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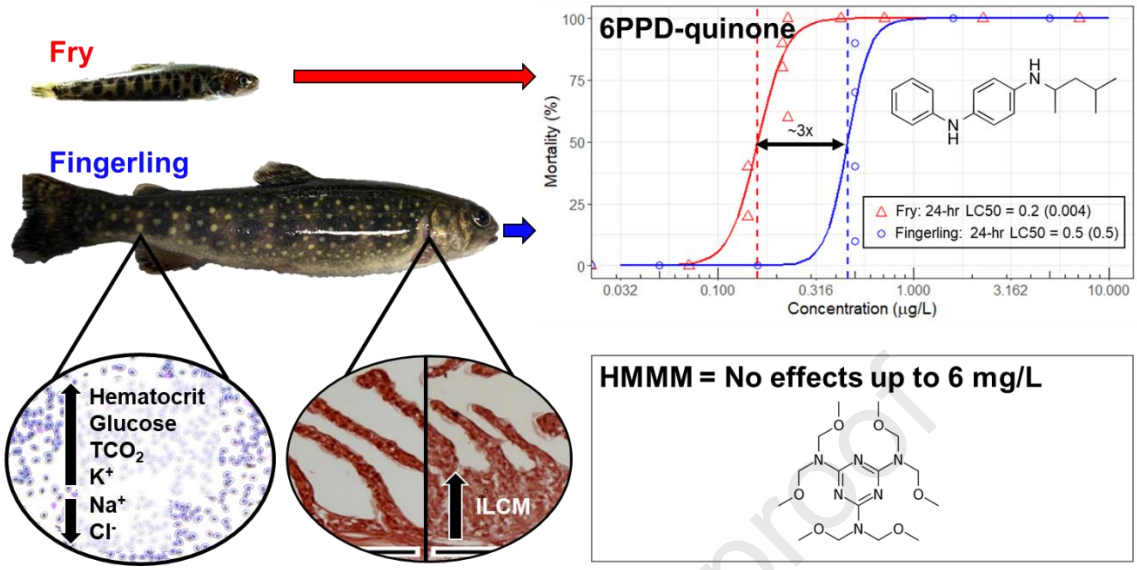
Key Words: 6PPD-quinone, tire leachate, Brook trout, HMMM, gill morphology

Synopsis: Environmentally relevant 6PPD-quinone exposures cause higher mortality rates in Brook trout fry than fingerlings, altered blood analytes and gill morphology are the likely mechanism of action.



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1. Abstract

Recent toxicity studies of stormwater runoff implicated N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine-quinone (6PPD-quinone) as the contaminant responsible for the mass mortality of coho salmon (*Oncorhynchus kisutch*). In the wake of this discovery, 6PPD-quinone has been measured in waterways around urban centers, along with other tire wear leachates like hexamethoxymethylmelamine (HMMM). The limited data available for 6PPD-quinone have shown toxicity can vary depending on the species. In this study we compared the acute toxicity of 6PPD-quinone and HMMM to Brook trout (*Salvelinus fontinalis*) fry and fingerlings. Our results show that fry are ~3 times more sensitive to 6PPD-quinone than fingerlings. Exposure to HMMM ≤ 6.6 mg/L had no impact on fry survival. These results highlight the importance of conducting toxicity tests on multiple life stages of fish species, and that relying on fingerling life stages for species-based risk assessment may underestimate the impacts of exposure. 6PPD-quinone also had many sublethal effects on Brook trout fingerlings, such as increased interlamellar cell mass (ILCM) size, hematocrit, blood glucose, total CO_2, and decreased blood sodium and chloride concentrations. Linear relationships between ILCM size and select blood parameters support the conclusion that 6PPD-quinone toxicity is an outcome of osmorespiratory challenges imposed by gill impairment.

2. Introduction

A recent study implicated N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine-quinone (6PPD-quinone) as the cause of mass mortality of coho salmon (*Oncorhynchus kisutch*) after stormwater runoff events (Tian et al., 2021). The parent compound, 6PPD, is included in tire rubber formulations as an anti-ozonate to prevent the premature breakdown and wear of tire rubber (Cataldo, 2019; Dorofeev and Zemskii, 2017). 6PPD-quinone is generated as a by-product of tire and tire wear particle oxidation (Seiwert et al., 2022). Since the discovery of the tire wear contaminant's toxicity to coho salmon, efforts have been made to determine the concentration of 6PPD-quinone in stormwater runoff and waterways near urban centers around the world, and to determine whether other frequently detected tire wear associated chemicals, like hexamethoxymethylmelamine (HMMM) (Johannessen et al., 2022a) (Johannessen et al., 2022b, 2021) similarly pose a hazard to aquatic life.

Developments in the methods used to measure 6PPD-quinone have driven increased quantification of the compound. In environmental samples collected from Nanaimo, BC, Canada, 6PPD-quinone concentrations ranged from 0.096- 0.112 µg/L in streams and 0.035-0.627 µg/L in stormwater runoff samples (Monaghan et al., 2021). In Leipzig, Germany, snowmelt and rainfall waste water treatment influent samples measured 6PPD-quinone concentrations of 0.105 and 0.052 µg/L, respectively (Seiwert et al., 2022). From the limited quantitative data available, 6PPD-quinone appears to be prevalent in a wide range of environmental matrices globally near busy roadways and urban centers (Challis et al., 2021; Hiki and Yamamoto, 2022; Huang et al., 2021; Johannessen et al., 2022a; Rauert et al., 2022; Wang

et al., 2022). Due to the proximity of freshwater habitat to these potential sources, there is concern that fish species other than coho salmon could be sensitive to this contaminant.

Though coho salmon are the most sensitive species found to date, phylogenetic similarity to coho salmon is not a good predictor of sensitivity to 6PPD-quinone and stormwater runoff. For salmonids, Sockeye salmon (*Oncorhynchus nerka*), Chum salmon (*Oncorhynchus keta*), Atlantic salmon (*Salmo salar)* and Arctic charr (*Salvelinus alpinus)* are not very sensitive to stormwater runoff or 6PPD-quinone, whereas Steelhead/Rainbow trout (*Oncorhynchus mykiss*), Brook trout (*Salvelinus fontinalis),* and Chinook salmon (*Oncorhynchus tshawytscha*) are much more sensitive (Brinkmann et al., 2022; Di et al., 2022; French et al., 2022; McIntyre et al., 2021). Of the limited non-salmonid fish species tested, zebrafish (*Danio rerio*), *Gobiocypris rarus, Oryzias latipes,* and white sturgeon (*Acipenser transmontanus*) were all insensitive to exposure (Brinkmann et al., 2022; Di et al., 2022; Hiki et al., 2021). Toxicity tests have also been performed with two freshwater crustaceans, *Daphnia magna* and *Hyalella azteca*, and they are also insensitive to 6PPD-quinone exposure[16].

The mechanisms of action responsible for the selective sensitivity of certain salmonids to 6PPD-quinone remains unclear. Studies on stormwater runoff as a whole have found that exposure increases hematocrit, decreases plasma sodium and chloride concentrations, decreases blood pH, and disrupts the blood-brain barrier in coho salmon (Blair et al., 2021; McIntyre et al., 2021)[,](Chow et al., 2019). Rainbow trout and Brook trout exposed to 6PPD-quinone also had increased hematocrit levels as well as increases in blood glucose (Brinkmann et al., 2022). 6PPD-quinone also decreases swim behavior and increases oxygen consumption in zebrafish, an

insensitive species (Varshney et al., 2022). *In vitro* studies with Rainbow trout gill cell lines suggest that disruption of mitochondrial respiration through electron transport chain uncoupling may also play a role in the selective toxicity of 6PPD-quinone (Mahoney et al., 2022). Changes in the blood, gill, and brain may all be drivers of the effects observed and may play a role in determining species-specific sensitivity to 6PPD-quinone exposure, but more research is needed.

While previous work has indicated that Brook trout are sensitive to 6PPD-quinone, additional work with this important species is warranted to independently validate those results, to expand on the understanding of potential mechanism of action, and to explore differences in sensitivity across life stages. Most toxicity tests performed on species sensitive to 6PPD-quinone exposure have been conducted using fingerling life stages of fishes, and no data are available on the comparative sensitivity of early life stages (e.g., fry). To fill this data gap, the purpose of this study was to determine if the sensitivity of Brook trout fry is similar to fingerlings, and to assess the impacts of tire wear contaminant exposure on blood chemistry and gill histology to determine a potential mechanism of action for toxicity. Both 6PPD-quinone and HMMM exposures were conducted, as 6PPD-quinone exposure at low concentrations have been shown to cause mortality to Brook trout fingerlings(Brinkmann et al., 2022), and HMMM has been measured in storm water runoff events near urban centers alongside 6PPD-quinone, with limited published toxicity data available. The data generated in the study will be used to better understand the potential risk of these contaminants in Brook trout habitat in close proximity to roadways and urban centers.

3. Materials Methods

3.1. *Organisms*

Brook trout (also called Brook char) fry and fingerlings were obtained from a commercial supplier (Brittany Hill Farms, Seeleys Cove, NB) in July 2022. Fish were acclimated at the Huntsman Marine Science Centre (St. Andrews, NB) in dechlorinated municipal freshwater for 2-3 weeks prior to the launch of any toxicity tests. During the acclimation and holding period, fish were fed a commercial fish food diet and were visually screened daily for disease and deformities. Fry were fed EWOS® #1 crumble (Cargill Incorporated, Surrey, BC, Canada) and were transitioned to 1.0 mm Nutra Spirit produced by Skretting (Vancouver, BC, Canada). The fingerlings were fed a mixture of EWOS 3.0mm Transfer and Skretting's 3.0mm Nutra Olympic feed. Fish were held on a 15:9 light: dark cycle for the duration of the acclimation period. All holding and care for the organisms prior to the trial and exposures were conducted according to the Department of Fisheries and Oceans Animal Care Committee protocol AUP 22-26. The negative control validity criteria for survival in all of the exposures was >80%, and this threshold was met and exceeded for all the experiments conducted in this study as there were no control mortalities in any of the trials.

3.2. *Test materials and exposure methods*

HMMM was acquired from a commercial supplier (95% purity; Alfa Chemistry, Ronkonkoma, NY, USA). The 6PPD-quinone used in this study was synthesized following the methods described in Monaghan et al(Monaghan et al., 2021). Stock solutions of both HMMM and 6PPD-quinone were prepared using dimethyl sulfoxide (DMSO) and stored frozen at -20°C until use.

Stock solutions were thawed overnight and added directly to the test vessels. A DMSO only control at the same concentration (% vol basis) as the greatest test concentration was tested with each trial, and the percent DMSO was less than 0.08% in all trials. All test vessels were aerated to ensure adequate mixing occurred after the addition of the stock.

3.2.1. Fry

HMMM exposures were conducted with nominal concentrations ranging from 180 – 6.600µg/L. Two replicate trials with 6PPD-quinone were conducted with the Brook trout fry using nominal concentrations ranging from 0.1 – 10 µg/L for 24hrs. Fry were fasted for 20-50 degree days (°C x number of days; equivalent to 2-5 days at 10°C) prior to exposure, and then were exposed in groups of 10 in aerated 1L (for the first trial) or 4L (for the second trial; to decrease fish biomass loading) glass jars filled with 0.8L or 3.5L of dechlorinated tap water (ranging in size from 0.7 – 1.9g, average biomass loading of 3.4 g/L) held in an environmental chamber at 10 ± 2°C. To analytically validate exposure concentrations, a surrogate jar with the highest exposure concentration per trial was prepared and held in the same manner as the exposure vessels and was sampled at trial launch (t = 0hrs). Additionally, two surrogate jars were also treated with the highest exposure concentration, one with and one without fish (the same size ones used in the trial) were sampled at the end of the trial (t = 24hrs). These surrogate vessels with and without fish were included to determine the impact of biological uptake or test vessel adsorption on HMMM and 6PPD-quinone concentrations over the course of the trial and were necessary to be separate from the exposure vessels due to the analytical volume requirement of 1-L. Fish were assessed for mortality and morbidity using 3 point scoring criteria. Fish were considered unaffected (score of 0) if they were swimming upright, appeared alert, and could

maintain equilibrium. Morbidity (score of 1) was categorized as fish that could not maintain equilibrium, were not activity swimming but had visible flashing or flaring of the operculum. Mortality (score of 2) was scored in individuals that had no visible movement and had no reaction after the caudal peduncle was gently probed. Mortalities were recorded and removed from the test vessel as soon as they were observed. Temperature, pH, dissolved oxygen (percent saturation), water hardness, and ammonia was measured in the control and highest exposure concentration at T0 and T24. In the first trial, effects were scored at 1, 4, 7 and 24hrs of exposure. Due to the rapid onset of effects observed in the first trial, we increased the observation frequency for the second trial to 1, 2, 3, 4, 6, and 24hrs of exposure.

3.2.2. Fingerlings

HMMM exposures were not conducted with the fingerlings as no effects were observed in the fry study. Two replicate 6PPD-quinone trials were also conducted with the Brook trout fingerlings with nominal concentrations ranging from 0.1 – 10 µg/L for 24hrs, mirroring the fry exposures. Fingerlings were fasted for 20-50 °C degree days (2-5 days) prior to exposure and were exposed in groups of 10 in aerated 208L steel drums lined with BPA-free low density polyethylene bags filled with dechlorinated tap water (ranging from 23.8 – 108.4g; average biomass loading of 4.5 g/L). The barrels were partially submerged in a flow-through water bath to maintain test temperature and were covered with fish nets held in place with metal clamps to limit escapes from jumping fish. In the first trial, a water chemistry sample was collected from each exposure concentration at T0. A replicate from the highest exposure concentration was then sampled again at 3, 6, and 24hrs of exposure to confirm exposure concentrations and quantify the loss of 6PPD-quinone over time in the barrel. Water chemistry samples in the

second trial were collected from all 3 replicates of the highest exposure concentration at both T0 and T24 to determine the inter-replicate variability in our trial. Dissolved oxygen, pH, alkalinity, hardness, temperature, and ammonia concentrations were measured at T0 in one barrel, and at T24 in a replicate of each treatment. Fish were assessed for morbidity and mortality using the same criteria described in the fry trials at 1, 3, and 24hrs of exposure in both trials.

3.2.2.1. Blood metrics and gill histology

The blood and gills were sampled from a subset of individuals in the fingerling exposures. Any surviving fish at the end of the 24hr exposure were euthanized using an overdose of TMS (tricaine methanesulfonate, Syndel, Nanaimo, BC; 400 mg/L), in a staggered manner to ensure an equal time between euthanizing and blood collection. Arterial blood was then collected using a 20-gauge needle inserted into the caudal artery and collected directly into a lithium-heparinized vacutainer. After collection, blood was gently swirled to minimize clotting in the vacutainer. Following blood collection, 100-200µL of blood was loaded into an i-STAT blood analyzer CHEM8+ cartridge (Abbott Point of Care Inc., Union City, CA, USA) using a 1mL syringe. The CHEM8+ cartridge was used to measure sodium, chloride, total CO_2, Anion gap, ionized calcium, glucose, urea nitrogen, creatinine, hematocrit, and hemoglobin concentrations in freshly collected blood samples (detection limits are available in **Supplemental Table 1**). The cartridges and analyzer were used according to the manufacturer's specifications, and cartridges have previously been used with fish blood samples to measure ion dysregulation(Chow et al., 2019). Blood glucose was also measured in a larger subset of individuals using the Contour Next Glucose Monitoring System (Bayer, Mishawak, IN, USA), and

hematocrit was measured using microhematocrit tubes. Each blood sample was allocated into 3 tubes and averaged to compile a final hematocrit reading. Hematocrit was also measured using the i-STAT analyzer. There was a linear relationship between the hematocrit values measured with the i-STAT cartridge and the microfuge tube method (**Supplemental Figure 1**), however, because the microhematocrit tube data had a much higher sample size, the hematocrit data presented in the results is based off of the microhematocrit tube data. To examine changes in blood cell count and morphology, blood samples were analyzed with a Beckman Coulter Counter Multisizer 4e (Indianapolis, IN, U.SA) with a 100µm aperture (**Supplemental Figure 3**). Blood smears were also prepared by fixing blood with methanol and staining cells using an eosin stain (**Supplemental Figure 3**).

After collecting blood samples, fish were sexed and gill arches on the left side were dissected from the fish. The gills were preserved and stored in 10% buffered formalin in 50mL plastic corning tubes until histology could be performed. Standard methods were then used to prepare 7 µm sections for hematoxylin and eosin staining. Digital images of the gill filaments were captured using light microscopy (20x magnification) and measurements made using ImageJ software (version 1.53)(Abramoff et al., 2004). Interlamellar cell mass (ILCM) height was measured from the base of the filament and expressed relative to the length of an adjacent lamella (i.e., percent of lamellar length; 5 ILCM measurements per fish).

3.3. *Analytical chemistry*

Water chemistry samples were collected in 1L polyethylene terephthalate (PET) bottles, frozen immediately after collection, and stored at -20°C. Details on the calibration methods, running

194 conditions, and the limit of detection (LOD) for the samples are provided in the Supplemental
195 Information. In brief, all samples were analysed using liquid chromatography-tandem mass
196 spectrometry (LC-MS/MS), using an AB Sciex 5500 QTRAP (Concord ON Canada) paired with an
197 Agilent 1100 Series LC and autosampler (Mississauga ON Canada), located in the Water Quality
198 Centre at Trent University. Analytes were detected using a quantitation method developed in
199 the AB Sciex Analyst 1.6.2 software. Triplicate laboratory blanks, consisting of high purity water,
200 were prepared and/or extracted and analysed, alongside each batch of samples. Spike and
201 recovery experiments were performed by spiking HMMM and 6PPD-quinone into high purity
202 water (50 µg/L) to evaluate the method; recoveries of both analytes were > 80%. The limit of
203 quantitation (LOQ) was 0.5 µg/L for 6PPD-quinone and 0.2 µg/L for HMMM.

204 ### 3.4. *Statistical Analysis*

205 All graphing and statistical tests were completed using R software(R Core Team, 2019). LC50
206 and LC10 values were calculated using log-logistic 2-parameter models, with the lower and
207 upper limits fixed to 0 and 100, in the drc package (Ritz et al., 2015).

208 The LT50 values (time at which 50% mortality occurs at a given concentration) were calculated
209 using 3-parameter Weibull models. The LC50 data at each of the time points measured were
210 then fitted to a first-order 1-compartment model described in equation (1) using the nlstools R
211 package (Baty et al., 2015). The following first-order one compartment model was used
212 (French-McCay, 2002):

213 $$(1)\quad LC_{50}(t) = LC_{50,\infty}\,[1-\exp^{(-\varepsilon\, t)}]^{-1}$$

The non-linear regression model in the package calculates the incipient value (i.e., LC50, ∞) where t describes the increasing exposure duration in units of hours, and ε describes the rate at which the organism accumulates damage/repairs in units of hr^{-1}. The same equation was applied to the LT50 data to determine the incipient time to lethality ($LT50_{\infty}$), which represents the minimum amount of time needed to observe 50% mortality, independent of concentration.

4. Results

4.1. *Exposure Characterization*

4.1.1. Water quality

Dissolved oxygen remained between 87 – 101 % air saturation in all the treatment groups tested. There were also no treatment or trial specific differences in pH (6.78 – 8.12; which varied between trial and treatments likely due to ammonia), water hardness (12 – 15 mg/L), and alkalinity (16 – 24 mg/L). The water temperature in the fry trial vessels (11.1 – 12.0°C) was slightly cooler than our fingerling trials (12.0 – 14.8°C) both pre- and post-exposure. Ammonia levels varied considerably between treatment groups and trials (0 – 0.53 mg/L), however all ammonia measurements were below ambient water quality criteria(United States Environmental Protection Agency, 2013).

4.1.2. Water chemistry

Due to the limit of quantification of the analytical technique used in this study, and the response of the Brook trout, only the highest exposure concentrations were quantified. The HMMM exposure concentrations remained consistent (~7% decrease) in the test vessel without fish over the course of the 24hr exposure, however, in the surrogate vessel containing fish

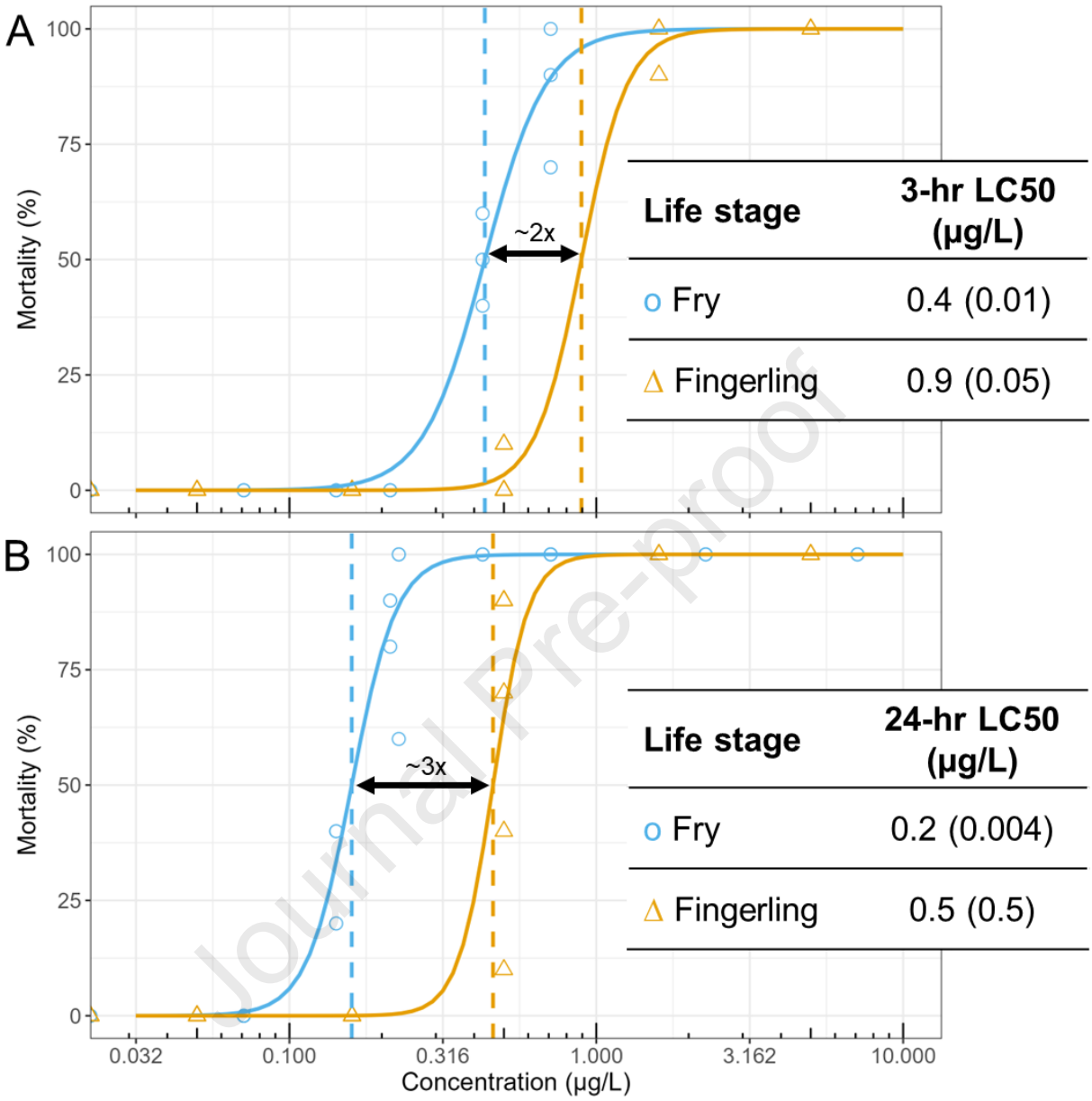
there was a ~30% decrease in exposure concentration over the 24hr period (**Supplemental Table 3)**. The 6PPD-quinone concentrations at the start of the exposure were on average 0.7x and 0.5x the expected nominal concentrations in the fry (glass jars) and fingerling (plastic bag lined drums), respectively. In the 6PPD-quinone fry trials the concentration in the glass jar containing fish was 18% lower than the paired vessel with no fish after 24hrs (**Supplemental Table 3**). In the plastic bag lined drums used for the fingerling studies there was no difference in exposure concentrations in the first 6 hours, however, there was a ~19% decrease in measured concentrations at 24hrs, representing a change in concentration from 5.3 µg/L to 4.4 µg/L. Due to discrepancy between the nominal and measured concentrations, the highest treatment level in two of the trials (1 fry and 1 fingerling) of 1.0 µg/L, resulted in concentrations being measured below the limit of quantitation (LOQ = 0.5 µg/L). In these cases, we used the measured data from the previous trials with the fry and fingerlings to estimate the concentrations based on the initial measurements being 0.7x (fry) and 0.5x (fingerling) that of the nominal. This correction factor was applied across all nominal concentrations that fell below the LOQ. In separate work we have tested 6PPD-qunione in these same exposure vessels at higher concentrations (3, 10, 30 µg/L owing to testing a less sensitive species) and have observed a consistent difference between measured and nominal (average 0.69x in jars, and 0.51x in plastic lined bags) across concentrations that was equivalent to what we observed in these Brook trout exposures. Due to the consistency of the relationship observed between measured and nominal concentrations, regardless of the concentration, we applied a correction factor of 0.7x and 0.5x to all nominal exposure concentrations for the fry and fingerling

exposures respectively. These measured and corrected values represent the initial concentration in the exposure solution and were used for subsequent data analysis.

4.2. *Biological effects*

4.2.1. Acute toxicity to fry and fingerlings

The onset of biological effects of 6PPD-quinone exposure began shortly after allocation of the test solution. Mortality was observed within 1-hr of exposure in both the fry and fingerling life stages. Prior to mortality occurring, fish showed signs of respiratory distress such as gasping, rapid opercular abduction rate, bursts of erratic swimming, and gill flaring. The fish would then begin to lose their ability to maintain an upright position in the water column and were scored as moribund. Morbidity preceded mortality, as the majority of moribund fish at the 1 and 2hr assessment were mortalities by the 3hr mark (**Supplemental Figure 2**). The majority of the observed mortalities occurred within the first 6hrs of exposure, after which any survivors remaining would persist until 24hrs. Fry appeared more sensitive to 6PPD-quinone exposure than fingerlings (**Figure 1**), and had ~2 fold lower LC50 values at 3hrs (0.4 ± 0.01 µg/L [standard error]; 95% confidence interval [CI] = 0.40 – 0.46) and ~3 fold lower LC50 values at 24hrs (0.2 ± 0.004 µg/L; 95% CI = 0.15 – 0.17) than the fingerlings (3hr LC50 = 0.9 ± 0.05 µg/L, 95% CI = 0.80 – 0.99; 24hr LC50 = 0.5 ± 0.05 µg/L, 95% CI = -0.99 – 1.58). The LC50 and LC10 values calculated for each of the assessment points are provided in the supplemental information (**Supplemental Table 4**).



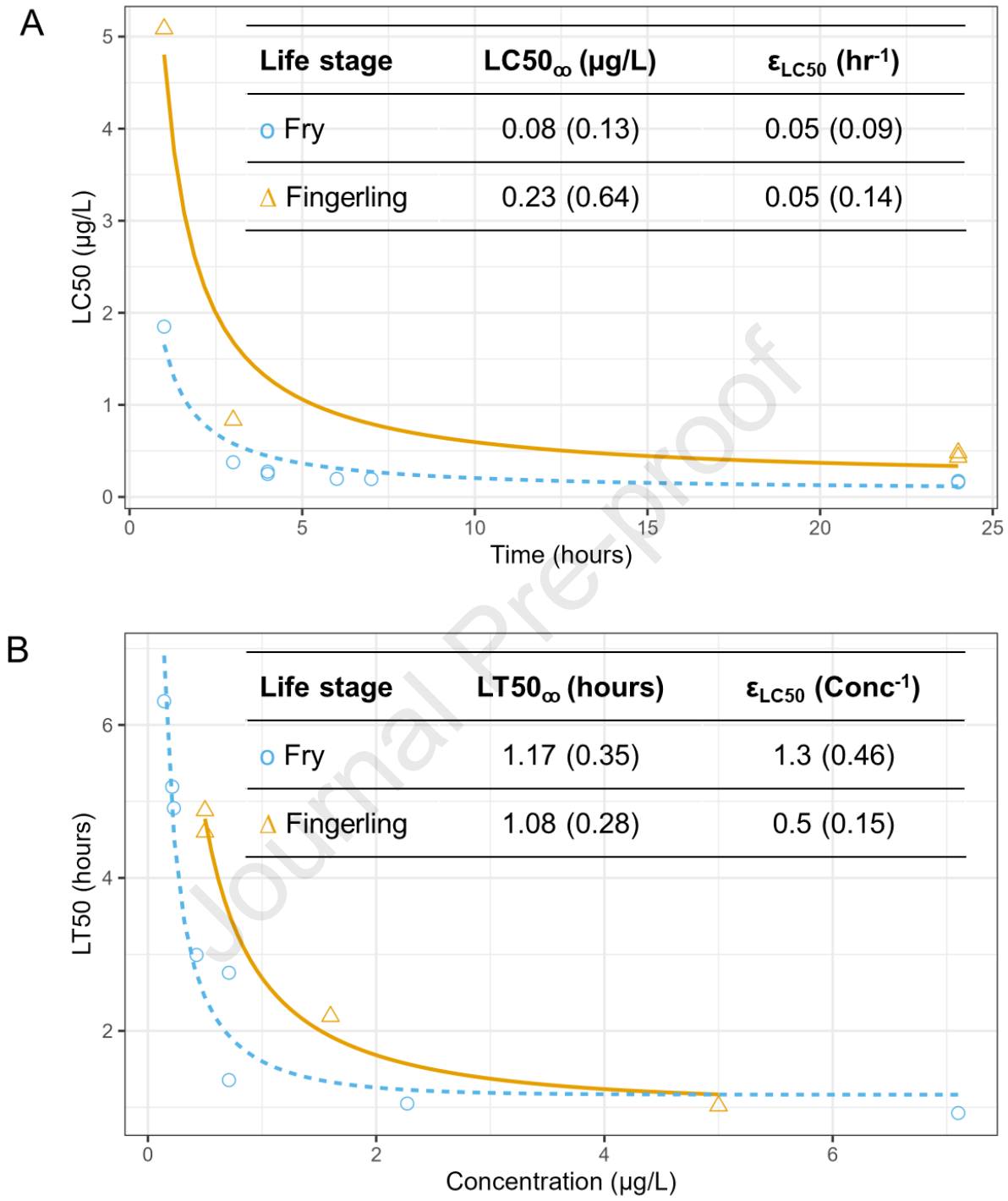
275

276 *Figure 1: Concentration response relationship for fry (blue circles) and fingerling (orange triangles) Brook*
277 *trout following 3 (A) and 24-hrs (B) of exposure to 6PPD-quinone. LC50 values are shown as the vertical*
278 *lines and presented with the standard errors beside the curves.*

279 4.2.2. Incipient Lethal Level

280 The incipient LC50 ($LC50_{\infty}$) represents the concentration at which the LC50 reaches an
281 asymptote such that there is no additional change in the value for additional exposure duration

(equivalent to the concentration where 50% of the population will be affected independent of exposure duration). Using the LC50s from each trial, the $LC50_{\infty}$ was calculated for each life stage (**Figure 2A**). The $LC50_{\infty}$ was smaller for Brook trout fry ($LC50_{\infty}$ = 0.08 ± 0.13 µg/L) than the fingerlings ($LC50_{\infty}$ = 0.23 ± 0.64 µg/L) however due to the large the standard error in the fingerling $LC50_{\infty}$ this difference was not significant (95% confidence interval for the $LC50_{\infty}$ ratio is 0.007 – 14.5). The epsilon (ε) values, which represents the time course of accumulation and damage of 6PPD-quinone was similar for both life stages (fry ε_{LC50} = 0.05 ± 0.09 hrs^{-1}; fingerling ε_{LC50} = 0.05 ± 0.14 hrs^{-1}), which suggests that though there appeared to be differences in life stage sensitivity, the rate at which effects are observed in exposed individuals is the same.



291

292 *Figure 2: LC50 over time (A) and LT50 (B) for the fry (blue circles, dashed line) and fingerling (orange*
293 *triangles, solid line) exposed to 6PPD-quinone. The incipient lethal values (and standard error) and the*
294 *corresponding epsilon (ε) values for each value and life stage are given in the insert.*

For each of the exposure concentrations used in the study that caused sufficient (i.e. >50%) levels of mortality, LT50 values were calculated to determine the time it took for 50% of the individuals in a treatment to die (**Supplemental Table 5**). The LT50 values from each trial were used to calculate the incipient time to lethality ($LT50_{\infty}$) (**Figure 2B**), which is the asymptote where the time to reach 50% mortality does not decrease with increasing exposure concentrations, representing a chemically and physiologically driven minimum amount of time needed to observe 50% mortality. The $LT50_{\infty}$ values were not statistically different between the fry ($LT50_{\infty}$ = 1.17 ± 0.35 hrs) and the fingerlings ($LT50_{\infty}$ = 1.08 ± 0.28 hrs) (95% confidence interval for the $LT50_{\infty}$ ratio is 0.68 – 1.72). These values further support the ε_{LC50} values, suggesting that the rate which damage occurs and is observed in exposed individuals is similar between the life stages.

4.1. *Fingerling Blood Chemistry*

There were no visible differences between the red blood cells of the control and highest concentration exposed fish, nor were there any differences in the size or concentration (number of cells per mL) of red blood cells (**Supplemental Figure 3).** Exposure to 6PPD-quinone did, however, alter some of the other blood parameters (**Figure 3**). There were concentration-dependant increases in hematocrit (**Figure 3A,** $p=0.015$), and blood glucose (**Figure 3B,** $p=3.7 \times 10^{-12}$), total CO_2 (**Figure 3C,** $p<0.001$**)**, and after 24hrs exposure to 6PPD-quinone. Blood chloride (**Figure 3D,** $p=3.1 \times 10^{-7}$) and sodium (**Figure 3E,** $p=1.6 \times 10^{-5}$) concentrations decreased in response to higher concentrations of 6PPD-quinone. Blood was only sampled from individuals

that survived the duration of the 24hr test, which may suggest that the sublethal effects on blood parameters observed here could be more severe in the mortalities that occurred due to exposure.

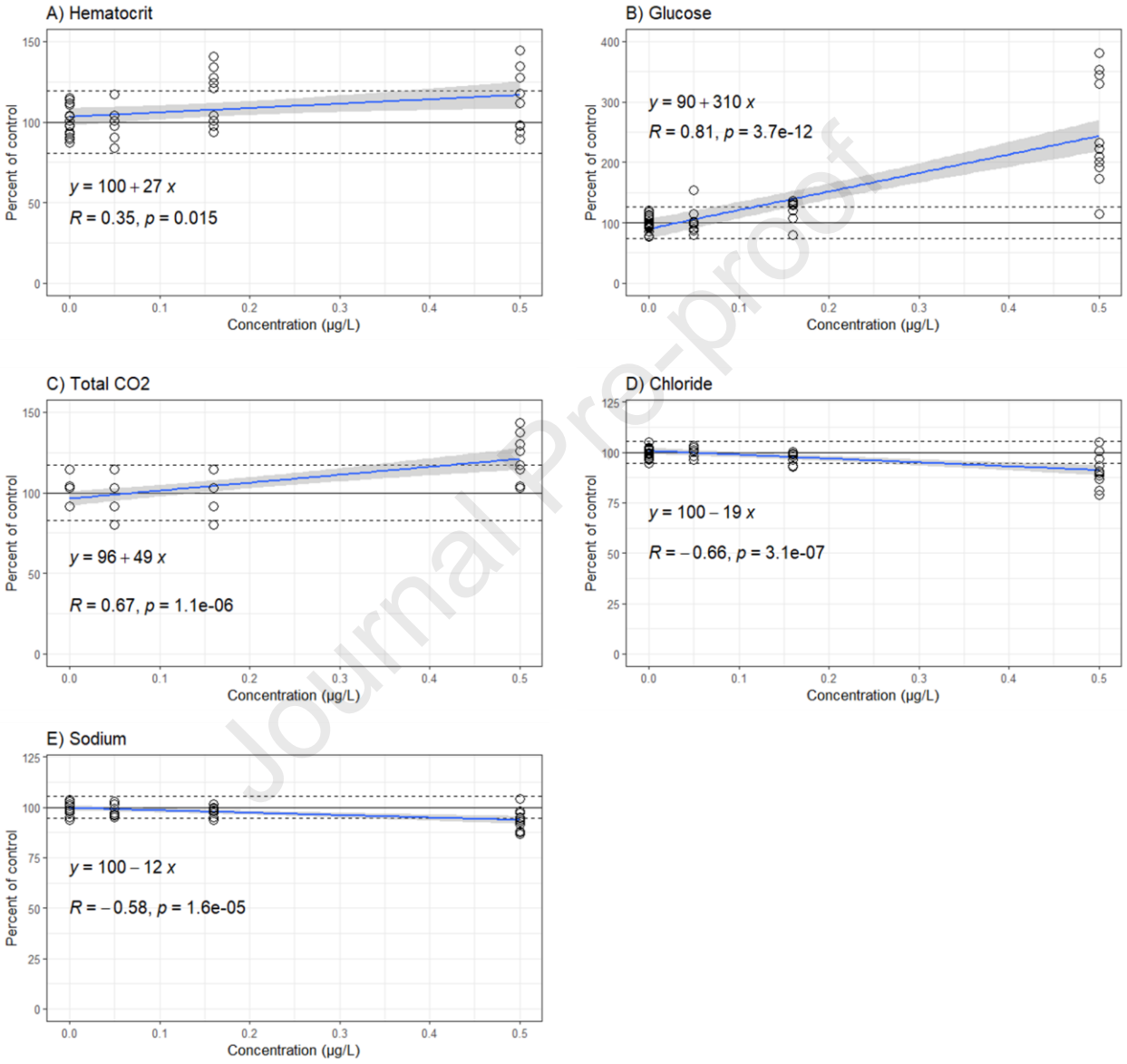


Figure 3: Impact of 6PPD-quinone exposure on the hematocrit (A), glucose (B), total CO_2 (C), chloride (D), and sodium (E) levels in Brook trout fingerling blood after 24hrs. Responses were normalized to control, and the data presented are pooled from both fingerling trials as there were no trial-specific differences in response. The solid horizontal line represents the mean of the control response, and the

dashed horizontal lines represent the range in control values ± 2 standard deviations. The Pearson correlation coefficient (R) and p values for the linear relationships presented are included on each of the corresponding panels. Shading represents the 95% confidence band around each relationship.

4.2. *Histology*

There was a significant increase in the mean relative interlamellar cell mass (ILCM) size in the 0.5 µg/L exposed fingerlings (21.1 ± 9.1% SD, n = 10) compared to controls (13.9 ± 4.5%, n = 10) (p = 0.045; Figure 4).

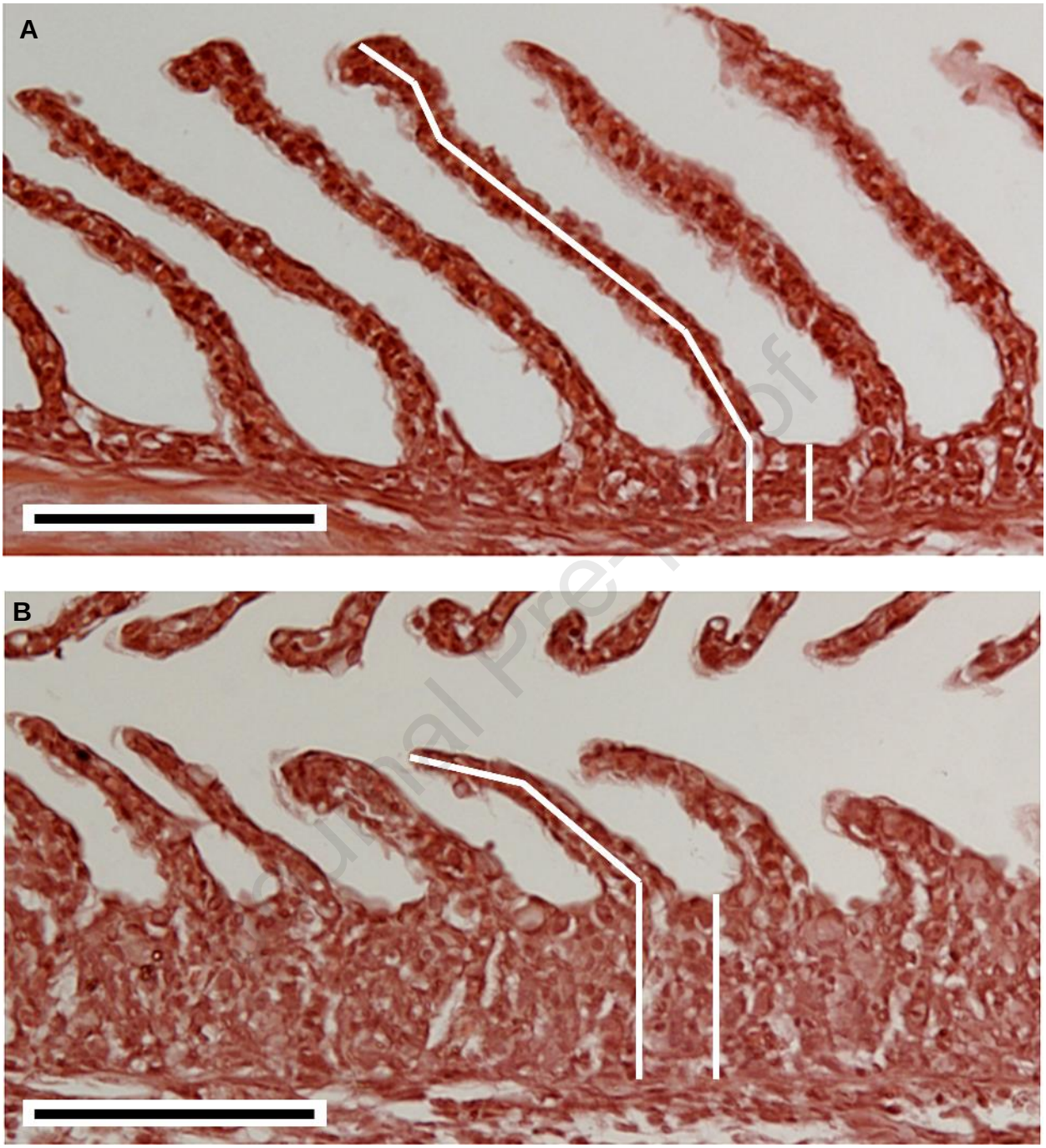


Figure 4: Lamellar length (longer line) and interlamellar cell mass (ILCM) height (shorter line) in representative micrographs from representative control (A) and 0.5 µg/L 6PPD-quinone exposed (B) fish. ILCM sizes are 11 and 39% of lamellar length for upper and lower micrographs, respectively; scale bar = 100 µm.

The ILCM values from the individual fish were not significantly correlated with fish size (total length and body mass) or hematocrit, however, they were significantly correlated with numerous blood plasma endpoints (**Figure**). Increases in blood glucose (**Figure 5A**) and total CO_2 (**Figure 5B**) correlated with increases in relative ILCM height (R = 0.64, p = 0.018, and R = 0.7 and p = 0.011, respectively). Increases in relative ILCM height also correlated with decreases in blood sodium (**Figure 5C)** and chloride (**Figure 5D**) at 24hrs of exposure (R = -0.67, p = 0.013, and R = -0.58 and p = 0.037, respectively).

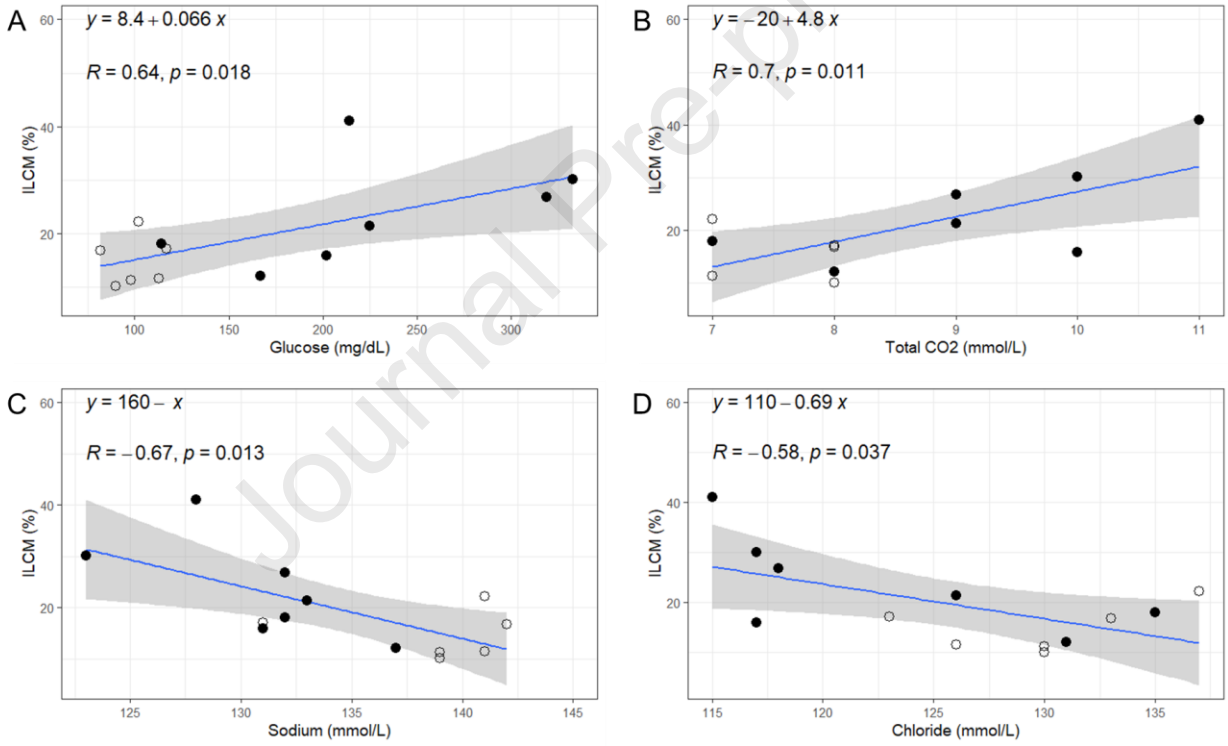


Figure 5: Correlation of ILCM with significant blood chemistry parameters, glucose (A), total CO_2 (B), sodium (C), and chloride concentrations (D). The open circles are control fish, and the filled circles are fish that were exposed to 0.5 µg/L 6PPD-quinone for 24-hours. The Pearson correlation coefficient (R) and p values for the linear relationships presented are included on each of the corresponding panels. Shading represents the 95% confidence band around each relationship.

5. Discussion

Since the discovery of 6PPD-quinone as the contaminant predominantly responsible for urban runoff mortality syndrome there has been a push to test the sensitivity of other fishes to tire wear particles, leachate, and compounds (e.g., HMMM). HMMM exposure caused no effects to fry Brook trout at concentrations as high as 6.6 mg/L, suggesting that acute toxicity may be attributed to only specific tire leachate contaminants. Brook trout fingerlings were considered one of the most sensitive species/life stage exposed to 6PPD-quinone to date (Brinkmann et al., 2022), and our fry and fingerling Brook trout data support these results. Despite the differences between our study and Brinkmann et al.(Brinkmann et al., 2022) in exposure set up (208-L steel drums lined with BPA-free low density polyethylene bags vs. 150-L fiberglass tank), analytical chemistry methods (LC-MS/MS vs. UHPLC-MS/MS), fish stock (Seeleys Cove, NB vs. Coleman, AB), and sample size (three replicates with n = 10 vs. two replicates with n = 4), our results are in very good agreement (24-hr LC50s of 0.5 and 0.59 µg/L in this study and Brinkmann et al.(Brinkmann et al., 2022) respectively) which supports the use of both exposure methods in generating comparable toxicity data. Our study expanded on the results presented in Brinkmann et al.(Brinkmann et al., 2022) by including Brook trout fry, and our study indicates that data collected from fingerling sized fish alone may underestimate the species sensitivity. In future studies, additional testing on earlier life stages may be required to ensure the data generated is protective of not only the fingerling life stage, but also more sensitive life stages like the fry .

Since the discovery of 6PPD-quinone as an emerging contaminant of concern, there have been multiple analytical methods developed for the quantification of this compound. One of the limitations of the methods used in this study is the relatively high limit of quantitation (LOQ). Due to the extreme sensitivity of Brook trout to 6PPD-quinone, the majority of our exposure concentrations were required to be below the LOQ of the instrument. With the growing interest in 6PPD-quinone studies there is likely to be improvements in the commercial availability of more sensitive techniques for 6PPD-quinone quantification.

The LC50 value obtained from the fry exposures indicate that Brook trout fry are one of the most sensitive species to 6PPD-quinone tested to date (most sensitive are coho with a 24-hr LC50 for fingerling sized fish of 0.095 ug/L(Tian et al., 2022), and 0.041ug/L for fry (Lo et al., 2023)) and that fry appear to be approximately 2-3 times more sensitive than fingerlings. This finding is not unexpected as early life stages are often more sensitive to exposure than adults. A study examining the impacts of chloramines found that Brook trout fry were the most sensitive to exposure, with alevins (i.e., yolk-sac fry) and fingerlings being more tolerant and having very similar sensitivity (Larson et al., 1977). A study examining the difference in copper sulphate toxicity in channel catfish (*Ictalurus punctatus*) yolk-sac fry and swim-up fry found that yolk-sac fry were 4.6 times more tolerant than the older swim-up fry stage. Fry used in our study were at the swim-up stage of development (i.e., actively feeding, and free-swimming), which may be one of the life stages most sensitive to contaminants during salmonid development.

Incipient LC50 values determine the exposure concentration at which only 50% of the population will die regardless of exposure duration, and the value of 0.08 µg/L for the fry is well

within measured environmental values (Challis et al., 2021). The asymptotic decrease in LC50 concentrations over time, was consistent between the two different life stages. Since epsilon values describe the rate of accumulated damage that occurs over the course of an exposure, it suggests that though overall sensitivity appears to be different, the rate of observed effects is the same. Incipient lethal levels and epsilon values have been used to describe the toxicokinetics of hydrophobic chemicals like polycyclic aromatic compounds (PACs)(French-McCay, 2002; Philibert et al., 2021; Redman et al., 2022), and the epsilon values calculated from the 6PPD-quinone Brook trout exposures are incredibly small in comparison. For PAC studies, epsilon values range from 0.43 – 1.6 $days^{-1}$ depending on the exposure set up and species tested (Bytingsvik et al., 2020; Philibert et al., 2021; Turner et al., 2021). The value of 0.05 $hour^{-1}$ reported in this study highlights the limited capacity of Brook trout fry to cope with the exposure and how quickly 6PPD-quinone damage accumulates in the target tissue.

Mirroring the epsilon values, the incipient LT50 ($LT50_{\infty}$) values were similar between the two life stages. LT50s are used to determine the amount of time needed to observe 50% mortality for a given concentration. The incipient LT50, calculated based on the LT50 values, can then be used to determine the minimum time required for effects to be observed regardless of exposure concentration, which was similar between the fingerling and fry. The non-linear model generated from LT50 values can also be used to estimate the exposure duration needed for a measured concentration to cause an effect. For example, applying this model to the measured concentrations from Challis et al.(Challis et al., 2021) in Saskatoon, SK, Canada it is estimated that exposure durations of 1.6, 2.4, and 11.9 hours would be required to cause 50% mortality in Brook trout fry for the 90^{th} centile (1.24 µg/L), mean (0.6 µg/L) and 50^{th} centile

(0.09 µg/L) of measured values. Peak exposure concentrations of 6PPD-quinone have been associated with storm water runoff(Challis et al., 2021; Johannessen et al., 2022b), which suggests exposures are more likely to be transient and linked with brief, pulsed, dynamic run-off events. The metrics determined through the application of these models to our LC and LT50 values provide critical inputs needed to understand the impact of exposure duration on observed effects with this novel contaminant.

Blood chemistry parameters were only measured in fish which survived the 24hr exposure. Dysregulation of ion transport was evident in 6PPD-quinone fish in a concentration dependent manner. This same effect has been observed in coho salmon exposures to stormwater runoff (Chow et al., 2019; McIntyre et al., 2018). The dose-dependent decrease in concentration of the dominant blood ions (sodium and chloride) in our 6PPD-quinone-exposed fish indicates a progressive loss of ability to maintain stable plasma ion levels, i.e., osmoregulatory distress, and is consistent with results observed in coho exposed to tire leachate (with up to 2.4 µg/L of 6PPD-quinone) in McIntyre et al. 2021(McIntyre et al., 2021). A concomitant increase in blood glucose demonstrates that the fish were trying to deal with this by mobilizing energy reserves to increase aerobic metabolism. Freshwater fish are hyperosmotic and therefore constantly losing ions by passive diffusion across any permeable surfaces in contact with the water. Maintaining a stable osmotic gradient and ion profile requires them to actively osmoregulate, accounting for a significant proportion of their resting metabolism, a situation that becomes less tenable when stressed and can ultimately lead to metabolic exhaustion and death. The increase in hematocrit and blood glucose due to 6PPD-quinone exposure observed in this study has been previously demonstrated with Rainbow and Brook trout(Brinkmann et al., 2022), and

parallel effects have been seen in select stormwater runoff studies with Brown trout(Meland et al., 2010). Juvenile (fingerling sized) coho salmon exposed to roadway runoff have repeatedly shown increases in hematocrit in response to exposure as well (Blair et al., 2021; Chow et al., 2019; McIntyre et al., 2018).

The increased size of the interlamellar cell mass (ILCM) in the surviving 6PPD-quinone-exposed fish provides further evidence of osmoregulatory distress. This remodeling of the gills slows passive diffusion of ions between the blood perfusing the lamellae and the water passing between them (Wood and Eom, 2021), and here may represent a compensatory response to limit ion loss in these surviving fish. Gill lamellae need a very large and permeable surface area to support respiratory gas exchange, but this then makes them a primary site for the loss of ions by diffusion and uptake of water by osmosis in freshwater fish. Healthy fish counter this through active osmoregulation. When this poses a serious metabolic challenge, they can decrease the amount of permeable surface area in contact with water by increasing the size of the protective ILCM. However, this also reduces the functional surface area for gas exchange to support aerobic respiration, an outcome commonly referred to as the 'osmorespiratory compromise' (Wood and Eom, 2021). The fish are now faced with increased oxygen demand (i.e., stress response) but a reduced ability to extract oxygen from the water. Our finding that ILCM size increased in direct proportion to the changes in blood sodium, chloride, glucose, and CO_2 levels, taken together with observations of gasping behaviors, gill flaring, and erratic swimming before the onset of mortality within 3-6 hours of exposure, provides compelling evidence for an inability to meet the aerobic demands while attempting (and failing) to osmoregulate adequately when exposed to 6PPD-quinone. The results of this study highlight

the importance of toxicity testing with early life stages of sensitive species and potential mechanism of action for toxicity for this novel contaminant.

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Highlights

- Brook trout fry and fingerlings were exposed to 2 different tire-wear contaminants.
- No effects were observed in HMMM fry exposures.
- Fry were 2-3x more sensitive to 6PPDq than the fingerling life stage.
- 6PPDq exposure resulted in concentration dependent changes in blood chemistry.
- 6PPDq changed the gill morphology, resulting in osmorespiratory compromise.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: