stormwater runoff pollution degrades water quality (Göbel et al., 2007) and poses a significant risk to the health of aquatic organisms (Burton et al., 2000). One example of "urban stream syndrome" has been referred to as "urban runoff mortality syndrome" (URMS) and has been used to reflect the premature death (40%–90%) of adult coho salmon (*Oncorhynchus kisutch*) returning to urban and semi-urban waterways in Washington State (USA; Scholz et al., 2011). The syndrome was first reported during monitoring of restored urban streams in Puget Sound (WA, USA) between 1999 and 2001, with unexplained prespawn mortality of adult coho following rain events. Fish exhibited a range of symptoms, including erratic surface swimming, gasping, fin splaying, and loss of orientation and equilibrium, ultimately leading to death within a few hours (Scholz et al., 2011). Whereas these effects were later linked to stormwater run-off and tire-associated contaminants (French et al., 2022; McIntyre et al., 2021; Peter et al., 2018; Spromberg et al., 2016), it wasn't until recently that Tian et al. (2021, 2022) identified the causal compound to

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be *N*-(1,3-dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine-quinone (6PPD-quinone), an ozonation product of the rubber tire antioxidant 6PPD. Initially, the reported 6PPD-quinone (6PPD-Q) coho median lethal concentration (LC50) was 790 ng/L, but this was revised downward to 95 ng/L after the availability of a commercial 6PPD-Q standard (Tian et al., 2021, 2022).

The acute toxicity of 6PPD-Q to other fish species has been explored. The following fish and invertebrate species displayed differing degrees of sensitivity: rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*), Arctic char (*Salvelinus alpinus*), white sturgeon (*Acipenser transmontanus*), brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*), white-spotted char (*Salvelinus leucomaenis pluvius*), southern Asian dolly varden (*Salvelinus curilus*), masu salmon (*Oncorhynchus masou masou*), zebrafish (*Danio rerio*), *Daphnia magna*, and *Hyalella azteca*; Brinkmann et al., 2022; Foldvik et al., 2022; Hiki & Yamamoto, 2022; Hiki et al., 2021). Interestingly, all were less sensitive than coho, including Arctic char and white sturgeon, two cold-water species whose 96-h LC50 values are estimated to be more than 12 700 ng/L (Brinkmann et al., 2022). The very high sensitivity of coho salmon to 6PPD-Q at a time of declining Pacific salmon populations in recent decades (Gustafson et al., 2007; Price et al., 2017; Walsh et al., 2020) has led many concerned parties (i.e., risk assessors, Environmental Quality Guideline specialists, conservationists, and Indigenous Peoples) to explore possible conservation and management policies.

Among the Pacific salmon species whose sensitivity to 6PPD-Q has not yet been established is Chinook salmon (*Oncorhynchus tshawytscha*). This species is important not only to First Nations, recreational anglers, and commercial fisheries, but also to the endangered Southern Resident killer whales that rely on this species as their primary prey (Ford et al., 2010). In Canada and the United States, many of the assessed populations (88% and 53%, respectively) of Chinook have been classified as at risk (Beamish, 2018; Committee on the Status of Endangered Wildlife in Canada, 2018; Environment and Climate Change Canada, 2021; National Oceanic and Atmospheric Administration Fisheries, 2022). In addition to contaminants, Chinook salmon face a number of threats, including climate change, habitat destruction, and overharvesting.

Many Chinook populations rear and/or migrate through urban-impacted waterways (Anzalone et al., 2022; Chalifour et al., 2020; Yeh et al., 2017), and thus may be exposed to 6PPD-Q during both early life history stages as they begin their lives in freshwater habitats, and as adults when they return to spawn. Age of salmon at seaward migration (when juveniles move from freshwater to the ocean) ranges from less than 1 to 3 years old for coho and less than 1 to 2 years old for Chinook (Quinn, 2018), indicating that a wide range of their time can be spent in freshwater systems (e.g., creeks, streams, rivers). It is unclear whether organism size or life stage alters the sensitivity to 6PPD-Q. However, size (i.e., weight)-related differences in sensitivity to other contaminants has been reported in a number of salmonid studies, in particular. For example, the reported LC50 values for two sizes of juvenile brown trout (*S. trutta*) were 868 and 354 µg/L (a 2.5-fold difference) between small fish that range from 0.148 to 0.423 g and larger juveniles ranging from 0.639 to 1.432 g (Diedrich et al., 2015). Similarly, small (2.0-g) Chinook and coho, as well as rainbow trout have been shown to be less sensitive than larger (10.0-g) juveniles to formalin, potassium permanganate, copper sulfate, acetic acid, and hydrogen peroxide (Taylor & Glenn, 2008). The previously reported coho LC50 was derived using juvenile fish age 1+ years, with a body weight between 30 and 64 g (Tian et al., 2022). To develop a more comprehensive understanding of the risk of 6PPD-Q to juvenile coho and Chinook survival in urbanized areas, research must also encompass a characterization of their sensitivity in the less than 1-year age range.

Our study investigated the acute toxicity of 6PPD-Q to newly feeding (~3 weeks post swim-up) juvenile Chinook and coho over a 24-h exposure. Mortality-based concentration–response curves were established, and a comparison of species sensitivity was explored. Our results will be used to inform the risk of this prevalent urban contaminant to juvenile Chinook and coho and to aid in the conservation, recovery, and management efforts of these much valued species.

MATERIALS AND METHODS

Fish source and culture

Juvenile Chinook and coho were obtained from the Chehalis River Hatchery (Agassiz, BC, Canada). Approximately 1 week after ponding (i.e., feeding for 1 week), fish were transported to the Pacific Science Enterprise Centre (West Vancouver, BC, Canada), and then reared for 2 weeks in 125-L flow-through inert glass-fiber tanks prior to 6PPD-Q exposure. As per guidance from Environment and Climate Change Canada's Biological Test Method: Acute lethality using rainbow trout (Environment Canada, 1990), rearing conditions included a minimum flow rate of 0.5 L/min, fish density of 1.28 kg/m^3 or less, a 16:8-h light:dark photoperiod, and fish were fed multiple times a day until satiation, targeting a maximum of 4% body weight/day. Well water was used for culturing and exposures (same source). An animal use protocol (# 21-020) was obtained from the Fisheries and Oceans Canada Pacific Region Animal Care Committee.

Chemicals and reagents

The 6PPD-Q (purity of 97.26%, lot #802502) was purchased from HPC Standards. Stock solutions of 6PPD-Q were prepared using absolute ethanol to achieve a final solvent concentration of 0.01% (v/v), which is consistent with previous 6PPD-Q coho toxicity studies (Tian et al., 2021, 2022). Stock solutions were prepared 24 h prior to exposure. For the Chinook exposure, serial dilution of a parent stock was not used throughout due to concerns regarding whether 6PPD-Q would sufficiently dissolve at high concentrations. Instead, three separate stock solutions for the three highest treatments were made by adding ethanol to 6PPD-Q and mixing each stock at room temperature using a stir plate for up to 3 h. The lowest concentration of the three stock solutions was used as a parent stock to create the remaining stock solutions using serial dilution. For the coho

exposure, a single parent stock solution was created, and subsequent stock solutions were created via serial dilution. All stock solutions were placed in sealed glass vessels and stored at −20 °C overnight. Prior to use, stock solutions were brought to room temperature and mixed using a stir bar to redissolve any precipitate that formed overnight.

Waterborne 6PPD-Q exposure experiments

Range finder studies using a simplified experimental setup ($n = 5$ fish/tank, no replicates) were conducted for each species to determine upper concentration ranges for the exposures. Test methods were adapted from the Environment and Climate Change Canada's rainbow trout acute lethality toxicity test method (Environment Canada, 1990). Fish were approximately 3 weeks post swim-up, and had wet body weight averages (±standard deviation) of 0.829 ± 0.148 and 0.433 ± 0.132 g for Chinook and coho, respectively. Due to density constraints, an $n = 11$ for Chinook and an $n = 14$ for coho/replicate tank were used. The test exposure volume was 18 L/replicate 20-L glass tank, resulting in fish densities of approximately 0.507 and 0.337 g/L for Chinook and coho, respectively. Exposures consisted of five concentrations of 6PPD-Q, one control (well water), and one solvent control (0.01% ethanol); all were tested in quadruplicate. Exposures were conducted under static conditions with continuous aeration, a 16:8-h light:dark photoperiod of and light intensity of 100–500 lux. Prior to use, glass tanks and pipettes (used for aeration) were cleaned in three steps using liquid detergent, 10% nitric acid, and acetone with distilled water rinses following each step. Temperature, dissolved oxygen, pH, and conductivity were measured at test initiation (0 h) and at test termination (24 h).

Fish were not fed for the duration of the exposure. A 24-h test duration was selected because it mimics a storm event, during which a pulse of 6PPD-Q is suddenly flushed from roadways into urban freshwater systems. Nominal test concentrations for the definitive study were 5716, 10 288, 18 519, 33 333, and 60 000 ng 6PPD-Q/L for Chinook and 11.9, 21.4, 38.6, 69.4, and 125 ng 6PPD-Q/L for coho. Fish were observed continuously for the first hour, and at 1.5, 3, 4, 5, 6, 22, and 23 h for the appearance of clinical signs including those consistent with 6PPD-Q exposure (i.e., erratic surface swimming, gasping, fin splaying, and loss of orientation and equilibrium [Chow et al., 2019; Scholz et al., 2011]); any signs were recorded. At test termination (24 h), fish were euthanized using an overdose of buffered tricaine methane sulfonate. Mortality, fish weight (g), and length (mm) were recorded. Average percentage of mortality for each treatment was calculated, and a two-parameter log-logistic model, binomial setup (R Core Team, 2022; Ritz et al., 2015) was used to estimate the LC5, 10, 25, and 50 for both species. Initial concentrations were used to develop dose–response curves.

To verify exposure concentrations, water samples for 6PPD-Q analysis were collected from two replicate tanks for each treatment at test initiation and termination. Ascorbic acid was added to each water sample as per SGS AXYS Analytical Ltd. (Sidney, BC, Canada) method MLA-118. Samples were shipped within 48 h of sampling to SGS AXYS Analytical Ltd. and held at 4 °C in the dark for 6–14 days until analysis. In addition, a preliminary exploration of potential changes in exposure water 6PPD-Q concentration without the presence of fish was conducted on three occasions. These three trials consisted of two replicate tanks with the same experimental conditions employed for the fish waterborne, static exposures just described including a 16:8-h light:dark photoperiod, with the exception of the first trial photoperiod, which was approximately 10:14-h light:dark. Water was subsampled for 6PPD-Q at test initiation and at 24 h (reflective of fish test termination). The concentrations evaluated in these fish-free exposure experiments were 100, 18 519, and 125 ng/L (nominal) during the first, second, and third trials, respectively.

Analytical determination of 6PPD-Q

After arrival at SGS AXYS Analytical Ltd. and prior to liquid–liquid extraction with dichloromethane, exposure water subsamples were spiked with isotopically labeled standard D5-6PPD-Q. Extracts were followed by ultraperformance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) analysis on a Waters ACQUITY UPLC I-Class System and Xevo TQ-S tandem Mass Spectrometer. Analytes were detected in positive electrospray ionized mode with multiple reaction monitoring (MRM) of the two most abundant product ions for each analyte. The precursor ion of 6PPD-Q is *m/z* 299.4 with two product ions (*m/z* 241.0 and 215.0). Two MRM transitions, Q1 (precursor ion) and Q3 (product ion), were used for simultaneous quantitation (299.4 → 241.0) and identification (299.4 → 215.0) of 6PPD-Q. Analyte separation was achieved on a Waters ACQUITY UPLC BEH C18, 1.7 μm, 2.1 × 50 mm column protected by an ACQUITY UPLC BEH C18 Vanguard Pre-column, 1.7 μm, 2.1- × 5-mm. Mobile phases consisted of 0.1% formic acid in UPLC water (solvent A) and 1:1 acetonitrile:methanol organic phase (solvent B). The starting mobile phase composition was 70% A, which was held for 1 min and increased to 100% B by 10 min. The mobile phase was maintained at 100% B for 2 min, and then returned to initial conditions by 13 min. The column was allowed to equilibrate for 1 min prior to the next injection.

Average concentrations of 6PPD-Q measured for samples taken at test initiation ($T = 0$ h) deviated by 39.3% ± 17.0% (16.0% – 63.0%) from nominal values for both fish species tested. Due to these differences, a holding time degradation study was conducted to assess loss of 6PPD-Q during the sample holding period (up to 14 days) at the Institute of Ocean Sciences (IOS), Fisheries and Oceans Canada (Sidney, BC, Canada). Well water from the toxicity study source was spiked with 6PPD-Q to yield nominal concentrations of 70, 700, 7000, and 70 000 ng/L. Solvent concentration in these samples was 0.01% ethanol (representative of fish exposure samples). Three samples of each concentration were measured at 8, 16, and 24 h and then daily for 14 days ($n = 51$/concentration). Samples were measured using LC–MS/MS) at IOS. Average loss of 6PPD-Q in water samples during the storage time study over

14 days was 40.3% ± 6.5% (Supporting Information, Table S1). No trends between concentration and 6PPD-Q percentage loss over 14 days were observed. These results indicate that significant loss of 6PPD-Q can occur in water samples during storage (at 4 °C in the dark).

To account for losses of 6PPD-Q prior to analysis in all water samples collected, results from the holding time degradation study were used to adjust the 6PPD-Q measured values. Holding time study results were natural log-transformed, and a linear regression was performed to determine the correlation between Ln(concentration) and day (Supporting Information, Figure S1), which is consistent with Hiki et al. (2021), who reported the loss of 6PPD-Q as a first-order reaction. An analysis of variance was conducted to determine whether the 6PPD-Q decrease over time was significant ($p = 0.0016$ for the lowest treatment and $p < 0.001$ for all others). Given the relationship and similar slopes of each regression line (range, −0.0311 to −0.0392), the average slope for all treatments was calculated (−0.0348) and used to estimate the adjusted concentration of 6PPD-Q in water samples at time of sampling (Equation 1). All water concentrations reported hereafter represent holding-time adjusted values.

$$\mathrm{LN(original\ reported\ value)} = -0.0348(\mathrm{day}) + \mathrm{LN(adjusted\ concentration)} \quad (1)$$

where original reported value is the concentration reported by SGS AXYS Analytical Ltd., and day is the number of days the sample was held in storage prior to extraction for analysis.

RESULTS AND DISCUSSION

6PPD-Q concentrations in exposure water

Test concentrations of 6PPD-Q were measured in two replicates/test concentration in both the coho and Chinook exposure experiments at initiation to determine whether nominal values were achieved, and after fish were exposed for 24 h to determine test concentrations at termination. The average concentrations of 6PPD-Q determined at test initiation ($T = 0$ h) deviated by 22.8% ± 16.9% (range: 3.2%–49.3%) from nominal values across both species (Supporting Information, Table S2). The average 6PPD-Q loss after 24 h was 41.0% ± 8.0% (range: 27.0%–46.1%) and 35.2% ± 16.1% (range: 18.4%–53.9%) in the tank water for Chinook and coho, respectively (Supporting Information, Table S3). These losses were slightly higher than what has been previously reported for cold-water fish. Brinkmann et al. (2022) reported an average loss of 14% (1.7% and 32% in high and low treatment groups, respectively) over 24 h in brook trout and rainbow trout exposures. For warm-water fish (exposure temperature 26 °C), Hiki et al. (2021) reported an average loss of 50.4% (range: 40.8%–74.1%) over 48 h for zebrafish and Japanese medaka (*Oryzias latipes*); however, it is unclear how much of this loss occurred during the first 24 h. The experimental parameters including (but not limited to) tank apparatus, fish density, test temperature, aeration (presence/absence), and water type, varied across these studies, which may have contributed to the variable losses reported for 6PPD-Q. As expected, there was greater loss of 6PPD-Q in the fish exposures (average 38.4% ± 11.8% across Chinook and coho) compared with water-only tanks (average 19.1% ± 5.8%). Indeed, in the water-only trials (no fish, $n = 2$/concentration) under a 16:8-h light:dark photoperiod, 6PPD-Q decreased by an average of 13.1% and 19.4% over 24 h at nominal concentrations of 18 518 and 125 ng/L, respectively (Supporting Information, Table S3). Similarly, during our first water-only trial (under a 10:14-h light:dark photoperiod of [$n = 2$]), 6PPD-Q decreased by 24.7% (over 24 h) at a nominal concentration of 100 ng/L. Although the toxicokinetics of 6PPD-Q are relatively unknown, there is evidence of uptake and bioaccumulation in fish, because 6PPD-Q has been detected in the tissue (homogenized whole body) of snakehead, weever, and Spanish mackerel purchased from a fish market (Ji et al., 2022). Thus, we cannot rule out interspecies differences in uptake (or adsorption) contributing to the differences in 6PPD-Q losses reported among studies.

Exposure control performance and water quality

Survival in the laboratory control was 100% for both Chinook and coho. Survival in the solvent control (0.01% ethanol) was 100% in the Chinook exposure ($n = 4$). In the coho exposure, fish in one solvent control replicate exhibited symptoms consistent with 6PPD-Q toxicity, and this resulted in a 24-h survival of 71.4%. A subsample of water was taken from the symptomatic tank and 6PPD-Q contamination (34.4 ng/L) was confirmed. Data from this contaminated replicate were excluded from all analyses. The remaining coho solvent replicates ($n = 3$) exhibited 100% survival.

All experimental water quality parameters measured were within suitable ranges. The average (±standard deviation) temperature during the Chinook and coho exposures were 13.6 ± 0.3 °C and 13.8 ± 0.3 °C, respectively. Dissolved oxygen remained at more than 86% for both exposures, and the pH ranged from 6.7 to 7.0 (Chinook) and 6.8 to 7.3 (coho). The well water used in the exposures had a hardness of 102 and 89.8 mg/L as $CaCO_3$ in the Chinook and coho exposures, respectively.

6PPD-quinone toxicity to newly feeding coho and Chinook

The present study provides further evidence that exposure to 6PPD-Q poses a serious risk to coho salmon. Results indicate that newly feeding (3 weeks post swim-up) juvenile coho are more sensitive to 6PPD-Q than previously reported for 1+-year-old fish and that sensitivity differed considerably between coho and Chinook (Figure 1). Juvenile coho were 3 orders of magnitude more sensitive to 6PPD-Q compared with juvenile Chinook, with an average of 7.1% survival in the highest treatment group (104.7 ng/L), and a 24-h LC50 of 41.0 ng/L (confidence interval [CI] of 33.6–48.5 ng/L; Table 1 and Figure 1A). Conversely, juvenile Chinook had an average survival rate of 61.4% in the highest treatment group (67 307 ng/L) and a 24-h LC50 of more than 67 307 ng/L (CI not calculable; Table 1 and Figure 1B).

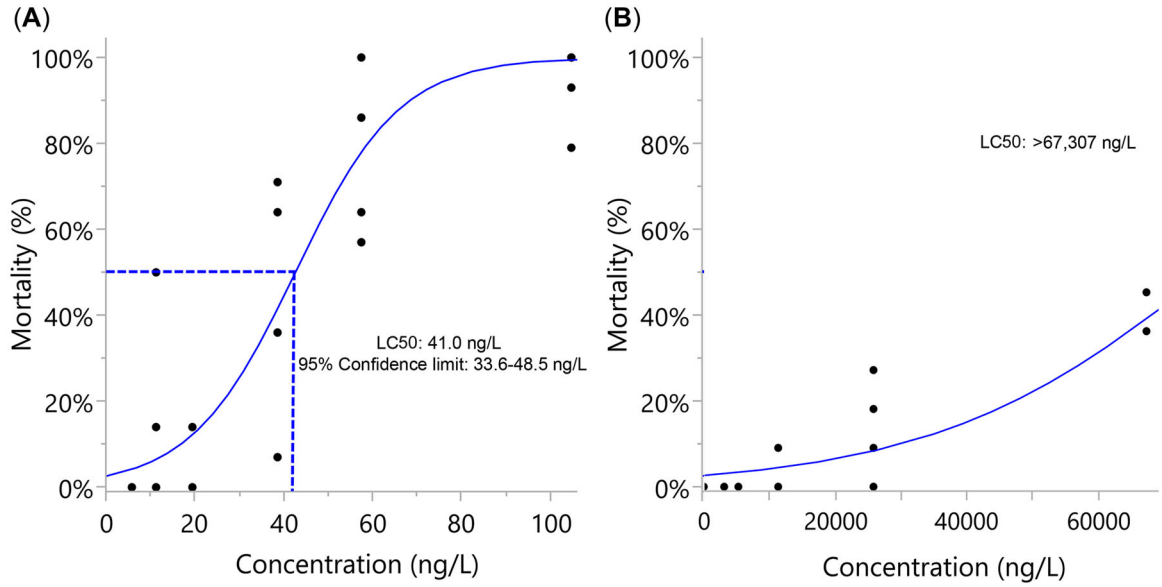

FIGURE 1: Concentration–response curves for 24-h juvenile (**A**) coho salmon and (**B**) Chinook salmon exposures to 6PPD-quinone. Results confirmed that juvenile Chinook are far less sensitive to 6PPD-quinone than coho. Measured concentrations at test initiation were used to calculate the dose–response curve. The median lethal concentration (LC50) was calculated using a log-logistic model.

These results demonstrate notable differences in the sensitivity of these two salmonid species to 6PPD-Q, with this comparative evaluation strengthened by identical experimental conditions and similar age/developmental stage. When the species sensitivity to 6PPD-Q is compared with other cold-water species, our findings support the growing notion that juvenile coho are a highly sensitive species, followed by brook trout, and rainbow trout (Table 1). Our findings from these two salmonids are consistent with those of other laboratory studies that found that urban stormwater runoff resulted in significant coho mortality (more than 92%), only limited Chinook mortality (0%–13%), and no signs of toxicity in chum (*Oncorhynchus keta*) or sockeye salmon (*Oncorhynchus nerka*; French et al., 2022; McIntyre et al., 2018). Juvenile coho in the present study were 2.3-fold more sensitive to 6PPD-Q than previously reported for coho salmon (Tian et al., 2022; Table 1). Different biological (e.g., life stage) and/or experimental conditions (e.g., water type, fish density) could have contributed to the variation in intraspecies sensitivity observed. For example, juvenile coho in the present study were significantly smaller (by 0.433 g), and hence younger, than those exposed in the Tian et al. (2022) study (30–64 g). Fish density (0.337 g/L) in the present study was 7- to 16-fold lower than that in Tian et al. (2022; density range: 2.57–5.48 g/L). The higher density in the Tian et al. study may have influenced coho sensitivity, because previous research investigating pulp and paper effluent toxicity reported lower toxicity (higher survival) with increasing fish density (Davis & Mason, 1973). Further research on the influence of age and different experimental conditions on 6PPD-Q toxicity to coho is needed.

The most common reported toxicity point estimate is the LC50, representing the concentration of a contaminant that is

TABLE 1: Early life stage juvenile coho in the present study were 2.3-fold more sensitive to 6PPD-Q than previously reported for coho salmon.

Species	LC50 (ng/L)	Life stage	Time (h)	Reference
Oncorhynchus kisutch (coho salmon)	41	Juvenile; ~3 weeks	24	Present study
Oncorhynchus tshawytscha (Chinook salmon)	>67 306[a]	Juvenile; ~3 weeks	24	Present study
Oncorhynchus kisutch (coho salmon)	95	Juvenile; 1+ year	24	Tian et al. (2022)
Salvelinus leucomaenis pluvius (white-spotted char)	510	Juvenile; <1 year	24	Hiki and Yamamoto (2022)
Salvelinus fontinalis (brook trout)	590	Juvenile; ~1 year	24	Brinkmann et al. (2022)
Oncorhynchus mykiss (rainbow trout)	1960	Juvenile; ~2 year	24	Brinkmann et al. (2022)
Salvelinus curilus (southern Asian dolly varden)	>10 000[a]	Juvenile; <1 year	24	Hiki and Yamamoto (2022)
Oncorhynchus masou masou (masu salmon)	>10 000[a]	Juvenile; <1 year	24	Hiki and Yamamoto (2022)
Salvelinus alpinus (Arctic char)	>12 700[a]	Juvenile; ~3 year	24	Brinkmann et al. (2022)
Acipenser transmontanus (white sturgeon)	>12 700[a]	Juvenile; ~4.5 year	24	Brinkmann et al. (2022)
Oryzias latipes (Japanese medaka)	>34 000[a]	Juvenile; 41 days	96	Hiki et al. (2021)
Danio rerio (zebrafish)	>54 000[a]	Embryo	96	Hiki et al. (2021)
Danio rerio (zebrafish)	308 670	Embryo	96[b]	Varshney et al. (2022)

[a]Value is greater than the highest concentration tested.
[b]Concentrations were measured for all studies, except for Varshney et al. (2022), which used nominal concentrations.
Several species' median lethal concentration (LC50) estimates are greater than the highest concentration tested, including our Chinook study. For ease of comparison with the present study, 24-h LC50 values were selected when available.

TABLE 2: Percentage of lethal concentration (LC): LC5, LC10, LC25, and LC50 6PPD-quinone mortality point estimates for newly feeding juvenile coho and Chinook salmon to demonstrate species sensitivity.

	Coho	Chinook
LC5 (ng/L)	16.6	12 614
95% Confidence limits	4.8–28.3	7293–17 934
LC10 (ng/L)	20.8	20 859
95% Confidence limits	9.3–32.3	14 750–26 967
LC25 (ng/L)	29.2	43 699
95% Confidence limits	19.5–38.9	37 397–50 000
LC50 (ng/L)	41.0	>67 307 ng/L
95% Confidence limits	33.6–48.5	NA

NA = not available.

predicted to cause 50% mortality (McCarty, 2012; Oris & Bailer, 1997). An LC50 could not be calculated for Chinook because more than 50% mortality was not observed in the highest test concentration. However, calculated lower point estimates (i.e., LC5, LC10, LC25) confirmed that juvenile Chinook are far less sensitive to 6PPD-Q than coho—by a factor of 824–1550 times (Table 2). Five other 6PPD-Q studies have reported species LC50 values that were greater than the highest test concentration in the present study (Table 1); however, all authors noted that at the highest concentration, no mortalities were observed. In our study, the Chinook LC5 is estimated at 12 614 ng/L (CI: 7293–17 934 ng/L) suggesting that Chinook is more sensitive to 6PPD-Q than Arctic char, white sturgeon (both no mortality at 12 700 ng/L; Brinkmann et al., 2022), southern Asian dolly varden, masu salmon (both no mortality at 10 000 ng/L; Hiki & Yamamoto, 2022), Japanese medaka (no mortality at 34 000 ng/L), and zebrafish (no mortality at 54 000 ng/L; Hiki et al., 2021). Varshney et al. (2022) reported a zebrafish 96-h LC50 of 309 000 ng/L; however, measured 6PPD-Q concentrations were not reported. Although additional research regarding 6PPD-Q solubility is needed, Hiki et al. (2021) have suggested that the limit of water solubility is approximately 100 µg/L (100 000 ng/L). Therefore, we adopted a precautionary approach, and have compared our results with those of studies that have reported toxicity values below the 100-µg/L concentration.

Both coho and Chinook in the present study exhibited typical 6PPD-Q symptomology (e.g., gasping, loss of equilibrium, erratic swimming), and fish that were symptomatic generally died from toxic injury. However, one coho proved the exception, showing symptoms in the final 90 min of exposure, and was euthanized at test termination. Variation in individual fish sensitivity to 6PPD-Q was observed for both species. Coho exhibited symptoms, and mortality occurred during the fourth hour of exposure at the highest test concentration (104.7 ng/L) with two of the four (50%) replicates exhibiting 100% mortality. In contrast, the first symptomatic Chinook were observed in the highest test concentration (67 307 ng/L) during the fifth hour of exposure, with no replicates in this concentration exhibiting 100% mortality. Across both species, however, some individual fish in the highest concentrations did not exhibit symptoms at any point during the 24-h exposure.

Environmental relevance

Although only a few studies have measured 6PPD-Q concentrations in creeks, rivers, and urban runoff (stormwater; Challis et al., 2021; Johannessen et al., 2021; Johannessen et al., 2021; Tian et al., 2021), the data reveal that environmental concentrations frequently exceed LC50 toxicity thresholds reported for coho in the present study and in that of Tian et al. (2022). In the Seattle (WA, USA) region, an area that is home to five species of Pacific salmon including Chinook and coho, 6PPD-Q concentrations in roadway runoff ranged from 800 to 19 000 ng/L (Tian et al., 2021), and coho-bearing water bodies ranged from less than 300 to 3200 ng/L (Tian et al., 2021), the highest of which exceeded our LC50 by 78-fold. A study based in Saskatoon (SK, Canada) found 6PPD-Q in 57% of the stormwater samples collected (12/21), and reported a mean concentration of approximately 600 ng/L (Challis et al., 2021). Samples taken during storm events in the fall and winter from the Don River in the Greater Toronto Area (ON, Canada) contained 6PPD-Q concentrations up to 2300 and 2850 ng/L (Johannessen et al., 2021), whereas the mean concentration during a summer rain event was 720 ng/L in grab samples from Highland Creek (Johannessen et al., 2021).

Together, these studies suggest that during storm events, 6PPD-Q concentrations in many urban regions pose a significant threat to coho survival. On the other hand, none of the receiving water concentrations reported thus far exceed our estimated Chinook LC5 of 12 614 ng/L. This finding suggests that environmental concentrations of 6PPD-Q may not present a significant risk to juvenile Chinook in terms of acute mortality during storm events. Further research is needed to determine the nature of the surprising divergence in species sensitivity between coho and Chinook, and to investigate whether as yet undetected sublethal effects of 6PPD-Q may be affecting Chinook salmon.

CONCLUSIONS

Our study reveals that exposure to 6PPD-Q poses a significant risk to juvenile coho salmon, with newly feeding fish exhibiting an LC50 that is 2.3 times lower than previously reported for 1+-year-old coho. Coho remain the most sensitive salmonid observed thus far, followed by brook trout, rainbow trout, and Chinook. Reported environmental concentrations of 6PPD-Q in aquatic environments frequently exceed LC50 estimates for coho, suggesting real-world consequences for this tire-related chemical. Our use of a storage time correction equation strengthened our LC50 value derivation, necessitated by a lack of published guidance regarding sample preservation methods prior to analysis. For many studies (laboratory or field based), the logistics of sampling, shipping time, and laboratory analysis require a minimum of several days. There exists a pressing need to elucidate an effective sample preservation method to avoid 6PPD-Q loss in various water types (e.g., surface water, dechlorinated municipal water). This will ensure accurate and comparable measurements of 6PPD-Q in future studies in the field and in the laboratory. Lastly, research

assessing the sublethal effects of 6PPD-Q will be critical to conducting a comprehensive evaluation of risk to the health of juvenile coho and Chinook in urban waterways.

Supporting Information—The Supporting Information is available on the Wiley Online Library at https:/10.1002/etc.5568.

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Conflict of Interest—The authors declare no conflicts of interest.

Author Contributions Statement—**Bonnie P. Lo**: Conceptualization; Methodology; Investigation; Data curation; Formal analysis; Project administration; Resources; Writing—original draft; Writing—review & editing. **Vicki L. Marlatt**: Conceptualization; Methodology; Investigation; Writing—review & editing; Supervision. **Xiangjun Liao**: Investigation; Data curation. **Sofya Reger**: Investigation. **Carys Gallilee**: Investigation; Data curation. **Andrew R.S. Ross**: Investigation; Data curation. **Tanya M. Brown**: Conceptualization; Funding acquisition; Methodology; Writing—review & editing; Supervision.

Data Availability Statement—All our data are presented in the article and Supporting Information. Raw data are available on request to the corresponding author (Tanya.Brown@dfo-mpo.gc.ca).

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Supporting Information

Figure S1. Holding time study revealed significant loss of 6PPD-Quinone during 14 days of sample storage (in the dark at 4°C). A linear regression was performed to determine the correlation between Ln(concentration) and day (R^2 provided below). An analysis of variance was conducted to determine if the 6PPD-Quinone decrease over time was significant (p-value = 0.0016 for the lowest treatment and <0.001 for all others).

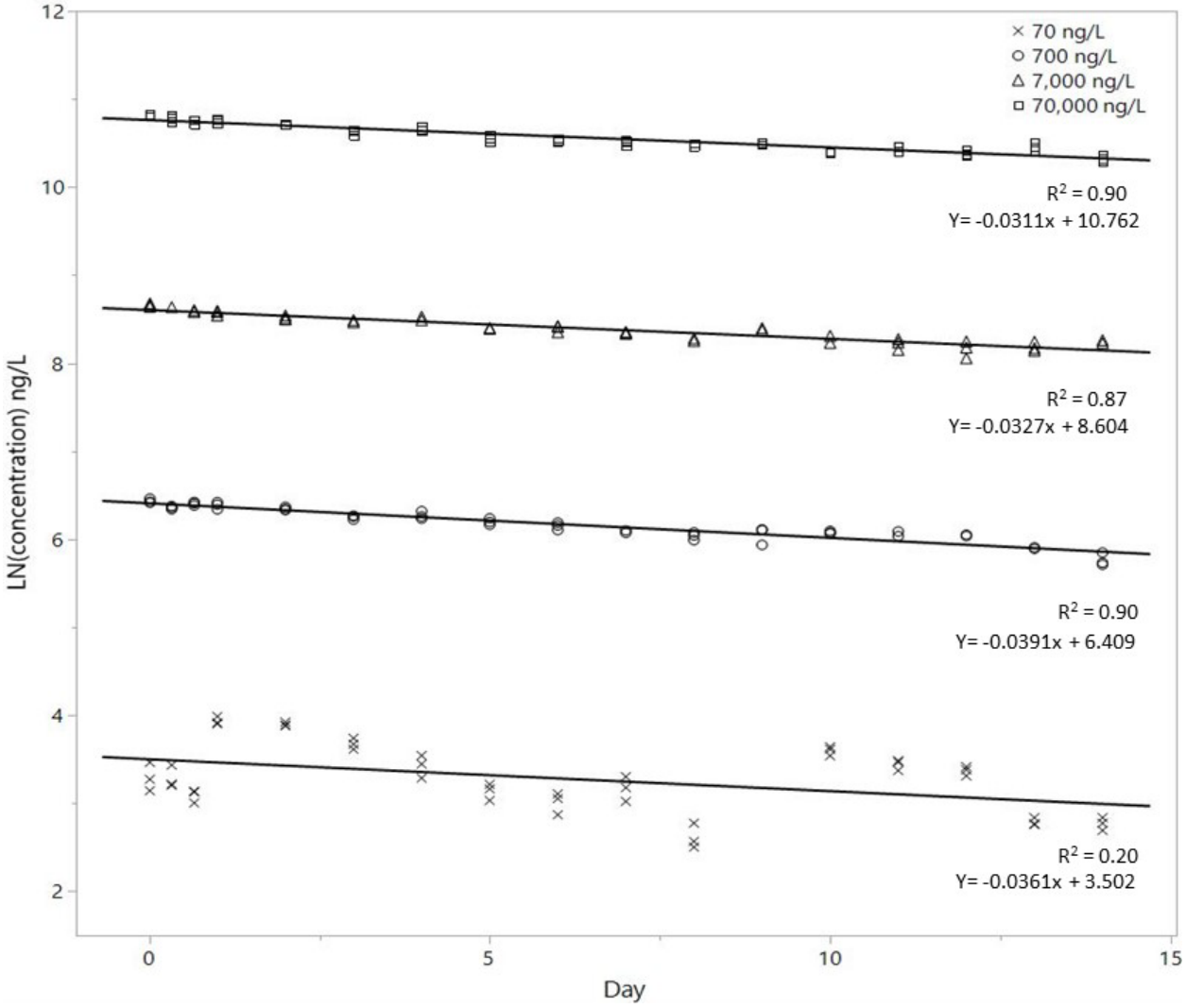

Table S1. Average ± SD loss of 6PPD-Quinone concentrations (ng/L) during the 14-day sampling holding period study. Samples were stored at 4°C in the dark; n=3.

	Measured concentration (ng/L)			
Time (day)	**Nominal 70 ng/L**	**Nominal 700 ng/L**	**Nominal 7,000 ng/L**	**Nominal 70,000 ng/L**
0	27.2 ± 4.4	624.1 ± 15.1	5,755.8 ± 111.0	49,549.5 ± 503.5
0.33	27.0 ± 3.6	578.7 ± 10.4	5,632.6 ± 17.6	48,048.0 ± 1,793.0
0.66	22.1 ± 1.6	605.6 ± 12.2	5,359.2 ± 61.1	46,623.5 ± 1,368.2
1	51.3 ± 2.3	593.7 ± 24.6	5,263.0 ± 142.6	46,893.0 ± 1,137.5
2	49.4 ± 1.1	571.7 ± 10.6	4,981.9 ± 112.2	44,775.5 ± 240.4
3	39.6 ± 2.5	518.2 ± 12.0	4,762.5 ± 66.7	41,156.5 ± 1,267.0
4	31.0 ± 3.9	529.8 ± 23.2	4,970.4 ± 113.4	42,619.5 ± 1,026.6
5	23.1 ± 2.1	492.8 ± 18.0	4,423.7 ± 30.6	38,346.0 ± 1,617.0
6	20.4 ± 2.5	469.7 ± 20.3	4,416.0 ± 164.3	37,614.5 ± 581.3
7	23.9 ± 3.4	442.4 ± 7.0	4,196.5 ± 46.7	36,459.5 ± 1,202.2
8	13.8 ± 2.0	419.3 ± 18.6	3,900.1 ± 66.7	35,381.5 ± 676.8
9	NA ± NA	426.2 ± 41.0	4,396.7 ± 67.7	36,113.0 ± 371.3
10	36.7 ± 1.9	436.6 ± 6.4	3,898.1 ± 220.5	32,763.5 ± 290.7
11	31.3 ± 1.9	425.8 ± 13.3	3,703.7 ± 229.4	34,265.0 ± 1,066.9
12	29.1 ± 1.5	421.9 ± 4.9	3,495.8 ± 332.0	32,301.5 ± 961.7
13	16.3 ± 0.7	365.6 ± 4.1	3,576.7 ± 190.6	34,958.0 ± 1,525.0
14	16.0 ± 1.2	319.9 ± 24.3	3,823.1 ± 80.8	30,569.0 ± 1,185.4

Due to equipment error, Day 9 measurements for the nominal 70 ng/L treatment were unavailable.
Note: Ascorbic acid was added to each water sample upon collection.

Table S2. 6PPD-Quinone nominal and measured exposure concentrations (ng/L) at test initiation (t=0 h) for coho and Chinook waterborne toxicity tests.

		6PPD-Quinone concentration (ng/L)		
Species	**Replicate**	**Nominal**	**Measured**	**% error**
coho	A	11.9	N/A	N/A
	B	11.9	11.3	5.3%
	A	21.4	19.6	8.6%
	B	21.4	19.3	9.7%
	A	38.6	37.3	3.2%
	B	38.6	39.9	-3.5%
	A	69.4	49.8	28.3%
	B	69.4	65.2	6.1%
	A	125.0	112.1	10.3%
	B	125.0	97.3	22.1%
Chinook	A	5,716	3,200	44.0%
	B	5,716	3,023	47.1%
	A	10,288	5,321	48.3%
	B	10,288	5,211	49.3%
	A	18,519	11,745	36.6%
	B	18,519	10,895	41.2%
	A	33,333	25,808	22.6%
	B	33,333	25,808	22.6%
	A	60,000	70,679	-17.8%
	B	60,000	63,934	-6.6%

N/A: Due to sampling error, only 1 replicate tank was measured.
Note: all measured concentrations are corrected for holding time loss.
% error = difference between measured and nominal concentration divided by nominal concentration x 100%

Table S3. Average ± SD 6PPD-Quinone nominal and measured exposure concentrations (ng/L) for the 24 h coho and Chinook waterborne toxicity tests. 6PPD-Quinone concentrations decreased over the 24 hour exposure period. 6PPD-Quinone concentrations were measured from two replicate tanks for each treatment.

	Nominal concentration (ng/L)	**Average concentration at test initiation (T= 0 h; ng/L)**	**Average concentration at test termination (T = 24 h; ng/L))**	**% decrease over 24 hours***
coho	12	11.3**	11.3**	0.0%
	21	19.5 ± 0.2	11.2 ± 2.6	42.7%
	38	38.6 ± 1.8	31.5 ± 0.8	18.4%
	69	57.5 ± 10.9	43.7 ± 17.7	25.6%
	125	104.7 ± 10.5	48.1 ± 2.1	53.9%
Chinook	5,716	3,112 ± 126	1,753 ± 545	44.0%
	10,288	5,266 ± 77	2,865 ± 115	45.6%
	18,519	11,320 ± 601	6,495 ± 1,059	42.3%
	33,333	25,808 ± 0	13,917 ± 2,716	46.1%
	60,000	67,307 ± 4,770	48,995 ± 691	27.0%
No fish	18,519	12,638 ± 19	10,983 ± 991	13.1%
(water only)	125	104.4 ± 1.1	84.1 ± 3.8	19.4%
	100	86.0 ± 20.5	64.8 ± 15.8	24.7%

*Percent decrease = average of decrease calculated for each replicate tank

**Due to subsampling error only one replicate tank was measured.