Bioavailability of 6PPD-quinone and testing alternative antiozonants

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Bioavailability of 6PPD-quinone

Background

The novel tire-derived chemical 6PPD-quinone is acutely toxic to juvenile coho salmon and has been implicated in annual mortality events of adult coho salmon returning to spawn in streams of the Pacific Northwest impacted by roadway runoff (Tian et al. 2020). Washington State has released a draft water quality standard for 6PPD-quinone that is based on laboratory-generated acute toxicity tests with juvenile coho. If differences in environmental variables and life stage modify toxicity for coho in the wild, proposed regulations may not be protective. We tested acute toxicity across a range of variables that can alter bioavailability of contaminants to fish: temperature, conductivity, pH, organic matter, water velocity, and life stage.

Methods

Chemicals and chemical analysis

Analytical grade 6PPD-quinone (97.3% purity) and penta-deuterated 6PPD-quinone (D5-6PPD-quinone) were purchased from HPC Standards, Inc. Molecular grade absolutely ethanol was used to make stock solutions of 6PPD-quinone (100 mg/L) and stock of D5-6PPD-quinone were made with methanol graded for liquid chromatography mass spectrometry. Exposure waters for analytical chemistry (200 mL) were spiked with 50 μL of D5-6PPD-quinone stock as an internal standard to account for potential losses of 6PPD-quinone between sampling and analysis. Quantification of 6PPD-quinone was conducted at the Kolodziej laboratory at the Center for Urban Waters (Tacoma, WA). Samples were extracted by solid phase extraction within 24 h of collection. Measurement used ultrahigh performance liquid chromatography (Agilent 1290 Infinity) coupled to a triple-quadrupole mass spectrometer (Agilent G6460A) with positive electrospray ionization as in previous studies (Tian et al. 2022). Agilent software was used to quantitate 6PPD-quinone concentrations by referencing mass spectroscopy signal intensity to internal standard (D5-6PPD-quinone) and a standard curve.

Fish source and care

Studies on habitat characteristics (water quality and velocity) were conducted on juvenile coho salmon from Voights Creek Hatchery, operated by the Washington State Department of Fish and Wildlife. Fish were reared at the Washington State University Puyallup Research and Extension Center in a recirculating aquaculture system under 12:12 light:dark regime. System

water was dechlorinated municipal water, subject to reverse osmosis and reconstituted with a commercial salt solution (Instant Ocean) adjusted to a neutral pH with sodium bicarbonate. Fish were fed commercial pellets (BioOregon *Biovita*) three times per week at 10% body weight per day.

For life stage studies, embryos were generated from gametes of spawning stage adult coho salmon that had returned to Voights Creek from the ocean. Embryos, and subsequent alevin and fry, were reared in aquaria or in incubation trays with system water until used in experiments. Fry were fed commercial pellets like the juveniles described above. Adult coho salmon were donated by the Puyallup Tribe of Indians from their Clark's Creek hatchery and were transported (<30 minutes) to WSU-Puyallup in a large tote (1000-L) with supplemental aeration.

Water quality experiments

A dose-response curve was generated for juveniles under 'standard laboratory conditions' (Table 1) for comparison with results generated under altered water quality conditions. To determine environmentally relevant ranges of each water quality parameter, we surveyed water quality data from 2021-2022 collected from freshwaters in three U.S states containing significant portions of coho salmon native range (Alaska, Oregon, and Washington), accessed through the National Water Quality Monitoring Council (NWQMC). Using averages from each sampling site to reduce bias from frequently sampled locations, we chose a range of each parameter across which to test toxicity of 6PPD-quinone (Table 1). To reduce the number of fish used in these experiments, many parameters were first tested at a low concentration (e.g., LC10) or a high concentration (e.g., LC90) depending on whether we expected that parameter to increase or decrease toxicity. Full dose-response curves were only generated when it appeared that the point estimate represented a departure from the standard conditions curve.

Table 1. Water quality parameters modified for testing 6PPD-quinone toxicity with juvenile coho salmon.

Parameter	**Field data median (interquartile range)**	**Values and 6PPD-Q concentrations tested**	**% of field range covered**
Temperature (°C)	12 (9-14)	5^{f}, 10^{f*}, 15^{LC10}, 20^{f}	94%
Conductivity (µS/cm)	103 (64-222)	$110^{\wedge\ LC10}$, 1100^{f*}	51%^
pH	7.7 (7.3-8.0)	6.0^{LC10}, 7.7^{f*}, 8.4^{LC90}	91%
Dissolved organic carbon (mg/L)	1.9 (1.2-3.0)	0.36^{f*}, 4.5^{p}	82%

^ In process of testing the 10th percentile, so will ultimately have covered 90% of range

* Standard laboratory condition

f Full dose-response curve generated

p Partial dose-response curve generated

LCx Parameter tested only at a concentration expected to produce this percent mortality

Life stage experiments

Early life stages

To determine sensitivity of embryos, we conducted experiments in two consecutive years. In Year 1, we reared embryos from fertilization through hatch exposing them to periodic pulses (approximately twice per week) of 500 ng/L 6PPD-quinone for 24-h. In all, there were 13 pulses across 56 days of development. Approximately 100 fertilized embryos were placed in each 9.5-L glass aquarium containing 7 L of system water that flowed at 59 mL/min into the bottom of each aquarium and out a mesh-covered drain hole near the top. During the 24-h pulses, influent water was switched from system water to system water spiked with 6PPD-quinone or with ethanol at the same concentration as used to carry the 6PPD-quinone (<0.1%). Survival was monitored across the 56 days, and embryos were sampled for morphometric at 34- and 41-days post fertilization (dpf).

In Year 2, to determine if sensitivity observed during Year 1 was dependent on the presence of the chorion and on the stage of early development, fish were subject to a single 24-h pulse exposure at one of three stages: early embryo (27 dpf), late embryo (37 dpf), and alevin (44 dpf). For each stage, there was a negative control (system water), a solvent control (system water plus ethanol), and 6PPD-quinone at 500 ng/L. For the two embryo stages, there were additionally replicates of embryos that had been manually dechorionated. After the 24-h exposure, fish were transferred to clean water and survival was monitored for up to 2 weeks. Exposures took place in 1-L glass beakers that were renewed daily with fresh control water after the first 24-h pulse. Additionally in Year 2, we generated concentration-survival curves for alevin (hatched) and fry (free-swimming) from 24-h exposures to concentrations of 100, 200, 300, 600, 1000 ng/L, with survival monitored for 120 hours (5 days).

We also generated concentration-survival curves for alevin and fry to compare with older juveniles. Ten fish were exposed in each of 4 glass aquaria (<1 g/L loading rate) at one of 5 concentrations or a clean water control for 24 h. Survival was enumerated at the end of the 24 h exposure.

Adults

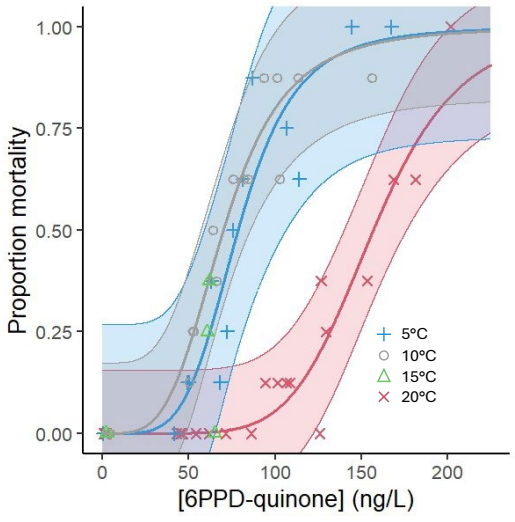
Adult coho salmon (fork length $\pm$ 95%CI: 49.0 $\pm$ 0.9 cm, weight: 1300 $\pm$ 80 g) were inserted into large PVC tubes (6-8” diameter) with aeration holes and removable gates on either end as in prior research on stormwater chemicals (Spromberg et al. 2016; McIntyre et al. 2018, 2023). Four tubes were placed in each 600-L HDPE exposure tank. Well aerated water was supplied to the head region of the fish through a tube connected to a recirculating pump inside each tank. Each exposure tank contained one concentration of 6Q or municipal water (conductivity: 228$\pm$16 μS/cm, pH 7.92$\pm$0.15) de-chlorinated with an in-line filter containing granulated activated carbon (CAMCO). Water was aerated by recirculation from 25 cm above the water level and by 15-cm air stone attached to an air compressor. Adults were monitored hourly for

the first 4 hours, at 8 h, 16 h, and 24 h. Fish found on their side inside a tube were transferred to an observation tank containing clean water. Individuals confirmed to show a complete loss of equilibrium were euthanized in MS-222 (400 mg/L). Juvenile coho salmon (weight: 8.8 [0.15] g, fork length: 8.8 [0.52] cm) were co-exposed in each adult exposure tank by enclosing 10 juveniles in a polyethylene mesh cage tied near the surface of each tank. Mortalities were enumerated at the end of the 24 h co-exposure.

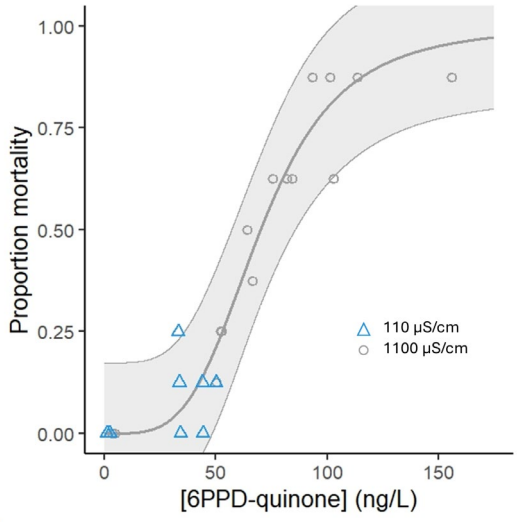
Results

Water quality experiments

We expected that toxicity might be increased at warmer temperatures; however, toxicity was only affected at the highest temperature tested. Estimated LC50s were 78.5±7.3 ng/L at 5°C, 70.1±4.3 ng/L at 10°C, and 157.0±7.3 ng/L at 20°C (Figure 1A). Testing at 15°C at the LC10 produced mortality rates similar to those at 5°C and 10°C, such that the reduced toxicity associated with elevated temperature is effectively limited to the highest temperatures coho would commonly encounter in the wild. Conductivity did not appear to alter toxicity; mortality at a low conductivity (110 µS/cm) tested at the LC10 was consistent with the mortality rate at the high conductivity (1100 µS/cm) of the standard conditions (Figure 1B). Mortality rates at lower and higher pH were also consistent with those at standard conditions (Figure 1C), as were mortality rates at a high concentration of DOC (Figure 1D).



A)



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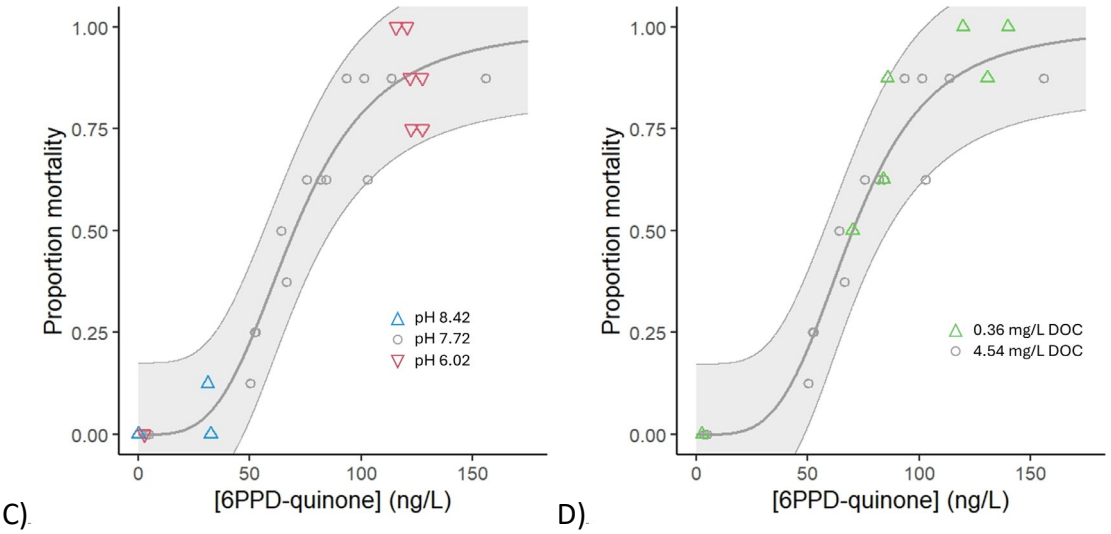

Figure 1. Concentration-survival data for juvenile coho exposed to various water quality conditions. A) temperature, B) conductivity, C) pH, D) dissolved organic matter. Error bands are for the prediction interval.

Life stage experiments

Embryos. Prior to hatching, embryos exposed to 6PPD-Q had smaller eyes at 34 dpf and 41 dpf, and were shorter at 41 dpf, but did not appear to have utilized less yolk (Figure 2). Survival over the 56-day experiment in Year 1 was impaired by pulses of 6PPD-quinone, but only after hatch (~43 dpf). Survival to 56 dpf was 67.2% for the 6PPD-quinone treatment and was 95.5% for controls (Figure 3). Manual dechorionation of embryos at 27 dpf increased sensitivity to a pulse of 6PPD-quinone, resulting in 33% survival 2 weeks after the exposure compared with 93% survival in the intact and dechorionated controls (Figure 4). The elevated mortality in dechorionated controls was coincident with the timing of natural hatch, which started at 38 dpf. Embryos with intact chorions exposed to the 6PPD-quinone pulse had an average survival of 83%, which we could not conclude was different from controls (n=3 replicates; Tukey post-hoc p=0.075).

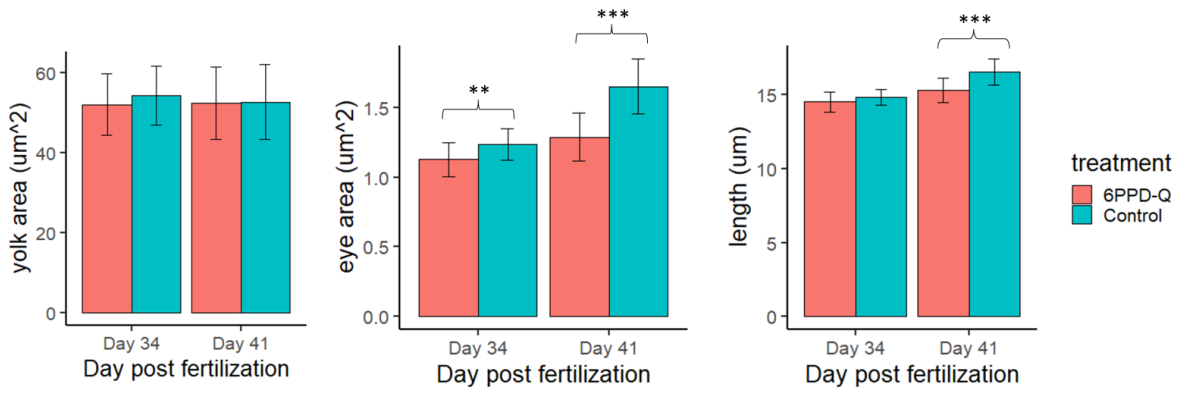

Figure 2. Morphometrics of coho embryos exposed approximately twice weekly to 24-h pulses of 6PPD-Q or control water.

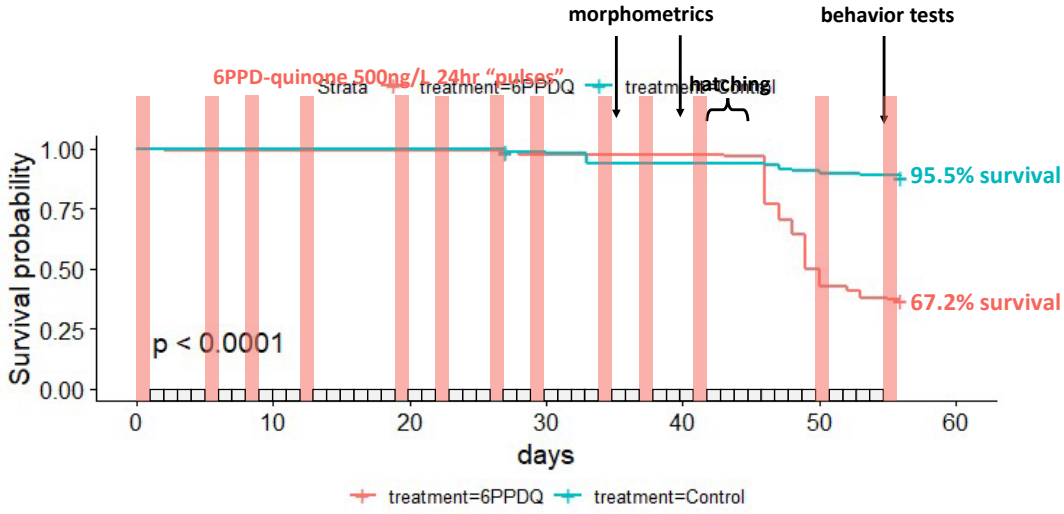

Figure 3. Kaplan-Meier survival curves for early life stage coho salmon exposed to 24-h pulses of 6PPD-quinone or clean water containing ethanol (solvent used for 6PPD-quinone).

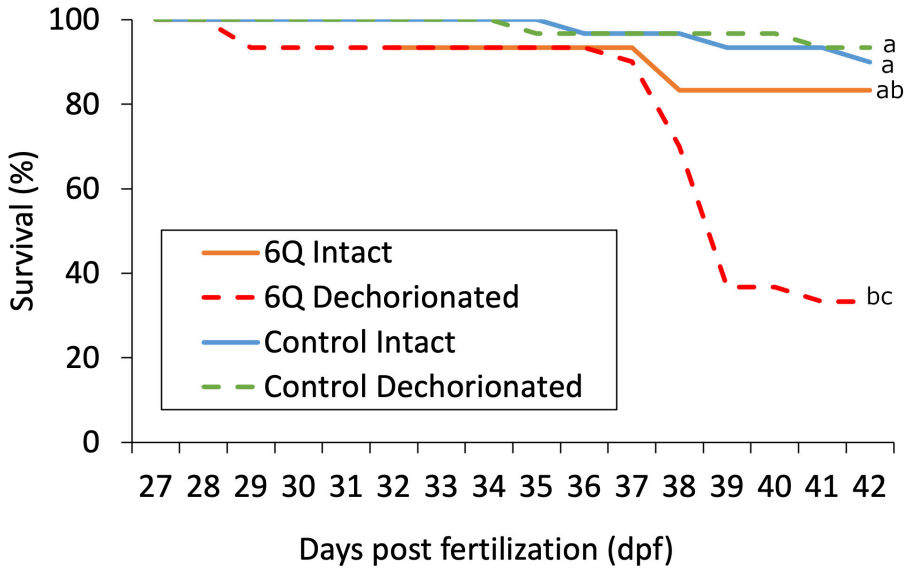

Figure 4. Average survival following exposure to a single 24-h pulse of 6PPD-quinone (500 ng/L) at 27 dph for embryos that were either intact or dechorionated. Three replicates of 10 embryos per treatment. Different letters indicate statistical divergence of survival (α=0.05). Natural hatch began at 38 dpf, with most hatched by 40 dpf.

Alevin and fry. Whereas pulses of 500 ng/L were not acutely lethal to coho embryos, alevin and fry were affected at that concentration. The estimated nominal LC50 for alevin was 566 ±127 ng/L (95% confidence interval) and was 192 ± 25 ng/L for fry. Alevin and fry life stages were less sensitive than older juveniles (Figure 5).

Adults. The sensitivity of fresh-water phase adult coho salmon was similar to that of co-exposed freshwater phase juveniles (Figure 6). Measured concentrations are not yet available but we expect them to be lower than in exposures focused on juvenile life stages; whereas juvenile life stage exposures were conducted in glass vessels at a loading rate <1 g/L, exposures with adults were conducted in HDPE and at a loading rate of 9 g/L, which was difficult to avoid for this round of testing. We expect that these differences in methodology will result in less bioavailable 6PPD-quinone during the exposure which may explain the much higher LC50s than in previous tests.

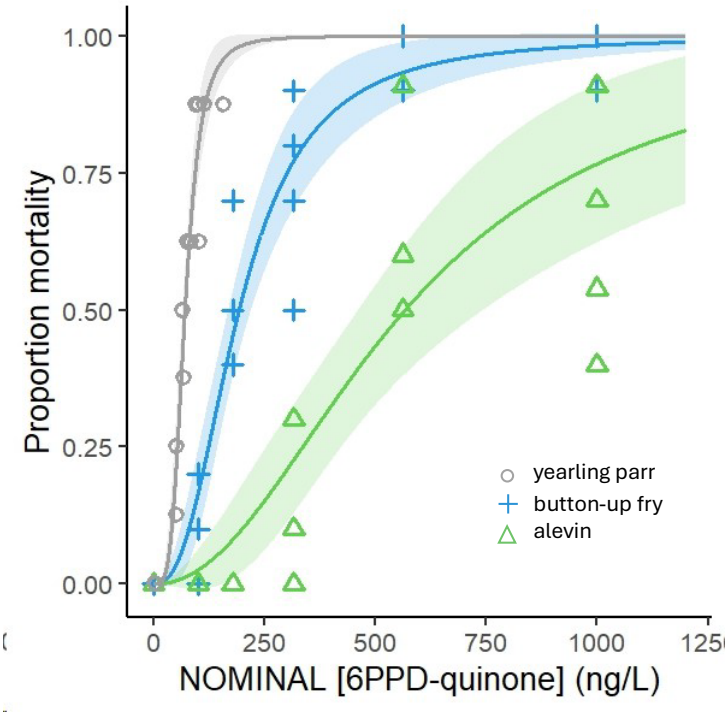

Figure 5. Mortality of three life stages of coho with log-logistic regression line and 95% confidence bands. Depicted 6PPD-quinone concentrations are nominal for alevin and button up fry, but quantitated by mass spectroscopy for yearling parr (103% of nominal).

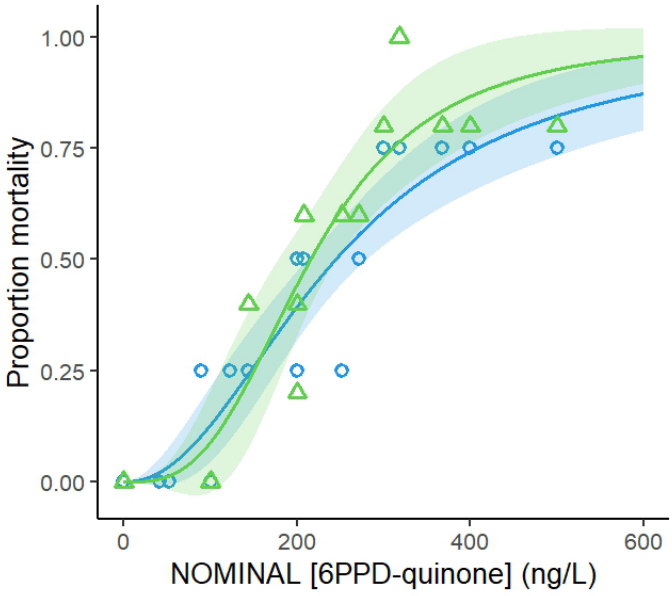

Figure 6. Mortality of juveniles (green, triangles) and adults (blue, circles), with log-logistic regression line and 95% confidence bands.

Conclusions

Temperature was the only tested water quality parameter that modified the toxicity of 6PPD-quinone for juvenile coho salmon. Toxicity was reduced at the maximum temperature tested (20°C), which was near the thermal maximum for coho (23-24°C; Richter & Kolmes 2005). A lack of significant effects of most temperatures, and of relevant ranges of pH, conductivity, and organic matter negates the need to consider site-specific criteria when setting regulations for 6PPD-quinone in aquatic environments. Coho salmon appear to have reduced sensitivity to 6PPD-quinone inside the chorion and in early life stages when yolk is present (dechorionated embryos, alevin, fry). While this effect is protective against acute mortality, sublethal impairments were still noted.

Acute toxicity of PD parent chemicals

Background

The antiozonant 6PPD is the parent compound of 6PPD-quinone. It is produced when ozone reacts with 6PPD – a sacrificial molecule added to tires to protect rubber polymers from attack by ozone. Other members of phenylene diamine (PD) family also have antiozonant properties and might serve as safer substitutes if less toxic than 6PPD and if they produce less toxic transformation products. We are using juvenile coho salmon to test the toxicity of potential alternatives and their ozone transformation products. At this time, we have completed testing of three parent compounds. Additional pilot tests are not included. We are collaborating with chemists at University of Washington who will be generating the ozonated mixtures of each parent compound.

Methods

Fish husbandry
Juvenile coho salmon were obtained as young-of-the-year from the Voigts Creek Hatchery (Orting, WA) and reared in a recirculating system at Washington State University Puyallup Research and Extension Center. Fish rearing water is reverse osmosis water reconstituted using an automatic dosing system. The rearing water is maintained at a pH of 7.4-7.8, a conductivity of 1250–1350 µS/cm, and a temperature of 10-12°C. The fish are reared in a 12 hour light : 12 hour dark cycle and are fed three times a week at 2.7% of body weight with commercial fish food (BioVita, Bio-Oregon).

Fish exposures
Exposures to determine relative toxicity of antiozonants to juvenile coho were acute (24-h) static tests performed following EPA guidelines (OCSPP 850.1075, USEPA, 2016). The test chambers contained 30L or 60L of water in temperature-controlled water baths.

Pilot tests were performed to determine appropriate concentration ranges for definitive testing. The pilot tests consisted of 3 concentrations and one control with 5 fish per tank. Definitive tests consisted of 5 concentrations and one control with 8 fish per tank and were repeated as needed to achieve sufficient replication across the relevant concentration range.

Exposure tanks were spiked with chemical stock and mixed with a glass stir rod and a clean beaker. Aeration was added and the tanks were allowed to equilibrate for 30 minutes. Water samples for chemistry analysis were collected 30 minutes after dosing. To allow sufficient mixing. Fish were added to the exposure tanks directly after chemistry sampling. Fish behavior was monitored hourly during daylight hours, and organisms were removed when moribund, defined as immobile on the bottom of the tank. The lengths and weights of individual fish were recorded.

At the end of 24 hours, survival was recorded for each tank and a second set of water samples were collected for chemical analysis.

Water chemistry

Analysis of water samples was performed to verify chemical concentrations during fish exposures. Water samples were collected from each tank at the beginning of exposures to measure initial chemical concentration in the exposure tanks. Samples were collected at the end of exposures to calculate average loss of test chemical over the 24-hour exposure.

Chemical concentrations for the parent compounds were analyzed using liquid chromatography with tandem mass spectrometry (LC-MS/MS). Each water sample was spiked with the internal standard (D5-6PPDQ). Within one hour after sampling, solid phase extraction was performed following the methods in Tian et al. (2022) and the eluent was evaporated with nitrogen from 10ml to 1ml to concentrate the sample for analysis. Each batch of samples was analyzed with a method blank and lab control standard for quality control. Analyses were performed at the Center for Urban Waters in Tacoma, WA.

Statistical analysis

Results from coho exposures were analyzed using the 'drc' package in RStudio to generate dose response curves and calculate median lethal concentrations (LC50s) for each compound (Ritz et al. 2015).

Results

From the time series experiment, 6PPD degraded at an exponential rate in water, which did not appear affected by the presence of aeration (Figure 7). At the end of 24 hours, approximately 30% of the initial mass of 6PPD remained. We plan to identify the major transformation products through non-target analysis which is not yet completed. Pilot tests with 6PPD acquired from different sources highlighted that impurities were an important source of toxicity, as the Flexsys 6PPD (95.9% purity) was considerably more toxic than the Cayman 6PPD (99.1% purity) (Figure 8).

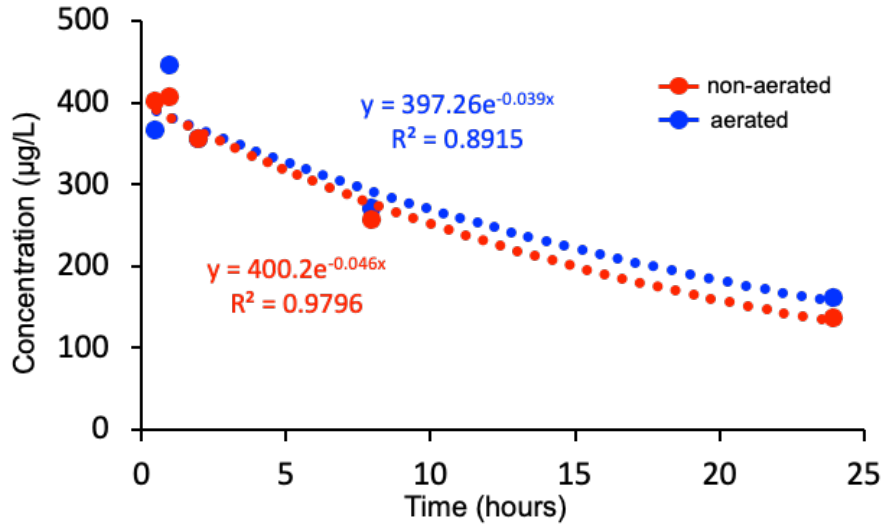

Figure 7. 6PPD time series test with repeated sampling over a 24-hour period. Tanks were dosed with a nominal concentration of 300 µ/L with one aerated and one non-aerated tank and no fish present. Both aerated and non-aerated tanks show significant loss of chemical over a 24-hour period. The combined average chemical loss between both data sets was 61.5%. Concentrations measured with LC-MS/MS.

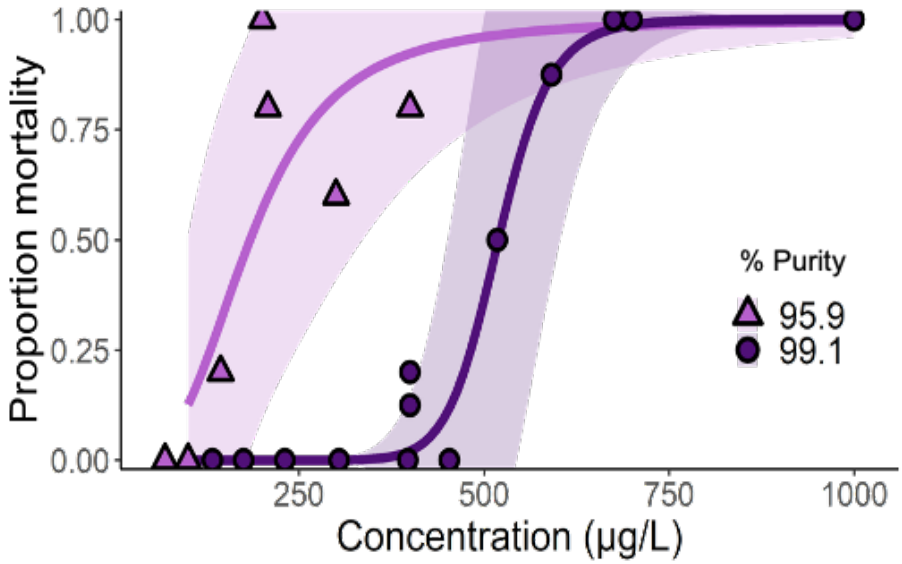

Figure 8. Concentration-response curves from 24-hour static exposures with juvenile coho salmon exposed to two purities of 6PPD. The high purity 6PPD (Cayman, definitive test) was less toxic than a lower purity 6PPD (Flexsys, pilot test), showing that impurities can be an important source of toxicity. All concentrations are nominal. Log-logistic regression lines and 95% confidence intervals are shown around each replicate of 8 fish (Cayman) or 5 fish (Flexsys).

Definitive tests with the first three antiozonant parent compounds (6PPD, 7PPD, and 77PD) showed significant differences in toxicity, with 77PD being much more toxic than 6PPD and 7PPD (Figure 9). The estimated LC50 was 159.6 ± 27.7 µg/L for 77PD, 509.5 ± 98.3 µg/L for 6PPD, and 706.5 ± 123 µg/L for 7PPD.

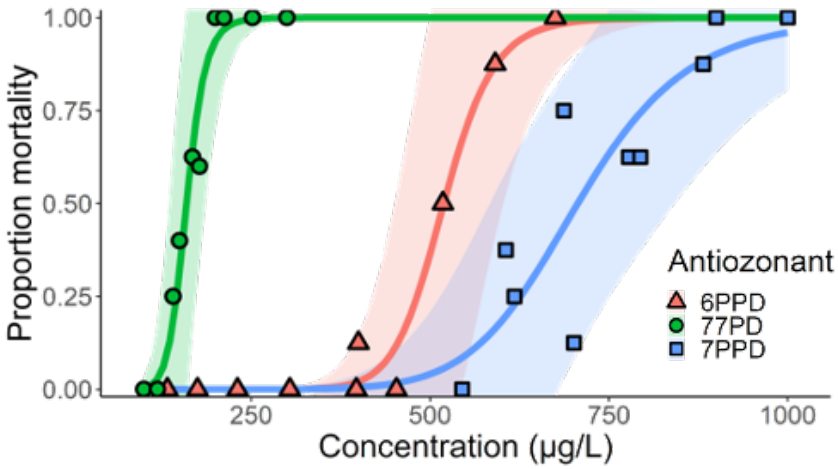

Figure 9. Concentration-response curves for 6PPD, 7PPD, and 77PD from 24-hour static exposures with juvenile coho salmon. All concentration values are nominal. Log-logistic regression lines and 95% confidence intervals are shown around each replicate of 8 fish.

Ongoing work

Additional antiozonants

Pilot tests have been conducted or are planned for: IPPD, 44PD, and CCPD. Additional antiozonants have been identified that will be tested subsequently.

Ozonation of parent compounds

6PPD and the potential alternative parent compounds will be ozonated to induce the formation of transformation products following the methods of Zhao et al. (2023). The ozonated compounds will then be used in coho fish exposures as previously described to determine if the ozonated chemical mixtures are more toxic than the parent compounds.

Conclusions

Our work so far has shown important differences in the toxicity of parent PD compounds. It has also highlighted challenges in measuring parent antiozonants.

Cited References

McIntyre, J. K., J. I. Lundin, J. R. Cameron, M. I. Chow, J. W. Davis, J. P. Incardona & N. L. Scholz (2018). Interspecies variation in the susceptibility of adult Pacific salmon to toxic urban stormwater runoff. *Environmental Pollution*, 238: 196.

McIntyre, J. K., J. Spromberg, J. Cameron, J. P. Incardona, J. W. Davis & N. L. Scholz (2023). Bioretention filtration prevents acute mortality and reduces chronic toxicity for early life stage coho salmon (*Oncorhynchus kisutch*) episodically exposed to urban stormwater runoff. *Science* of the Total Environment, 902.

Spromberg, J. A., D. H. Baldwin, S. Damm, J. K. McIntyre, M. Huff, C. A. Sloan, B. F. Anulacion, J. W. Davis & N. L. Scholz (2016). Widespread coho salmon spawner mortality in western U.S. urban watersheds: Lethal stormwater impacts are reversed by soil bioinfiltration. *Journal of Applied Ecology*, 53: 498.

Tian, Z. Y., H. Zhao, K. T. Peter, M. Gonzalez, J. Wetzel, C. Wu, X. Hu, J. Prat, E. Mudrock, R. Hettinger, A. E. Cortina, R. G. Biswas, F. V. C. Kock, R. Soong, A. Jenee, B. Du, F. Hou, H. He, R. Lundeen, A. Gilbreath, R. Sutton, N. L. Scholz, J. W. David, M. C. Dodd, A. Simpson, J. K. McIntyre & E. P. Kolodziej (2021). Ubiquitous tire rubber-derived chemical induces acute mortality in coho salmon. *Science*, 371(6525): 185.

Zhao, H. N., X. M. Hu, M. Gonzalez, C. A. Rideout, G. C. Hobby, M. F. Fisher, C. J. McCormick, M. C. Dodd, K. E. Kim, Z. Y. Tian & E. P. Kolodziej (2023). Screening p-Phenylenediamine antioxidants, their transformation products, and industrial chemical additives in crumb rubber and elastomeric consumer products. *Environmental Science & Technology*.