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Bioretention filtration prevents acute mortality and reduces chronic toxicity for early life stage coho salmon (*Oncorhynchus kisutch*) episodically exposed to urban stormwater runoff

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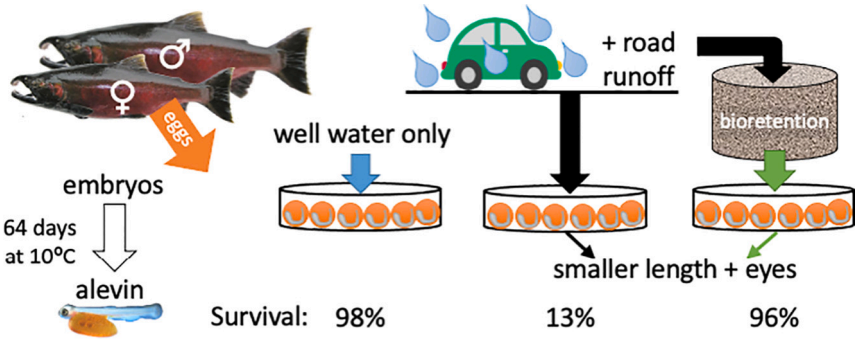
HIGHLIGHTS

- Juvenile and adult coho salmon die from brief (<24h) exposure to roadway runoff
- Are early life stages similarly sensitive, affecting species recovery?
- Coho embryos were intermittently exposed to road runoff during development
- Embryo survival in runoff was high (>90%) but 87% of alevin (hatched) died
- Bioretention filtration prevented all mortality and reduced sublethal toxicity

GRAPHICAL ABSTRACT



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ABSTRACT

As the human population of western North America continues to expand, widespread patterns of urban growth pose increasingly existential threats to certain wild stocks of Pacific salmon and steelhead (*Oncorhynchus* sp.). Rainfall previously absorbed into the soils of forests and grasslands falls instead on pavement and other hardened surfaces. This creates stormwater runoff that carries toxic metals, oil, and many other contaminants into salmon-bearing habitats. These include freshwater streams where coho salmon (*O. kisutch*) spawn in gravel beds. Coho salmon embryos develop within a thick eggshell (chorion) for weeks to months before hatching as alevins and ultimately emerging from the gravel as fry. Untreated urban runoff is highly toxic to older coho salmon (freshwater-resident juveniles and adult spawners), but the vulnerability of the earliest life stages remains poorly understood. To address this uncertainty, we fertilized eggs and raised them under an episodic stormwater exposure regimen, using runoff collected from a high-traffic arterial roadway from 15 discrete storm events. We monitored survival and morphological development, as well as molecular markers for contaminant exposure and

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cardiovascular stress. We also evaluated the benefit of treating runoff with green infrastructure (bioretention filtration) on coho salmon health and survival. Untreated runoff caused subtle sublethal toxicity in pre-hatch embryos with no mortality, followed by high rates of mortality from exposure at hatch. Bioretention filtration removed most measured contaminants (bacteria, dissolved metals, and polycyclic aromatic hydrocarbons), and the treated effluent was considerably less toxic – notably preventing mortality at the alevin stage. Our findings indicate that untreated urban runoff poses an important threat to early life stage coho salmon, in terms of both acute and delayed-in-time mortality. Moreover, while inexpensive management strategies involving bio-infiltration are promising, future green infrastructure effectiveness research should emphasize sublethal metrics for contaminant exposure and adverse health outcomes in salmonids.

1. Introduction

Stormwater runoff from roads, parking lots, and similar impervious surfaces is a ubiquitous source of pollution to aquatic ecosystems. Storm events mobilize complex mixtures of chemical contaminants, some of which have been a focus of ecotoxicological study for decades (e.g., metals, petrochemicals) while others have only recently been identified as emerging concerns for the health of aquatic species (Du et al., 2017; Peter et al., 2018, 2020, 2022). A prominent example of an emerging contaminant is *N*-(1,3-dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine (6PPD), an anti-degradant chemical added during tire manufacturing primarily to prevent tire rubber from cracking when exposed to ozone. As 6PPD migrates to the surface of tires over the lifetime of the tire, it is transformed by ozone into a quinone derivative (6PPD-quinone) that readily dissolves into water (Monaghan et al., 2021) and has recently been shown to cause widespread die-offs of coho salmon (*Oncorhynchus kisutch*; Tian et al., 2021; McIntyre et al., 2021).

The urban runoff mortality syndrome now attributed to 6PPD-quinone was initially characterized in adult coho salmon returning from the ocean to spawn in restored urban watersheds. Early field studies revealed a very high premature mortality rate for spawners across numerous runoff-dominated stream networks (>60 % mortality; Scholz et al., 2011). Importantly, the urban runoff mortality syndrome is not limited to adult spawners, but also affects juvenile coho. Specifically, untreated roadway runoff is also acutely lethal to juvenile (freshwater stage) coho salmon over a timescale of a few hours (McIntyre et al., 2015; Chow et al., 2019), producing a distress syndrome identical to that of adults (Chow et al., 2019). Moreover, acute mortality from runoff exposure has also been confirmed in other Pacific salmonids, including juvenile Chinook salmon (*O. tshawytscha*) and steelhead trout (*O. mykiss*) (French et al., 2022), expanding concern over stormwater impacts to additional species of economic, ecological, and cultural importance.

Several distinct wild population segments of coho, steelhead, and Chinook salmon are currently protected under the U.S. Endangered Species Act (ESA) throughout California and the Pacific Northwest. The conservation of these protected species is generally managed at the population-scale, which necessitates a consideration of cumulative losses across all life stages from exposure to toxic urban runoff. Conventional demographic models typically aggregate estimated losses across distinct life stages. For salmonids, vulnerable stages encompass adults in freshwater to release their gametes (spawners) and their offspring (embryos) that hatch (alevins) and then emerge from gravel nests (fry), subsequently rearing as juveniles (fingerlings) before migrating to the ocean (smolts). Despite a growing body of toxicological information for the juvenile and adult life stages, less is known about impacts to embryos and alevins. Accordingly, initial population models (Spromberg and Scholz, 2011) are likely to underestimate aggregate risk.

A related conservation goal is the promotion of mitigation strategies that reduce non-point source contaminant loadings to receiving waters (i.e., salmonid spawning and rearing habitats). Given the importance of 6PPD for the structural integrity and safety of tires currently used throughout the world, it may be more than a decade before non-toxic alternatives are in use. At the same time, initial modeling of the urban mortality syndrome (Spromberg and Scholz, 2011) suggests local salmon population extinctions over a time course of a few years to a few decades. There is therefore an intensive and ongoing societal effort to identify mitigation strategies that can capture and remove 6PPD-quinone, and many other toxics, before they reach rivers, lakes, and estuaries. Bioretention is a green stormwater infrastructure technology that uses an engineered soil media to treat runoff pollutants (Liu et al., 2014; Tirpak et al., 2021). We previously showed that bioretention can prevent mortality of coho salmon spawners (Spromberg et al., 2016) and juveniles (McIntyre et al., 2015), but corresponding benefits for early life stages have not been examined.

The chemical complexity of stormwater runoff and the potential for interactions between chemicals are major sources of uncertainty with respect to anticipating toxicity to fish embryos. Runoff from urban roadways contains mixtures of petroleum-derived polycyclic aromatic hydrocarbons (PAHs), a subset of which are well known to cause developmental defects in fish and other vertebrates (reviewed by Incardona, 2017; Incardona and Scholz, 2018). Therefore, the potential for PAH toxicity in fish spawning habitats is common to both oil spill injury assessments and urban stormwater management. By extension, much of what is currently known about environmental risks from PAHs is derived from the global oil spill literature – e.g., determinations of fisheries losses in the aftermaths of the 1989 and 2010 *Exxon Valdez* and *Deepwater Horizon* oil spills in U.S. waters, respectively.

Numerous studies related to oil spills have shown that certain PAHs induce the metabolizing capability of CYP1A, a mixed function oxygenase of the cytochrome P450 superfamily (Uno et al., 2012). The induction of CYP1A is also a sensitive indicator of sublethal detoxification stress in fish embryos experimentally exposed to urban runoff, including zebrafish (*Danio rerio*; McIntyre et al., 2016a) and Pacific herring (*Clupea pallasii*; Harding et al., 2020). Moreover, tricyclic PAHs such as phenanthrene – pervasive in both crude oil and stormwater – are known to specifically disrupt the normal physiological function of fish heart muscle cells (Brette et al., 2014, 2017; Vehniainen et al., 2019; Kompella et al., 2021; Al-Moubarak et al., 2021). Fish embryos exposed to PAHs in complex environmental mixtures – whether as weathered crude oil or urban runoff – displayed nearly indistinguishable defects in cardiac development and function (McIntyre et al., 2016a; Harding et al., 2020). Moreover, stormwater-associated cardiovascular dysregulation occurred in concert with upregulation of the gene *nppb* (McIntyre et al., 2016a), which encodes a B-type natriuretic protein used as a biomarker for cardiac stress in humans (Hama et al., 1995; Liang et al., 2003) as well as fish exposed to mixtures of PAHs (Edmunds et al., 2015; McIntyre et al., 2016b).

The current study had two primary objectives. The first was to generate lethal and sub-lethal toxicity information for salmonid early life stages to fill a data gap for population-level decision support tools that are presently guiding the management of ESA-listed stocks. To this end, we exposed coho salmon to stormwater runoff from fertilization (embryo stage) until just after hatch (alevin stage). To emulate discrete and episodic rainfall events, runoff was collected from multiple storms. In addition to survival, we monitored developmental abnormalities via morphometrics and changes in the expression of *cyp1a* and *nppb* genes as molecular biomarkers of exposure and injury. The second goal was to

evaluate the effectiveness of soil bioretention treatment, as quantified by reducing or eliminating mortality and morphometric and molecular indicators of exposure and sublethal toxicity. Notably, our experiments were performed in 2014–2016, so our findings are discussed in the context of the subsequent identification of 6PPD-quinone (Tian et al., 2021), as well as the transferability of oil spill-related exposure and injury biomarkers for assessing salmonid health in rapidly urbanizing watersheds.

2. Experimental methods

2.1. Coho embryo fertilization and husbandry

Adult coho salmon were spawned on November 23, 2015 by personnel at the Grovers Creek Salmon Hatchery (Indianola, WA, USA) operated by the Suquamish Tribe. Milt from 11 males was collected in individual plastic cups and added simultaneously to a bucket containing the eggs from 8 ripe females. Fertilized eggs were placed in cups made from acrylonitrile butadiene styrene (ABS) pipe (Schedule 40; 1.5″ high, 3″ diameter) with mesh bottoms (fiberglass window screen). Following water hardening, the eggs placed in each cup ($\bar{x} = 90$, SD = 9) formed a monolayer atop the screen.

Embryos were reared in vertical flow incubator stacks in a covered area outdoors. An individual stack consisted of eight trays. The upper 4–5 trays in each stack were left empty to allow for settling of fine sediments and other particulates. Nine cups of eggs were distributed randomly in each of the lower trays. Each stack served as a distinct treatment (Fig. S1): undiluted stormwater runoff (100 %; R100), the same runoff filtered through bioretention columns (F100), runoff diluted by 50 % with clean water (R50), and a clean water control – a mix of well and spring water supplying the normal operation of the hatchery. Sufficient eggs were available to fill four replicate trays for the R100 and F100 treatments, and three trays in R50 and control treatments.

Daily fluctuations in ambient water temperature were monitored with data loggers (Hobo U22, Onset Computer Corporation, Borne, MA, US) placed in the top tray of each stack and programmed to record on a 2-h time step. Cup position was rotated within each tray during development to prevent potential location bias. Beginning at the eyed stage (29 days post-fertilization; dpf), each stack was treated with formalin by hatchery personnel (1500 mg/L for 15 min/day) to prevent fungal growth due to unfertilized or dead embryos. This husbandry approach yielded high survival rates among controls (>90 %).

2.2. Episodic runoff exposure simulation

Stacks received flow-through (7.6 L/min) control water between exposure days to simulate transient exposures to runoff during rain events in urban watersheds. Roadway runoff was collected opportunistically from 15 discrete storms from a busy urban arterial road in Seattle, WA (Spromberg et al., 2016). For each rainfall event, stormwater was transported in a large stainless steel tote (250 gal; 946 L) to the hatchery. On exposure days, exposure water supplied to the top of each stack was switched from flow through hatchery water to recirculated water from a stainless-steel sump dedicated to that stack. Sumps were filled with 115 L of exposure water (control, R50, R100 or F100). Runoff for the F100 treatment was filtered through bioretention media as described previously (Spromberg et al., 2016). Briefly, undiluted runoff (R100) was dispensed by peristaltic pump through silicone tubing at 2 L/min to 4 drums (55-gal) containing bioretention media. Media was a mixture of sand and compost (60:40 % by volume, 46 cm deep), overlying a gravel drainage layer (30 cm), and covered with wood bark mulch (8 cm). The bioretention system was constructed during autumn 2014 when it was used to treat ~2500 L of roadway runoff from the same collection source, for studies with adult salmon (Spromberg et al., 2016). During a pilot year of the current study (Nov 2014–Jan 2015), an additional ~850 L was filtered, followed by an additional ~1700 L (Nov 2015–Jan 2016). The effluent (F100) was collected in high density polyethylene (HDPE) buckets and used to fill the sump supplying the F100 stack. When sumps were full, conventional water quality was recorded for each treatment (temperature, pH, dissolved oxygen) and samples were collected for water chemistry analyses (see Methods Section 2.5 Water chemistry).

To start the exposure, the flow-through input of clean water was turned off and stacks were allowed to drain (approximately 5 min). A recirculation pump (QuietOne 3000, LifeGard Aquatics, Santa Fe Springs, CA, US) in each sump was turned on (7.6 L/min) to supply treatment water to the top tray of each stack, which cascaded down and finally drained from the bottom tray into the respective sump. Approximately 7 L of hatchery water was retained in each stack when it was drained, amounting to a dilution of nominal stormwater concentrations by approximately 6 %. Each of the 15 individual episodic exposures was 24–48 h in duration and interspersed throughout the 64 days of coho embryonic development in our study [1–64 days post-fertilization (dpf); Table S1]. The cumulative exposure was 45 % of the development period. At the end of each exposure, recirculation pumps were turned off, stacks allowed to drain, and clean hatchery water flow-through (7.6 L/min) was restored.

2.3. Embryo sampling

To ensure that embryos were not disturbed during the sensitive stage of gastrulation, sampling did not begin until 22 dpf (Dec 15). Embryos were then sampled weekly for seven weeks. For each sampling event, one cup was removed from each of three trays for each treatment (stack). Cups were transported in Salmon Ringer solution (3.2 mM $CaCl_2$, 2.4 mM $NaHCO_3$, 3.8 mM KCl, 135 mM CaCl) into the adjacent indoor laboratory where each cup was photographed, and all embryos enumerated and scored (hatched or unhatched, live or dead). Ten live embryos were randomly selected for cardiovascular and morphometric analysis from each of the three replicate cups and manually dechorionated. The degree of physical manipulation necessary to remove the thick chorion of salmon caused frequent cardiovascular defects, rendering ensuing cardiovascular metrics unreliable. As a result, morphometrics analysis included only embryo length and eye area.

Dechorionated embryos were embedded in 3 % methylcellulose, with their left side exposed, and assessed for developmental stage (Velsen, 1980; Danner, 2003; Boyd et al., 2010) using a stereomicroscope (Nikon SMZ-800, Nikon Corporation). Each embryo was photographed for later analysis of morphometrics. The first embryo from each replicate group of ten was placed in a 2.0-mL microcentrifuge tube (safe-lock Eppendorf™) after imaging, frozen in liquid nitrogen, and stored at −80 °C for quantitative analysis of *cyp1a* and *nppb* expression.

2.4. Quantitative gene expression (RT-qPCR)

The relative expression patterns of the target genes *cyp1a* and *nppb* were calculated using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) following established guidelines (Bustin et al., 2009; Bustin and Nolan, 2017). RNA was extracted from embryos using a Zymo Direct-zol™ RNA purification kit (Zymo Research, Irvine, California, US). Briefly, embryos were homogenized in TRIzol® (Life Technologies, Carlsbad, California, US) with a 5 mm stainless steel bead (Qiagen, Venlo, The Netherlands) and TissueLyser II (Qiagen). The sample fraction relative to TRIzol was <5 % by volume (v/v). Lysed samples were phase separated with 10 % v/v of 1-Bromo-3-chloropropane (BCP). A complementary DNA (cDNA) was synthesized from each RNA molecule using reverse transcriptase (RT) from a Superscript IV kit (ThermoFisher Scientific, Waltham, Massachusetts, US) and oligo dt(20) primers (IDT, Integrated DNA Technologies, Inc., San Diego, California, US) to initiate the RT reaction from the 3′ poly-A tail of each

messenger RNA. After synthesis, the cDNA was diluted and stored in Invitrogen yeast tRNA (ThermoFisher Scientific) at 1 μL of cDNA reaction to 100 μL of 0.1 mg/mL tRNA (1:100).

RT-PCR assays were performed on an ABI Viia™ 7 Real-Time PCR system with Fast SYBR (ThermoFisher Scientific), using 10 μL of buffer with 10 ng of cDNA per reaction. All primer sets were designed from a consensus sequence of orthologous genes from the following species: *O. kisutch*, *O. mykiss*, *O. gorbuscha*, *O. nerka*, and *O. tshawytscha*, and are applicable for use in all of these species. Amplicons produced by each primer set were validated for a single product via melt curve and gel purification and sequenced via Sanger sequencing to confirm gene identification. Primer efficiency was tested by applying primer sets to coho cDNA pooled across each treatment (not individual samples). Standard curves with acceptable slopes were produced (Nolan et al., 2006).

The relative abundance and fold-change of *cyp1a* and *nppb* were calculated from conventional Ct (cycle threshold) values after normalization to five ubiquitously expressed candidate reference genes (*rxrba*, *spop*, *ef1a*, *mtm1*, and *wdtc1*; Table S2). The reference genes were selected from a consensus of algorithms based on correlation (BestKeeper; Pfaffl et al., 2004), ranks (GeNorm; Vandesompele et al., 2002), and ratios (NormFinder; Andersen et al., 2004) using RefFinder (Xie et al., 2012). The consensus-based approach showed that expression levels of *rxrba*, *spop*, and *ef1a* remained constant across all developmental samples, irrespective of experimental treatment. Normalized expression levels (ΔCt) for each sample were then calculated by subtracting the Ct value for each target gene by the geomean of the three selected reference genes per sample [Ct(target gene) – Ct(geomean ref gene) = ΔCt]. Relative expression levels (ΔΔCt) were normalized to the mean ΔCt for *cyp1a* and *nppb* across all control samples [ΔCt(target) – ΔCt(controls) = ΔΔCt]. The normalized ΔΔCt data were expressed graphically as 2^-ΔΔCt representing fold-change from controls (Schmittgen and Livak, 2008).

2.5. Water chemistry

On the first day of each of the 15 discrete stormwater exposures, water from each treatment was sampled for analysis of dissolved metals (As, Cd, Cr, Cu, Ni, Pb, Zn), dissolved organic carbon (DOC), nutrients (ortho-phosphate, nitrite+nitrate (NO_X)), total suspended solids (TSS), fecal coliform (FC) and polycyclic aromatic hydrocarbons (PAHs). Samples were collected in a stainless-steel cup from each sump just prior to beginning recirculation and dispensed into containers provided by Am-Test Laboratories (Kirkland, WA). Samples were kept on ice until delivery to the laboratory for analysis within 24 h. Additional water samples for analysis of polycyclic aromatic hydrocarbons (PAHs) were collected in amber glass containers to which 10 % methylene chloride was added as a preservative following Sloan et al. (2006). Concentrations of 19 parent and 23 alkylated PAHs (Table S3) were analyzed by GC–MS at the National Oceanic and Atmospheric Administration (NOAA) Northwest Fisheries Science Center (Seattle, WA) following established protocols (Sloan et al., 2014).

2.6. Data treatment and statistical analyses

All statistical analyses were conducted in R programming language with RStudio (2020), using $\alpha = 0.05$ to determine statistical significance unless otherwise noted. Programming code is presented in square brackets.

2.6.1. Water quality

Undetected analytes were assigned a value of ½ the detection limit, except for individual PAH congeners (assigned a value of zero). The contaminant removal performance of the bioretention system was calculated as the concentration of measured analytes in filtered effluent (F100) relative to their concentration in untreated influent runoff (R100), or C_{EFF}/C_{INF}. Values >1 indicated net export from bioretention whereas a value <1 indicated net retention by bioretention. The percent of the 15 storm events for which there was net retention was calculated as well as the median of the ratio C_{EFF}/C_{INF}. Finally, for each analyte, effluent concentration was regressed on influent concentration [$\log_{10}(-C_{EFF}) \sim \log_{10}C_{INF}$] to determine whether influent concentration played a role in the degree of treatment. Treatments with non-detects (either inflow or outflow) were not analyzed.

2.6.2. Embryo thermal experience and development

Water temperature is an important determinant of developmental rate in fish. Thermal experience of the embryos was analyzed using a linear mixed effects model (*lmer* function in *lmerTEST* package) with average daily temperature as the dependent variable, treatment (Control, R50, R100, F100) as a fixed factor, and exposure condition (flow-through or recirculating) as a random factor: [Temp ~ Treatment + (1|Exposure)]. A dependency of temperature on exposure condition was explored by Student's *t*-test (*t.test* function): [Temp ~ Exposure]. Developmental stage across sampling periods was assessed by linear mixed model with ATU and tray position as random factors: [StageNum ~ Treatment + (1|ATU) + (1|Tray)].

2.6.3. Embryo survival

Survival of fertilized embryos was assessed across sampling days using ANOVA with proportion alive as the dependent variable and treatment and sampling date (dpf) as fixed factors: [Survival ~ Treatment*dpf]. Due to a significant interaction between treatment and date, treatment effects were interpreted for each date by Tukey HSD post-hoc analysis.

2.6.4. Embryo morphometrics

For each weekly sampling event, values for each individual were normalized to the average value for controls on that date, to account for allometric growth. Across the study as a whole, the effect of each exposure treatment was assessed relative to controls by a linear mixed model, with treatment as a fixed factor, and ATU and tray position as random factors: [Morphometric ~ Treatment + (1|ATU) + (1|Tray)]. To assess the outcome at the end of embryo development, a linear mixed effect model was used to determine the effect of treatment for just the final sampling day (64 dpf), with tray position as a random factor: [Morphometric ~ Treatment + (1|Tray)], followed by a Tukey HSD post-hoc analysis.

2.6.5. Quantification of biomarker mRNAs

Expression levels for *cyp1a* and *nppb* were analyzed for embryos exposed to runoff before and after bioretention-treatment (R100 and F100), relative to controls, using both a linear mixed effects model (*lmer*) and ANOVA (*aov*). The *lmer* model accounted for differences in transcription due to sampling date (dpf), to determine if there was an overall effect of exposure on gene induction as ΔΔCt: [ΔΔCt ~ Treatment + (1|dpf)]. The ΔΔCt data for *cyp1a* was log-transformed to meet normality. The two-way ANOVA used treatment and sampling date (dpf) as factors to assess whether there were differences in transcription related to sampling day: [ΔΔCt ~ Treatment*dpf]. Subsequently, treatment effects were assessed for each sampling day with an ANOVA followed by Tukey HSD because of a significant interaction between treatment and dpf (described in Results Section 3.5 Upregulation of molecular biomarkers for contaminant exposure and cardiac stress). To account for the numerous resulting comparisons, a false discovery rate approach (Narum, 2006) was used when assessing differences among treatments. The adjusted critical values were $p = 0.034$ for *cyp1a* and $p = 0.046$ for *nppb*. The high variability among *nppb* transcription in R100 on the final sampling day (COV = 75 %) suggested a difference in individual response. To further explore an effect of treatment for R100, the three individual samples from Day 64 were compared with controls using a Z-test.

3. Results

3.1. Urban runoff water quality improved by pre-treatment with soil column infiltration

Collected runoff was chemically similar to runoff from the same source reported in previous studies (McIntyre et al., 2015; Spromberg et al., 2016). Bioretention was effective at reducing concentrations of most contaminants of concern (Table 1); 50–100 % of samples showed net removal of bacteria, solids, ammonia, most dissolved metals, and total PAHs in runoff. The highest rates of removal were for PAHs, with concentrations in effluent at just 3 % of influent concentration (Table 1). Removal of dissolved As, Ni, DOC, Mg, and nutrients was less efficient (7–33 % of samples); bioretention media appeared to be a source of these pollutants into treated runoff, as indicated by ratios of effluent to influent concentration (C_{EFF}/C_{INF}) that were predominantly >1 (Table 1). For four parameters, effluent concentration was correlated with influent concentration, suggesting that the amount of treatment depended on influent concentration (Fig. 1). For fecal coliform bacteria (FC), influent and effluent concentrations were positively correlated, indicating relatively less treatment of bacteria when influent concentrations were higher. Despite this, there was a net reduction in FC concentration for 14/15 events (Table 1), with median effluent concentration 37 % of the influent concentration. In contrast, DOC, As, and ortho-P concentrations in effluent concentration were negatively correlated with influent concentration, indicating relatively more retention at higher influent concentrations (Fig. 1, Table S4).

3.2. Stormwater exposures did not cause developmental delay in embryonic coho

Water temperature was not significantly different among treatments, accounting for exposure condition ($F(3,267) = 0.0359$, $p = 0.991$), despite a 2 % difference in accumulated thermal units (ATU) for embryos between treatments (Table S5). There was a significant difference in temperature among exposure conditions ($t(57) = 6.13$, $p < 0.001$); exposure waters were cooler during recirculating exposures than during flow-through conditions (Table S5). Embryos were well into organogenesis (Stage >18) at the time of first sampling (22 dpf; Table S6). Accounting for ATUs and tray position across the study period, treatment did not have an effect on developmental stage ($F(3,47) < 0.001$, $p = 1.000$).

3.3. High rates of mortality among runoff-exposed alevins at hatch

Survival in the control treatment remained high (>90 %) for most of the experiment duration (Fig. 2B). The effect of treatment on survival was highly dependent on sampling date (interaction: $F(3,73) = 7.824$, $p < 0.001$). For the first six sampling events, survival in runoff treatments was not different from controls (Tukey post-hoc: $p = 0.248$–0.883). On the final sampling date, embryos were mostly hatched (Fig. 2A), and survival was low in R50 (29 %) and R100 (13 %) compared with controls (98 %) (Tukey post-hoc: all $p < 0.001$). In contrast, embryo survival in filtered runoff (F100) was similar to controls (96 %) (Tukey post-hoc: $p = 0.993$). Importantly, most embryos hatched between the penultimate and final sampling events (Fig. 2A), an interval that included the final two exposures. Consequently, many coho at the final point of data collection had recently hatched directly into unfiltered or filtered runoff.

3.4. Surviving alevins showed reduced body length and eye size

Treatment affected embryo length and eye area (Length $F(3,24) = 4.93$, $p = 0.008$; Eye $F(3,24) = 6.74$, $p = 0.002$). Across the study as a whole, embryos were significantly shorter with smaller eyes in the R100 treatment compared to controls that did not receive runoff (Fixed Effects: p(length) = 0.006, p(eye) = 0.014). Bioretention treatment (F100) did not prevent all impairments (Fig. 3); embryos exposed to treated water had smaller eyes ($p = 0.004$) relative to controls for the study as a whole. Differences between treatments were most pronounced in surviving alevin (hatched) at the end of the study. At 64 dpf, surviving alevin exposed to R100 were 13 % shorter with 16 % smaller eyes than controls (both Tukey HSD: $p < 0.001$). Deficiencies were less pronounced in embryos exposed to filtered runoff (vs controls, Tukey HSD: $p \leq 0.001$), though not completely prevented (Fig. 3). F100 alevin were only 6 % shorter and with eyes 11 % smaller than controls, a statistically significant improvement for length (vs. runoff Tukey HSD: $p < 0.001$).

Table 1
Water quality parameters measured across all episodic events for well water (control), 100 % runoff (R100), and 100 % runoff filtered through bioretention (F100). Values are median (min-max). % $C_{EFF}/C_{INF} < 1$ is the percent of events with net removal; C_{EFF} = effluent (filtered) concentration; C_{INF} = influent (runoff) concentration. DL = Detection Limit.

Type	Parameter	Units	DL	Control	Runoff	Filtered	% $C_{EFF}/C_{INF} < 1$	*MEDIAN* C_{EFF}/C_{INF}
Bacteria	FC	CFU/100 mL	1	1 (<1–150)	400 (100–2800)	190 (29–650)	93 %	0.37
	E. coli	CFU/100 mL	1	1 (<1–120)	350 (100–2520)	171 (29–500)	93 %	0.38
Conventional	pH	std	0.1	7.0 (6.6–7.3)	6.9 (6.7–7.4)	7.0 (6.6–7.2)	33 %	1.0
	SSC	mg/L	0.2	<0 (<0–4)	160 (34–480)	43 (10–140)	73 %	0.51
	TSS	mg/L	1	<0.2 (<0.2–2)	170 (40–670)	36 (12–81)	80 %	0.34
	DOC	mg/L	0.5	2.4 (1.6–9.0)	4.7 (2.6–14)	21 (6.9–53)	20 %	4.9
	Alkalinity	mg/L $CaCO_3$	1	74 (58–82)	24 (14–50)	62 (18–140)	20 %	3.0
	Hardness	mg/L $CaCO_3$	0.05	89 (76–93)	38 (24–340)	68 (17–920)	27 %	1.7
	Ca	mg/L	0.05	16 (14–30)	12 (6.9–120)	17 (5.4–230)	33 %	1.2
	Mg	mg/L	0.01	12 (3.1–13)	1.9 (1.2–11)	6.4 (0.8–85)	13 %	3.3
	Ammonia	mg/L	0.01	0.04 (0.01–0.13)	0.81 (0.39–6.8)	0.04 (0.02–0.21)	100 %	0.04
Nutrients	NOx	mg/L	0.025	1.58 (0.72–3.2)	0.28 (0.13–2.5)	3.4 (0.01–19)	7 %	7.8
	Ortho-P	mg/L	0.005	0.051 (<0.005–0.1)	<0.005 (<0.005–0.3)	0.53 (<0.005–1.1)	0 %	126
Dissolved Metals	As	µg/L	0.02	1.3 (0.52–1.7)	0.70 (0.42–3.2)	2.7 (1.1–7.2)	13 %	2.3
	Cd	µg/L	0.025	<0.025	0.07 (<0.03–1.4)	0.05 (0.01–0.12)	67 %	0.74
	Cr	µg/L	0.05	0.92 (0.33–1.6)	1.4 (0.73–3.2)	1.4 (0.20–2.5)	67 %	0.79
	Cu	µg/L	0.1	0.8 (0.3–1.8)	9.4 (4.6–61)	12 (3.6–25)	53 %	0.95
	Pb	µg/L	0.05	<0.05	0.09 (<0.05–0.26)	0.19 (<0.05–1.02)	40 %	2.8
	Ni	µg/L	0.05	6.0 (1.4–14)	1.1 (0.64–8.7)	3.7 (0.79–8.1)	13 %	2.5
	Zn	µg/L	0.5	<0.5 (<0.5–2.9)	67 (35–294)	8.9 (1.7–29)	100 %	0.17
Aromatics	TPAH	µg/L	na	0.1 (0–1.2)	10.1 (2.5–33.8)	0.2 (0.1–1.6)	100 %	0.03

FC = fecal coliform; SSC = suspended sediment concentration; TSS = total suspended solids; DOC = dissolved organic carbon; NOx = nitrate+nitrite; TPAH = sum of all polycyclic aromatic hydrocarbons.

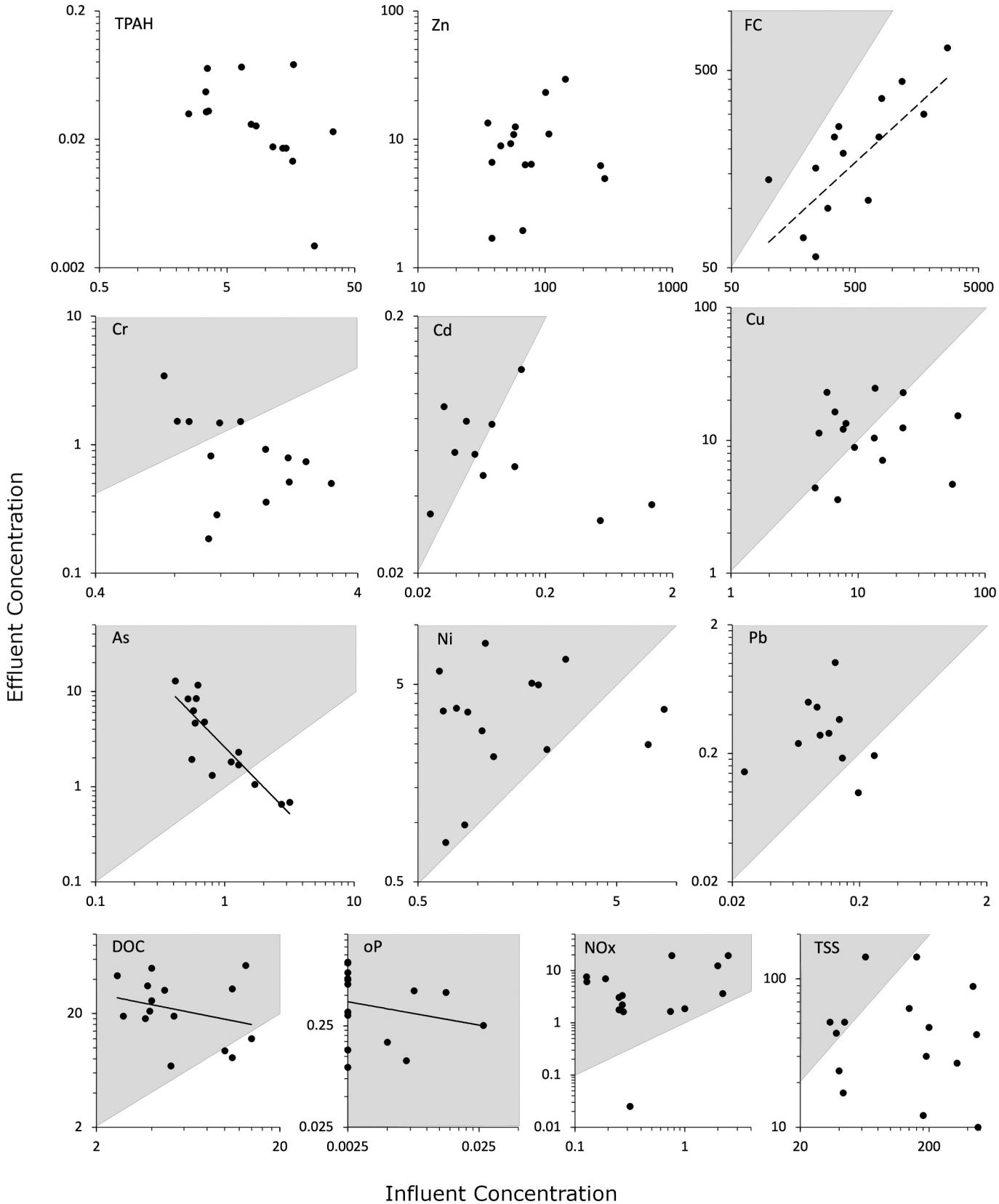

Fig. 1. Contaminant concentrations in influent runoff (C_{INF}) and treated effluent (C_{EFF}) waters from the bioretention system. Shaded polygons enclose events for which there was net export of the analyte in effluent water from the bioretention system. Significant linear regressions of effluent concentrations on influent concentrations are shown as solid ($p < 0.01$) or dashed ($p < 0.1$) lines. Regression statistics are shown in Table S4. Positive correlations indicate relatively less treatment at higher influent concentration (FC) whereas negative correlations indicate relatively more retention at higher influent concentration (DOC, As, oP). Units are mg/L for total suspended solids (TSS), ortho-P (oP), dissolved organic carbon (DOC), nitrate+nitrites (NOx), CFU/100 mL for fecal coliform (CF), or μg/L for metals and PAHs.

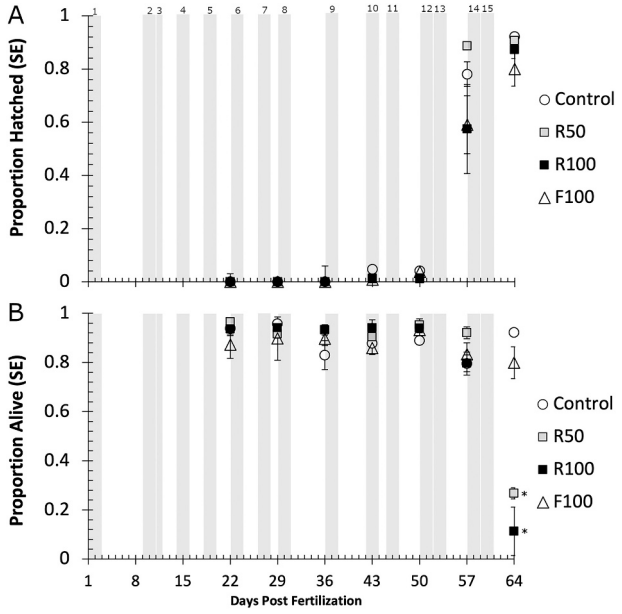

Fig. 2. Average A) hatching and B) survival for coho embryos across weekly sampling events. Shaded vertical bars indicate recirculating exposure periods of 24–48 h, numbered sequentially. The x-axis applies to both panels. Error bars are one standard error of the mean of n = 3 replicates containing 68–112 ($\bar{x}$-bar = 90) embryos. Asterisks indicate survival significantly different ($p < 0.05$) from control on Day 64.

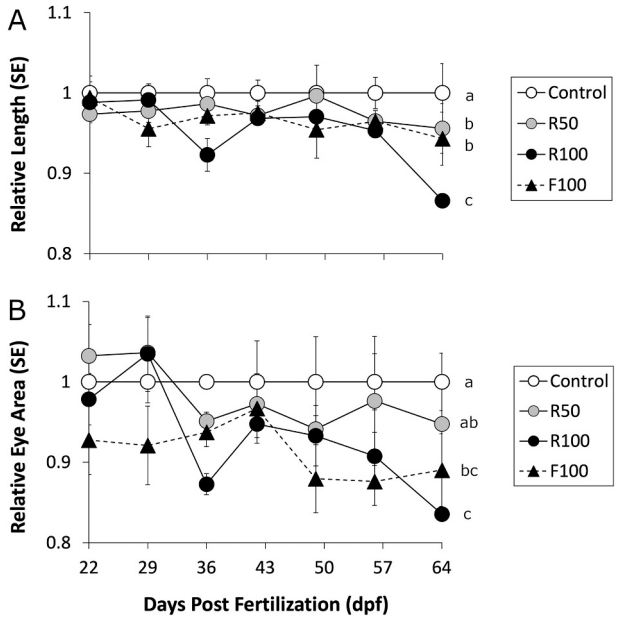

Fig. 3. Average A) embryo length and B) eye area relative to same-day controls for each treatment across the study (clean water control, 50 % or 100 % runoff (R), or filtered 100 % runoff (F100)). Error bars are one standard error of the mean for the 10 embryos imaged from each of three replicates per treatment. Letters indicate statistical significance for treatments at 64 dpf. For each metric, treatments that share a letter are not statistically different (Tukey's post-hoc).

3.5. Upregulation of molecular biomarkers for contaminant exposure and cardiac stress

Transcripts for *cyp1a* and *nppb* were assessed in three embryos from each of the control, 100 % runoff, and filtered runoff treatments (Fig. 4). The linear mixed effects model detected a study-wide effect on

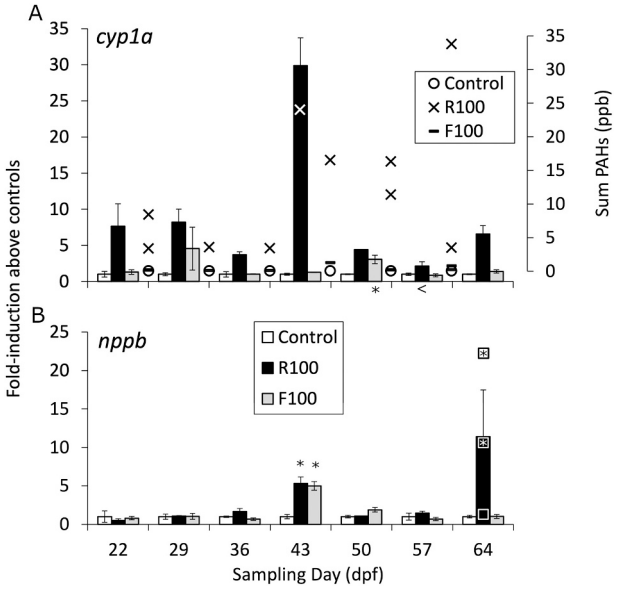

Fig. 4. Gene expression for each sampling day, showing the mean (n = 3) and standard error of the mean for clean water controls, episodic runoff exposure (R100), and episodic exposure to filtered runoff (F100). One sampling day (Day 43) was concurrent with an exposure. A) Induction of *cyp1a* (bars, legend in Panel B), with sum of PAH concentrations in episodic exposure waters for each treatment (symbols, legend in Panel A). There were one to two exposures between most sampling dates. The symbol '<' indicates the only day that *cyp1a* was not significantly induced in R100 above controls. The asterisk indicates the only unique day on which F100 was different from controls. B) Induction of *nppb*. On Day 64, *nppb* values for the three unique samples for the R100 treatment are additionally shown, as squares. Asterisks indicate samples (or treatments on Day 43) significantly induced above controls.

transcripts of *cyp1a* ($F(2,48) = 49.1$; $p < 0.001$); with R100 > F100 > Control (Tukey's comparison: $p \leq 0.002$), suggesting that the overall increase in *cyp1a* transcripts caused by runoff was not completely prevented by filtration (Fig. 4A). There was also a study-wide effect on induction of *nppb* ($F(2,50) = 4.68$; $p = 0.014$), attributed to R100 ($p = 0.011$) but not F100 ($p = 0.510$). Explicitly considering transcription on different sampling days, there was a significant interaction between treatment and sampling date for both *cyp1a* ($F(1,12) = 2.446$, $p = 0.018$) and *nppb* ($F(1,12) = 2.222$, $p = 0.031$) indicating that transcription was upregulated on some but not all dates for both genes. Transcription of *cyp1a* was upregulated on six of the seven sampling days (not Day 57) in embryos episodically exposed to runoff ($0.001 \leq p \leq 0.034$; Fig. 4A), with the highest value (30-fold upregulation) relative to controls on 43 dpf. This sampling date occurred during an exposure to runoff with one of the highest measured concentration of aromatic hydrocarbons (TPAH = 24.1 μg/L; Table 1). On other dates, upregulation of *cyp1a* was 2–8-fold higher than controls. In contrast, embryos episodically exposed to filtered runoff did not show significant upregulation of *cyp1a* on any individual sampling date ($0.053 \leq p \leq 1.000$). Induction of the cardiac stress gene *nppb* was elevated 5-fold above controls for both runoff ($p = 0.008$) and filtered runoff ($p = 0.010$) on Day 43. On the last sampling date (64 dpf), *nppb* induction was on average 11-fold higher than controls (Fig. 4B). The response was highly significant for two of the three sampled individuals (Z-stats = −17.25, −22.6; $p < 0.001$).

4. Discussion

Untreated runoff from roads, parking lots, and similar hardscapes represents a geographically widespread, and growing threat to Pacific salmon and steelhead conservation. We have shown here that the earliest coho salmon life history stages (embryos and newly-hatched

alevins) are sensitive to chemical contaminants in stormwater. Together with an expanding body of evidence for stormwater-driven mortality in adult spawners (Scholz et al., 2011; Spromberg et al., 2016; McIntyre et al., 2018) and freshwater-resident juveniles (McIntyre et al., 2015; Chow et al., 2019; French et al., 2022), our findings demonstrate the importance of evaluating losses across the entire coho salmon life cycle. The embryolarval information gap is an obstacle to ongoing efforts to estimate aggregate reductions in lifetime survival and reproductive success (i.e., individual fitness) for wild coho populations at risk from ongoing urbanization in the western U.S. We also show here that simple and inexpensive bioinfiltration substantively reduces the bioavailability and corresponding toxicity of contaminants in conventional urban runoff to coho salmon early life stages, in accordance with positive green infrastructure outcomes previously reported for older juveniles (McIntyre et al., 2015) and adults (Spromberg et al., 2016).

4.1. Sublethal toxicity during embryogenesis is consistent with PAH exposure

Previous studies of fish organogenesis, based on continuous exposures to petrogenic PAH mixtures containing ∑PAH levels in the ranges of stormwater samples used here, would be expected to impact cardiac function and morphology. Threshold concentrations in water and tissue are low for these impacts. For example, latent impacts on heart development were observed in pink salmon embryos exposed to crude oil-derived mixtures as low as ∑PAH 9.8 μg/L (222 ng/g tissue) and herring embryos exposed to stormwater with ∑PAH 7.5 μg/L (71 ng/g tissue) (Incardona et al., 2015; Harding et al., 2020). Coho salmon embryos in the current study were exposed to stormwater with ∑PAH ranging 2.5–34 μg/L. While we were unable to assess direct impacts on cardiac function and development due to technical constraints, the episodic exposures resulted in upregulation of both *cyp1a*, a biomarker of exposure to PAHs and other aromatic organic contaminants (Uno et al., 2012), and *nppb*, a biomarker of cardiac stress, consistent with cardiac injury from stormwater exposure documented in other species (McIntyre et al., 2016a, 2016b; Harding et al., 2020). Similar to our findings here, smaller eyes and shorter larvae are commonly observed following exposures to environmental mixtures enriched in PAHs (Hose et al., 1996; Colavecchia et al., 2007; McIntyre et al., 2014; McIntyre et al., 2016a, 2016b; Magnuson et al., 2020) as well as single PAH compounds (particularly phenanthrene; Incardona et al., 2004).

Whereas crude oil containing higher PAH concentrations reduced eye development and body size of Atlantic haddock embryos secondary to reduced cardiac function (e.g., Sorhus et al., 2021), oil-derived chemical mixtures containing lower PAH concentrations did not impact length or eye size of herring embryos (Incardona et al., 2021). In contrast, exposure to stormwater runoff reduced both growth metrics in herring, at similarly low PAH concentrations (Harding et al., 2020). This difference in response is likely related to compositional differences between the two environmental mixtures. For example, stormwater is relatively enriched in higher molecular weight PAHs that have not been studied extensively in salmonids or herring, in addition to other aromatic contaminants that could contribute to these impacts. This includes 6PPD-quinone and other tire-derived chemicals, which were recently shown to reduce eye size in embryolarval zebrafish (Varshney et al., 2022; Chang et al., 2023). Our observed effects on eye and body size may also be an indirect consequence of distributed physiological demands associated with detoxifying and eliminating the much more complicated stormwater mixture. Specifically, *cyp1a* induction is likely indicative of an upregulation of protective metabolic pathways (Weinrauch et al., 2021) in the skin and other tissues, which would invariably divert maternally-derived energy resources away from growth.

In contrast to previous live imaging of translucent fish embryos, technical constraints related to the tough chorion of coho salmon prevented a detailed analysis of heart chamber orientation, rhythm, and output during early development. Consequently, determinations of early-onset cardiotoxicity will necessarily await future studies that circumvent the challenges posed by the salmon egg membrane. However, potential latent impacts of embryonic exposure to cardiotoxicants should be accessible in coho that survive post-hatch (e.g., Incardona et al., 2015; Gardner et al., 2019). Consequently, future stormwater toxicity assessments should focus on the fine structure of the post-hatch ventricle and corresponding reductions in cardiac output at later, free-swimming life stages.

4.2. Acute lethality after hatch is consistent with 6PPD-quinone toxicity

Despite high survival prior to hatching, coho salmon alevin (post-hatch) experienced high rates of mortality following exposure to urban road runoff. Studies exposing salmonids to various contaminants supports increased susceptibility post-hatch versus during embryo stages (Vanleeuwen et al., 1985; Vuorinen and Vuorinen, 1987; Viant et al., 2006), which could reflect toxicokinetics related to the chorion, or the ontogeny of molecular/cellular targets. This sensitivity timeline agrees with previous studies demonstrating acute mortality of coho juveniles (McIntyre et al., 2014; Chow et al., 2019) and returning adults (Scholz et al., 2011; Spromberg et al., 2016; McIntyre et al., 2018) exposed to runoff from this same source and subsequently attributed to 6PPD-quinone – a highly toxic anti-degradant from tires (Tian et al., 2022) discovered after our study was conducted. That the observed alevin mortality was driven by 6PPD-quinone is further supported by recent single-chemical exposure studies that show 6PPD-quinone is acutely lethal to early life stages of coho salmon (Lo et al., 2023) at concentrations consistently measured in runoff from the source used in this study (Tian et al., 2022). Furthermore, significant mortality was recently observed for coho salmon exposed to 6PPD-quinone for a few hours during hatching (Greer et al., 2023). The available evidence therefore suggests 6PPD-quinone is the primary driver of the salmonid urban runoff mortality syndrome across all free-swimming life stages (alevins, juveniles, and adults).

4.3. Implications for environmental monitoring in salmon spawning habitats

The present study is an outgrowth of two decades of applied stormwater research designed to understand how deteriorating water quality in urbanizing watersheds might compromise the viability of wild Pacific salmon and steelhead (McCarthy et al., 2008). An underlying goal is to improve and expand the toolbox for *in situ* assessments of salmon environmental health in habitats impacted by runoff, now and in the future. Monitoring is essential to adaptive management. For example, in the late 1990s, dying adult coho salmon in restored urban streams set the research community on a search for causal and potentially emerging contaminants underlying observed fish kills, which led eventually to the discovery of 6PPD-quinone in roadway runoff. Field surveys remain the most direct and accurate approach to quantifying the urban mortality syndrome, although the phenomenon is generally easier to document for large adult carcasses relative to dead juveniles. Nevertheless, field surveys have yielded watershed-by-watershed mortality estimates for coho salmon (Scholz et al., 2011; Feist et al., 2017), thereby informing extinction risk projections (Spromberg and Scholz, 2011), and vulnerability mapping for wild coho salmon across the entire Puget Sound Basin (Peter et al., 2022; Ettinger et al., 2021; Feist et al., 2011, 2017).

Less well understood are runoff-driven toxicity pathways that cause indirect mortality from sublethal stress, often in combination with other habitat stressors. Sublethal risks remain a major management concern for ESA-listed species, and extend beyond coho salmon to other Pacific salmonids, including species for which 6PPD-quinone does not induce mortality (e.g., sockeye and chum). In the context of generalized contaminant exposure, we anticipated an upregulation of *cyp1a*, a well-established and highly sensitive biomarker that is responsive to many aromatic contaminants including PAHs, PCBs, and potentially 6PPD-

quinone. Despite this biomarker promiscuity, the relationship between *cyp1a* induction and delayed mortality has been studied extensively in salmon embryos exposed to PAHs in crude oil following the 1989 Exxon Valdez disaster. A main conclusion drawn from those earlier studies is that any significant measurable upregulation of *cyp1a* in developing salmon is an indicator of metabolic stress, and corresponds to significant reductions in marine survival at later life stages (Carls et al., 2005). Thus, as previously discussed in the context of green infrastructure science (McIntyre et al., 2016a), *cyp1a* expression should serve as a useful indicator of chemical exposure and sublethal injury, both for baseline/trends monitoring in urban waterways and for assessing the biological effectiveness of pollution control strategies (Lapointe et al., 2022).

4.4. Protective benefits of soil infiltration extend to coho early life stages

Bioinfiltration of roadway runoff prevented acute mortality of coho alevin, similar to studies with older coho juveniles (McIntyre et al., 2015) and adults (Spromberg et al., 2016). This is consistent with bioretention media retaining the majority of applied 6PPD-quinone during stormwater treatment (Rodgers et al., 2023). Bioretention treatment also prevented most induction of the cardiac injury biomarker *nppb* and all induction of the aromatic hydrocarbon exposure biomarker *cyp1a*. The lack of response of exposure and cardiac injury biomarkers in filtered water agrees with effective elimination (>93 % reduction) of PAHs by bioretention treatment, similar to previous studies of PAH-containing stormwater treated by bioretention (McIntyre et al., 2016a, 2016b). Filtration through bioretention did not prevent all effects for coho embryos; the smaller length and eye area noted for embryos exposed to runoff were only partially prevented by filtering stormwater. This agrees with prior assessments of bioretention treatment that showed a still significant, but reduced, impact on eye size for zebrafish embryos exposed to filtered roadway runoff (McIntyre et al., 2014). Bioretention filtration resulted in clear benefits to water quality and drastic improvement in the survival of coho alevin, although studies are needed to assess whether there are long-term impacts from developmental defects still present after bioretention filtration.

4.5. Implications for assessing risks to vulnerable coho populations

Previous population modeling, albeit limited in scope, strongly suggests that wild coho cannot withstand the high rates of observed mortality in Puget Sound watersheds. Adult spawner mortality rates can be expected to drive local extinctions on a timescale of a few years to a few decades (Spromberg and Scholz, 2011). Given that unfiltered stormwater has subsequently been shown to be lethal also to juvenile coho (McIntyre et al., 2015; Chow et al., 2019), and alevins (present study), the current modeling is likely to substantively underestimate actual extinction risk, given losses to these other freshwater life stages. For example, in the present study, we observed an increase in mortality at hatching (64 dpf) from ~10 % in clean water controls to ~90 % following exposure to stormwater. Moreover, the earlier population decline projections did not consider sublethal toxicity, or interactions between stormwater contaminants and other stressors. At the same time, GIS-based vulnerability mapping has shown that large geographical segments of lowland Puget Sound are already compromised by poor water quality (Peter et al., 2022; Ettinger et al., 2021; Feist et al., 2017), a trend that is accelerating with increasing development and corresponding expansion of the regional transportation grid. Future salmon recovery is premised on an adequate supply of cool, clean water across watershed scales. To date, with a few notable exceptions (Spromberg and Scholz, 2011; Baldwin et al., 2009; Lundin et al., 2019), the clean water requirements for wild salmon populations have not been assessed using models or related decision-support tools, particularly where exposures to complex environmental mixtures of toxics are the norm. As evidenced by our current findings, stormwater toxicity to salmonid early life stages should be integral to future life-cycle modeling in large western river basins where people and salmon coexist, now and in the future.

CRediT authorship contribution statement

Jenifer McIntyre: Conceptualization, Funding acquisition, Project administration, Methodology, Investigation, Data analysis, Writing – original draft preparation. Julann Spromberg: Conceptualization, Methodology, Investigation, Writing – Reviewing and Editing. James Cameron: Methodology, Investigation, Data analysis, Writing – original draft preparation. John Incardona: Conceptualization, Methodology, Investigation, Writing – Reviewing and Editing. Jay Davis: Conceptualization, Funding acquisition, Project administration, Writing – Reviewing and Editing. Nat Scholz: Conceptualization, Funding acquisition, Resources, Writing – Reviewing and Editing.

Declaration of competing interest

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.165759.

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