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# Zebrafish and clean water technology: Assessing soil bioretention as a protective treatment for toxic urban runoff

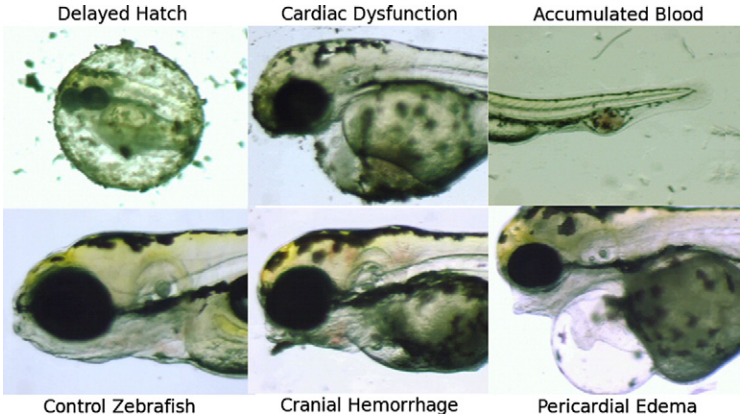
J.K. McIntyre [a,*], J.W. Davis [b], J.P. Incardona [c], J.D. Stark [a], B.F. Anulacion [c], N.L. Scholz [c]

[a] Washington State University Puyallup Research & Extension Center, 2606 W Pioneer Ave, Puyallup, WA 98371, USA
[b] U.S. Fish & Wildlife Service Washington Fish & Wildlife Office, 510 Desmond Dr. SE, Lacey, WA 98503, USA
[c] NOAA–NMFS Northwest Science Center, 2725 Montlake Blvd E, Seattle, WA 98112, USA

## HIGHLIGHTS

- Green stormwater infrastructure (GSI) is used to reduce and treat urban runoff.
- Biological effectiveness of GSI technologies like soil bioretention is not known.
- Zebrafish embryos acutely exposed to urban runoff developed a suite of symptoms.
- Included developmental delay, microphthalmia, and cardiac dysfunction
- Bioretention treatment of runoff prevented mortality and most sublethal toxicity.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Urban stormwater contains a complex mixture of contaminants that can be acutely toxic to aquatic biota. Green stormwater infrastructure (GSI) is a set of evolving technologies intended to reduce impacts on natural systems by slowing and filtering runoff. The extent to which GSI methods work as intended is usually assessed in terms of water quantity (hydrology) and quality (chemistry). Biological indicators of GSI effectiveness have received less attention, despite an overarching goal of protecting the health of aquatic species. Here we use the zebrafish (*Danio rerio*) experimental model to evaluate bioinfiltration as a relatively inexpensive technology for treating runoff from an urban highway with dense motor vehicle traffic. Zebrafish embryos exposed to untreated runoff (48–96 h; six storm events) displayed an array of developmental abnormalities, including delayed hatching, reduced growth, pericardial edema, microphthalmia (small eyes), and reduced swim bladder inflation. Three of the six storms were acutely lethal, and sublethal toxicity was evident across all storms, even when stormwater was diluted by as much as 95% in clean water. As anticipated from exposure to cardiotoxic polycyclic aromatic hydrocarbons (PAHs), untreated runoff also caused heart failure, as indicated by circulatory stasis, pericardial edema, and looping defects. Bioretention treatment dramatically improved stormwater quality and reversed nearly all forms of developmental toxicity. The zebrafish model therefore provides a versatile experimental platform for rapidly assessing GSI effectiveness.

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* Corresponding author at: 2606 W Pioneer, Puyallup, WA 98371, USA. Tel.: +1 206 369 1832.
*E-mail address:* jen.mcintyre@wsu.edu (J.K. McIntyre).

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## 1. Introduction

Stormwater in urban areas contains a complex mixture of contaminants that originate from atmospheric deposition and runoff from roofs, roads, parking lots, and other impervious surfaces. Automobiles are a predominant source of pollution, and runoff contains elevated levels of metals and petroleum hydrocarbons (Sansalone and Buchberger, 1997; Shinya et al., 2000; Stein et al., 2006) from exhaust and wearing of vehicle tires and brake pads (Aatmeeyata and Sharma, 2010; Rogge et al., 1993). In urban watersheds, stormwater is usually conveyed without treatment to the nearest water body, and non-point source pollution thus represents a widespread and important threat to ecological resiliency.

Chemical contaminants in road runoff, particularly during the so-called first flush phase, can be acutely toxic to model aquatic organisms commonly used for water quality bioassays (Dorchin and Shanas, 2013; Kayhanian et al., 2008; Marsalek et al., 1999; Mayer et al., 2011). In the wild, runoff from paved surfaces is also causing the widespread and recurring deaths of adult coho salmon that return each fall to spawn in urban creeks in the Pacific Northwest of the US (Feist et al., 2011; Scholz et al., 2011).

Green stormwater infrastructure (GSI), or low-impact development, is designed to mimic the hydrologic and filtration capacity of an undeveloped landscape. Soil bioretention, green roofs, and permeable pavements are all examples of GSI techniques (Ahiablame et al., 2012; Dietz, 2007). In urban and urbanizing areas of the USA, GSI is increasingly being used to improve water quality and prevent the types of biological decline that are characteristic of the urban stream syndrome (Walsh et al., 2005). However, there has been little research to date on GSI effectiveness, and the protective benefits for aquatic species and communities are not well understood.

Developing fish embryos are particularly vulnerable to the harmful effects of chemical contaminants and have long been a focus for toxicity screening (McKim, 1977). More recently, model species such as the zebrafish (*Danio rerio*) have provided an increasingly sophisticated experimental context for evaluating the developmental toxicity of individual chemical constituents in stormwater, including metals (Linbo et al., 2006) and polycyclic aromatic hydrocarbons (Incardona et al., 2004, 2006, 2011). However, real-world urban stormwater runoff contains a more complicated mixture of these and other compounds. The aggregate impacts of environmentally realistic mixtures on fish early life stages, before and after treatment with GSI, have not been investigated.

In the present study we exposed zebrafish embryos to urban runoff collected during six distinct storm events from a high traffic volume arterial in Seattle (Washington, USA). In addition to embryolarval survival (96 h), we assessed the effects of stormwater on the form and function of the heart, the integrity of the lateral line, and the normal development of the swim bladder and other structures. To determine whether contaminants in stormwater become less bioavailable upon freezing, we compared the toxicity of water samples that were fresh or previously frozen. Lastly, after characterizing the baseline toxicity of urban runoff, we used experimental soil columns (bioretention) to treat runoff from a single storm, to evaluate the extent to which this common GSI technique can improve water quality and reverse the suite of toxic responses observed across all storm events.

## 2. Methods

### 2.1. Highway runoff collection and storage

Stormwater was collected at the beginning of six distinct storms between August 2011 and September 2012, following dry periods of 5–50 d. Runoff was captured at the National Oceanic and Atmospheric Administration's Northwest Fisheries Science Center (NWFSC; Seattle, WA, USA) from downspouts draining an elevated urban highway with high traffic density (annual average daily traffic = 94,000 vehicles in 2011, 67,000 in 2012). A diverter (Rain Harvesting, AquaBarrel, Gaithersburg, MD, USA) collected the first flush into glass carboys (19 L for the Aug 2011–May 2012 events, 250 L for the Sep 2012 event). Coarse detritus was pre-filtered with a window screen to prevent clogging of the collection apparatus. Runoff from the Sep 2012 event was diluted to a final volume of 410 L with roof runoff collected at the Washington State University Puyallup Research and Extension Center (WSU-P; Puyallup, WA, USA). Dilution was necessary because the Sept 2012 rain event (0.3 mm) did not generate sufficient runoff for the volumes of untreated and treated runoff required in concurrent juvenile coho exposures. Runoff (150 mL) was frozen at −20 °C in amber glass bottles (250 mL) within hours of collection and thawed the day of toxicity testing.

### 2.2. Chemical analyses

Runoff was analyzed for conventional water chemistry parameters, dissolved and total metals, and a suite of PAHs. All samples were transported on ice to the analytical laboratory within 24 h of collection. Conventionals were analyzed by ARI Laboratories (Tukwila, WA, USA) using the US Environmental Protection Agency (EPA) methods. All alkalinity was in the form of bicarbonate. Metals (total and dissolved) were analyzed by Frontier™ Global Sciences (Woodinville, WA, USA) by inductively coupled plasma mass spectrometry (ICP-MS). Samples for PAH analysis were preserved with methylene chloride within 24-h of collection. Sixteen PAH compounds and their alkylated homologues (Table S1) were analyzed by gas chromatography/mass spectrophotometry (GC/MS) at the NWFSC following established NOAA protocols (Sloan et al., 2014). Embryo medium chemistry was previously characterized by Linbo et al. (2009).

For the bioretention test (Sep 2012), chemical changes in runoff between the pre-and post-cistern homogenization (see Section 2.7) were assessed by comparing the pre-cistern composite water sample with the triplicate samples collected after homogenization. Statistical differences in chemical concentrations among untreated (Runoff) and treated (No Plants, Plants) runoff were tested by univariate ANOVA with a Bonferroni post-hoc. All statistics were performed in SPSS v. 21 (IBM) with $\alpha = 0.05$.

### 2.3. Zebrafish early life stage exposures

Wild-type (AB) zebrafish were cultured and spawned at the NWFSC using previously described methods (Linbo, 2009). Groups of embryos ($n = 15$) were distributed at 2–4 h post-fertilization (hpf) into triplicate glass petri dishes (60 × 15 mm) and exposed to 10 mL highway runoff (exposed) or embryo rearing medium (controls). Test waters were brought to room temperature prior to an exposure over 1–2 h by placing glass bottles containing runoff (or embryo medium) in a tepid water bath (~25 °C). Remaining solutions were kept overnight at 4 °C and re-warmed the following day for solution renewal at 24 h. Embryo medium was synthetic freshwater made from 1 g of Instant Ocean Sea Salt® dissolved in 1 L of distilled water. Embryos and larvae were incubated at 28.5 °C until test termination at 48 or 96 h. Because exposure began at ≤4 hpf, exposure time (h) and age (hpf) are used interchangeably. In addition to assessing undiluted stormwater for each storm (100% runoff), a dilution series was evaluated using runoff from the Oct 2011 storm in separate 48 h exposures.

Solutions were renewed daily following visual examination for embryo survival, developmental stage, hatch rate, morphological or functional anomalies, and swim bladder inflation. At the end of the exposure interval, unhatched embryos were manually dechorionated, anesthetized in tricaine methane sulfonate (250 μg/L MS-222; Sigma), mounted in 2% methylcellulose, and photographed with a Fire-i 400 digital camera (Unibrain Inc., San Ramon, CA, USA) mounted on a Nikon SMZ800 stereomicroscope.

### 2.4. Survival and morphometry

Individual embryo standard length, eye size (ocular area), and pericardial area were determined from digital images. Pericardial area is a measure of fluid accumulation (edema) in the pericardial space, as an indicator of heart failure and other forms of cardiac dysregulation (Antkiewicz et al., 2005). Eye size is an indicator of craniofacial malformations and is related to cardiovascular defects (Incardona et al., 2004). Length and area measurements were performed using Image J software (http://rsbweb.nih.gov/ij/).

Zebrafish daily survival over 96 h in runoff or control water was assessed by a univariate General Linear Model (GLM) for each storm event. Sublethal endpoints (hatch rate at 48 h, swim bladder inflation at 96 h, length, pericardial area, and eye size) were analyzed by multivariate GLM with sublethal endpoints as dependent variables for each storm event (Aug 2011, Oct 2011, Jan 2012, Feb 2012, May 2012, Sep 2012) and time point (48 h and 96 h). For dilutions of Oct 2011 runoff, sublethal effects at 48 h were analyzed by multivariate GLM with a Dunnett post-hoc analysis. Average survival of control replicates was always ≥96% with low incidence of sublethal abnormalities.

### 2.5. Lateral line neurotoxicity

To screen for potential neurotoxicity, we examined mechanosensory neurons in the lateral line of larval zebrafish exposed to runoff from the Aug 2011, Oct 2011, and Jan 2012 storms. Fish at ages 72 or 96 hpf were exposed to runoff for 3 h, anesthetized, labeled with 0.05% DASPEI dye, and the number of fluorescently-labeled lateral line neurons in the O2 neuromast overlying the otic vesicle was counted as previously described (Linbo et al., 2009). Cells in this neuromast take up DASPEI beginning at 34 hpf (Raible and Kruse, 2000). An exposure duration of 3 h is more than sufficient to cause effects in the presence of neurotoxic contaminants (Linbo et al., 2006). Neuron counts were averaged for 17–33 fish per treatment per storm event. Significant differences between runoff-exposed and control treatments were assessed by independent *t*-test.

### 2.6. The toxicity of fresh vs. frozen runoff

We assessed the influence of storage on stormwater sample integrity by exposing zebrafish embryos to fresh and frozen runoff from the Oct 2011 storm event. At 96 hpf, length, pericardial area, eye area, and swim bladder inflation were measured. Whereas the trial with fresh runoff was initiated on the day of collection (Oct 20, 2011), the trial with stored runoff was initiated more than two months later (Jan 9, 2012). For each metric, averages of triplicates exposed to runoff were compared with the same for controls using a ratio of runoff/control values. This ratio for the frozen runoff test was compared with the same ratio for the fresh runoff test (frozen/fresh). Standard errors of the mean of the ratios were used to represent the variability among triplicates.

### 2.7. Bioretention treatment

Runoff from the Sep 2012 storm event was homogenized in a high density polyethylene (HDPE) cistern (1135-L) equipped with adductors and an outflow valve connected to a distribution pump. Grab samples were collected to measure changes in water chemistry due to contact with the cistern. After homogenization in the cistern, 102 L of untreated runoff was distributed to six glass of carboys (17 L each). The remaining runoff was distributed through Teflon tubing for treatment in 12 bioretention columns.

Bioretention column construction, conditioning, and plantings are described in Palmer et al. (2013). Briefly, for each column, a 121.7 cm cylindrical length of PVC (36.5 cm diameter) was attached to a 1.3 cm PVC baseplate (total volume 127 L) and filled with 61 cm of mixed bioretention soil medium (63.8 L) that was 60% sand, 15% compost, 15% shredded bark, and 10% drinking water treatment residuals (WTRs), by volume. WTRs are a byproduct of flocculation during the treatment of drinking water. The WTRs in this study were amorphous aluminum hydroxides from the Anacortes Water Treatment Plant at Mount Vernon, WA and were originally added to the bioretention soil medium for improved phosphorus control (Palmer et al., 2013). The soil medium was underlain with 30 cm (31.9 L) of drainage layer (3/4″ Seattle Type 26 sandy gravel), and overlain with a shallow ponding layer (15.9 L). Half of the columns were left barren while the other half were planted in December 2011 with *Carex flacca*, a deep-rooted sedge tolerant of wet and dry conditions. Columns were maintained in a greenhouse at 16–21 °C on a minimum 14:10 light:dark regime and watered to saturation and allowed to drain once per week.

Each column received an input of 22 L of untreated runoff over one hour, equivalent to a 5-mm rain event over 4.3 $m^2$. This corresponded to a treatment area 2.5% of the effective contributing area. After passing through each experimental column, the treated stormwater effluent was collected via slotted 3.80-cm diameter PVC pipes at the base of the drainage layer. Effluent from each column was collected over three hours into 19-L glass carboys placed on ice. Runoff from two carboys was combined in 35 L glass aquaria ($n = 3$ per treatment) for water sampling followed by toxicity testing with juvenile coho salmon (not reported here). Treatments included untreated runoff ('Runoff'), plus runoff treated with bioretention alone ('No Plants'), or bioretention with plants ('Plants'). Samples from replicate aquaria (50 mL/aquarium) were composited in amber glass jars (250 mL) and frozen (−20 °C) until zebrafish testing.

### 2.8. Atrial regurgitation

For experiments evaluating the effectiveness of bioretention as a GSI technique (Sep 2012 storm), atrial regurgitation was monitored as a measure of cardiac function. Specifically, the movement of blood into the atrium and in the peripheral vasculature of zebrafish larvae was observed at 48 hpf. Larvae were scored as 'normal' if no backflow of blood through the atrial valve was visible, 'regurgitation' if mild to severe regurgitation was evident, and 'circulatory stasis' if there was no movement of blood through peripheral vessels. The effect of treatment on these three scoring categories was tested using a multivariate GLM with a Dunnett post-hoc test.

## 3. Results

### 3.1. Variation in the chemical profile of highway runoff

Runoff collected from the urban highway in Seattle contained complex mixtures of contaminants, including metals and PAHs, together with elevated hardness, nutrients, total suspended solids (TSS), and dissolved organic matter (DOC). For each parameter, concentrations varied among runoff events, both in absolute magnitude and in relation to each other (Table 1). Pacific Northwest surface waters (lakes and streams) are typically soft, containing less than 60 mg/L $CaCO_3$ (Briggs and Ficke, 1977). By comparison, elevated concentrations of Ca and Mg in highway runoff resulted in hardness values ≥180 mg/L, falling into the EPA hardness classification of 'very hard' (Briggs and Ficke, 1977). Total ammonia, ortho-phosphate (ortho-P), TSS, TOC, and DOC also tended to be elevated relative to ambient surface water conditions. Antecedent dry period (ADP) was strongly correlated with ortho-P and DOC ($p < 0.01$), and modestly correlated with Cu, Pb, total PAHs, TSS, and DOC ($0.01 < p < 0.05$). The proportion of total metals in the dissolved phase varied among events, but on average was the lowest for Pb (3%) and the highest for Cu (54%). Dissolved concentrations were generally in the order Zn > Cu > Ni > Pb > Cd (Table 1). Total metal concentrations appear in Table S2.

**Table 1**
Water chemistry parameters for stormwater runoff used in baseline toxicity and bioretention effectiveness testing.

| Parameter | Units | RL[a] | Method | Aug 2011 | Oct 2011 | Jan 2012 | Feb 2012 | May 2012 | Sep 2012[b] | Post-cistern[c,d] | No Plants[d] | With Plants[d] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ADP[e] | Days | n.a. | n.a. | 30 | 7 | 5 | 7 | 15 | 50 | 50 | 50 | 50 |
| Zn[f] | ug/L | 2 | ICP-MS FGS-054 | 494 | 181 | 2210 | 59.5 | 318 | 821 | $887_a$ | $8_b$ | $9_b$ |
| Cu[f] | ug/L | 1 | ICP-MS FGS-054 | 300 | 107 | 23.1 | 86.45 | 251 | 89.1 | $102.3_a$ | $28.5_b$ | $30.9_b$ |
| Ni[f] | ug/L | 1 | ICP-MS FGS-054 | 1.51 | 7.86 | 6.67 | 6.71 | 13.7 | 6.6 | $6.8_a$ | $4.7_b$ | $4.5_b$ |
| Pb[f] | ug/L | 0.4 | ICP-MS FGS-054 | 22.0 | 0.461 | U | U | 1.22 | 2.95 | $2.82_a$ | $0.25_b$ | $0.33_b$ |
| Cd[f] | ug/L | 0.2 | ICP-MS FGS-054 | 0.49 | 0.242 | 5.39 | U | 0.45 | 0.31 | $0.28_a$ | $U_b$ | $U_b$ |
| TPAH | ug/L | 0.1 | NOAA GC/MS | 22.6 | 10.3 | 2.3 | 8.5 | 9.1 | 4.09 | $1.58_a$ | $0.09_b$ | $0.08_b$ |
| pH | Std units | 0.01 | EPA 150.1 | 6.87 | 6.98 | 6.79 | 7.72 | 7.03 | 6.56 | $6.9_a$ | $6.71_b$ | $6.56_{ab}$ |
| Alkalinity | mg/L CaCO3 | 1 | SM 2320 | n.m. | 41.4 | 74.5 | 85.1 | 131 | 63 | $59_a$ | $115_b$ | $105_b$ |
| Ca | mg/L | 0.05 | 6010C | n.m. | 58.2 | 279 | 54 | 85.2 | 32.50 | $32.17_a$ | $51.37_b$ | $43.10_c$ |
| Mg | mg/L | 0.05 | 6010C | n.m. | 7.42 | 23.4 | 10.7 | 8.03 | 4.98 | $4.95_a$ | $9.54_b$ | $7.81_b$ |
| Hardness | mg/L CaCO3 | n.a. | calculated | n.m. | 180 | 790 | 180 | 250 | 100 | $100_a$ | $167_b$ | $140_c$ |
| TSS | mg/L | 10 | SM 254D | 880 | 215 | 133 | 221 | 238 | 89 | $65_a$ | $25_b$ | $37_b$ |
| Nitrate | mg N/L | 0.1 | EPA 300.0 | n.m. | n.m. | n.m. | n.m. | n.m. | 3.5 | $3.4_a$ | $9.1_b$ | $3.6_a$ |
| N-Ammonia | mg N/L | 0.01 | EPA 350.1 M | n.m. | 2.47 | 3.52 | 4.73 | 5.65 | 3.2 | $3.0_a$ | $0.14_b$ | $0.09_b$ |
| Ortho-P | mg P/L | 0.008 | EPA 365.2 | n.m. | 0.149 | 0.011 | 0.129 | 0.522 | 0.029 | $0.024_a$ | $0.021_a$ | $0.028_a$ |
| Total P | mg P/L | 0.008 | EPA 365.2 | n.m. | n.m. | n.m. | n.m. | n.m. | 0.719 | $0.649_a$ | $0.208_b$ | $0.251_c$ |
| TOC | mg/L | 1.5 | EPA 9060 | n.m. | 98.9 | 40.8 | 104 | 226 | 105 | $103_a$ | $60_b$ | $62_b$ |
| DOC[f] | mg/L | 1.5 | EPA 9060 | 400 | 80.8 | 26.5 | 89.6 | 219 | 96 | $96_a$ | $58_b$ | $58_b$ |

U = below reporting limit.
n.a. = not applicable.
n.m. = not measured.
[a] Lower reporting limit.
[b] Runoff diluted 40% with rural roof runoff.
[c] Post-mixing in HDPE cistern.
[d] Average of triplicate samples.
[e] Antecedent dry period.
[f] Passed through 0.45 μm filter.

Total PAHs in runoff used in toxicity testing ranged from 2 to 23 μg/L (Table 1). The PAH profile for each storm event was similar and dominated by 3-ring (phenanthrenes) and 4-ring (chrysenes and fluoranthenes) compounds (Fig. S1). Alkylated homologue distribution was fairly uniform, although fluorenes and dibenzothiophenes were consistently sloped — i.e., homologues with a greater number of alkyl substitutions were more abundant than those with fewer substitutions or the parent compounds. The PAH profiles fit a predominantly combustive pattern of origin, as indicated by the relative enrichment of higher molecular weight PAHs (i.e., 4 or more rings) and an elevated pyrogenic index of 0.55 (Wang et al., 1999). Thus, vehicle exhaust and atmospheric deposition were likely significant sources of PAHs in runoff (vs. oil and grease). Low levels of perylene suggest little or no PAH contribution from biogenic sources. Total PAH was strongly correlated with dissolved Pb and TSS ($p < 0.01$).

Notably, PAHs were not stable in cold storage over time. Rather, concentrations rapidly diminished in unpreserved samples stored in dark amber glass at 4 °C. In a pilot test, runoff collected in July 2011 had an initial TPAH level of 6 μg/L, but showed a 96% drop in concentration after 7 d in cold storage. The loss was presumably due to microbial activity, and affected both low molecular weight and high molecular weight PAHs.

The diluted Sep 2012 runoff had similar levels of metals and conventional water chemistry constituents as previous events (Table 2). Total PAHs were lower (1.6 μg/L) than the range for the earlier storms due to adsorption by the HDPE cistern (pre-cistern sample TPAH = 4.1 μg/L). Henceforth, untreated Sep 2012 runoff will refer to homogenized post-cistern samples.

### 3.2. Untreated runoff causes mortality and a range of sublethal developmental defects

Highway runoff was acutely lethal to zebrafish embryos (Fig. 1) following 96 h exposures for the storms in Aug 2011 ($F(1,4) = 676.55$, $p < 0.001$), May 2012 ($F(1,4) = 62.46$, $p = 0.001$), and Sep 2012 ($F(1,4) = 16.00$, $p = 0.016$), but not in Oct 2011 ($F(1,4) = 4.50$, $p = 0.101$), Jan 2012 ($F(1,4) = 4.97$, $p = 0.090$, or Feb 2012 ($F(1,4) = 0$, $p = 1$). For the two storms that caused 100% mortality (Aug 2011, May 2012), developmental delay was evident by 24 hpf, as gauged by conventional staging for zebrafish ontogeny (Kimmel et al., 1995).

Beyond acute lethality, untreated runoff from the different storms also caused a varied but consistent suite of sublethal developmental abnormalities, including delayed hatch (48 hpf), lack of swim bladder inflation (96 hpf), reduced growth, pericardial edema, and small eyes (microphthalmia) (Table 2). Hatch rate at 48 hpf was 0% for all storms, and significantly different from controls for four of the six runoff events. Swim bladder inflation at 96 hpf was also significantly reduced, with a median inflation rate of 2% in runoff-exposed zebrafish relative to 82% for controls. Cardiac abnormalities were also common. Defects in heart shape included enlarged or improperly looped chambers and linear (vs. looped) "string" hearts. Effects on cardiac function included pooling

**Table 2**
Summary of sublethal effects of highway runoff on zebrafish development at two time points (hpf = hours post fertilization) during exposure (began by 4 hpf). Values are mean ratio of treatment value to control value for triplicates. For example, average hatch rate of embryos in Jan runoff for 96 h was 72% of average control hatch rate.

| Event | hpf | Hatched | Swim bladder | Length | Pericardial area | Eye area |
|---|---|---|---|---|---|---|
| Aug | 48 | $0^*$ | n.a. | n.m. | n.m. | n.m. |
| Oct | 48 | $0^*$ | n.a. | 0.99 | $1.64^{**}$ | $0.80^{**}$ |
| Jan | 48 | 0 | n.a. | $0.99^{**}$ | $1.26^{**}$ | 0.96 |
| Feb | 48 | $0^*$ | n.a. | $0.97^*$ | $1.30^*$ | $0.76^{**}$ |
| May | 48 | $0^*$ | n.a. | $0.87^*$ | $1.75^{**}$ | $0.54^{**}$ |
| Sep | 48 | 0 | n.a. | $0.99^{**}$ | $1.37^*$ | $0.83^{**}$ |
| Aug | 96 | DEAD | DEAD | DEAD | DEAD | DEAD |
| Oct | 96 | 0.95 | $0.03^{**}$ | $0.95^{**}$ | $1.54^*$ | $0.72^{**}$ |
| Jan | 96 | $0.72^*$ | $0.02^{**}$ | $0.93^*$ | 1.40 | $0.79^{**}$ |
| Feb | 96 | 1.00 | $0^{**}$ | $0.89^{**}$ | $3.60^{**}$ | $0.61^{**}$ |
| May | 96 | DEAD | DEAD | DEAD | DEAD | DEAD |
| Sep | 96 | 0.93 | $0^{**}$ | $0.91^{**}$ | $1.44^*$ | $0.68^{**}$ |

n.a. Not applicable at this time point.
n.m. Not measured at this time point.
DEAD All embryos dead by this time point.
* $0.05 > p > 0.01$.
** $p < 0.01$.

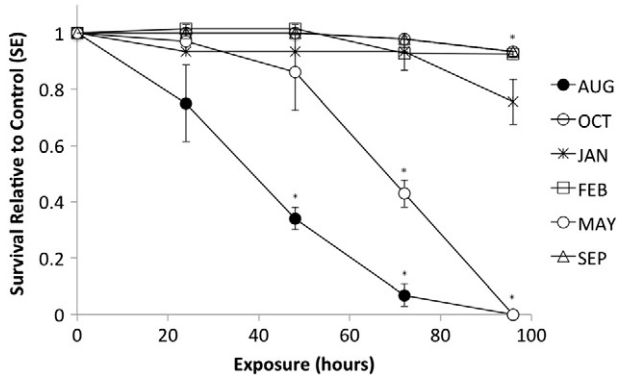


**Fig. 1.** Daily survival of zebrafish embryos reared in stormwater for six runoff events. Error bars are ± one standard error of the mean for triplicate samples. Asterisks denote significant differences from daily control survival.

of blood in the caudal-ventral region of the trunk, increased atrial regurgitation, and circulatory stasis (lack of peripheral circulation). Although not quantified, these abnormalities were often associated with pericardial edema (Fig. S2).

To evaluate toxic potency, diluted runoff from one storm event (Oct 2011; assessed at 48 hpf, no mortality) was still sufficient to significantly delay hatching ($F(4,15) = 17.281$, $p = 0.0002$), increase pericardial edema ($F(4,15) = 10.941$, $p = 0.001$), and decrease eye size ($F(4,15) = 19.550$, $p = 0.001$), albeit with no effect on length ($F(4,15) = 0.769$, $p = 0.570$). The most sensitive endpoints were hatch timing ($p = 0.0004$) and eye area ($p = 0.015$) at dilutions of 95% (5% runoff) and 90% (10% runoff), respectively (Fig. 2).

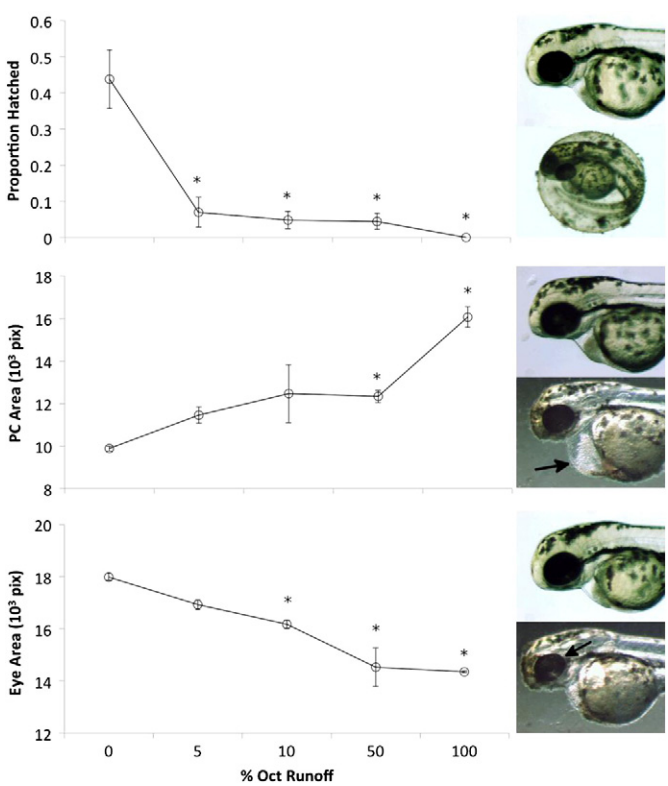


**Fig. 2.** Sublethal effects in zebrafish embryos after 48 h exposure to dilutions of Oct 2011 runoff. Exposures were to embryo medium (control) or dilutions containing 5% to 100% highway runoff (R). Sublethal effects included delayed hatch (top panel), increased pericardial (PC) area (middle panel), and decreased eye size (bottom panel). Asterisks indicate significant differences from control. Error bars are ± one standard error of the mean of triplicates. Images are typical individuals from control or undiluted runoff exposures (100%). Arrows point to either pericardial edema (middle panel) or microphthalmia (bottom panel).

**Table 3**
Counts of mechanosensory neurons at the O2 neuromast in zebrafish exposed for 3 h to control water or stormwater runoff.

| Runoff | Control | Runoff | hpf[a] | R/C[b] | t[c] | df[d] | p[e] |
|---|---|---|---|---|---|---|---|
| Aug 2011 | 12.7 | 12.6 | 96 | 0.99 | 0.171 | 38 | 0.865 |
| Oct 2011 | 9.0 | 8.7 | 72 | 0.97 | 1.358 | 34 | 0.183 |
| Jan 2012 | 10.4 | 9.4 | 72 | 0.90 | 1.533 | 33 | 0.135 |

[a] Age (in hours post fertilization) of zebrafish larvae at time of exposure.
[b] Ratio of runoff to control neuron count.
[c] Statistic for independent *t*-test.
[d] Degrees of freedom.
[e] Statistical significance.

### 3.3. No indication of peripheral neurotoxicity

We found no evidence of neurotoxicity to the lateral line system of zebrafish following 3 h exposures to three distinct highway runoff events (Aug 2011, Oct 2011, Jan 2012). For each storm, the numbers of fluorescently labeled mechanosensory neurons in the O2 neuromast were within 10% of controls (Table 3; $p \geq 0.135$).

### 3.4. No appreciable loss of toxicity in stormwater samples stored at − 20 °C

Baseline toxicity in the form of reduced length, small eyes, pericardial edema, and uninflated swim bladders was similar in zebrafish embryos exposed to fresh and frozen highway runoff collected during the Oct 2011 storm (Fig. 3). The ratios for length, pericardial edema, and eye size were 0.99, 0.93, and 1.03, respectively. Proportionally more of the embryos that were exposed to stored runoff showed swim bladder inflation (ratio = 4), but this measure was highly variable among replicates and thus less reliable than the other three toxicity indicators.

### 3.5. Bioretention treatment improves water quality and prevents developmental toxicity

Filtration through the experimental bioretention columns reduced the concentrations of dissolved metals, specifically Zn (99%), Cu (72%), Ni (31%), Pb (91%) and Cd (>95%) in treated runoff (Table 1). Similarly, bioretention treatment reduced total PAH concentrations by 95%. The presence of plants in the bioretention soil did not further increase the removal of metals or PAHs compared to soil columns without plants. Bioretention also influenced all conventional water chemistry parameters with the exception of ortho-P (Table 1). Notably, treatment reduced levels of ammonia, phosphorus, suspended solids, and organic matter.

Relative to embryos incubated in untreated runoff from the same Sep 2012 storm, the health of zebrafish exposed to runoff filtered through the experimental soil columns was significantly improved in nearly all respects. As with the other storms, untreated runoff had a

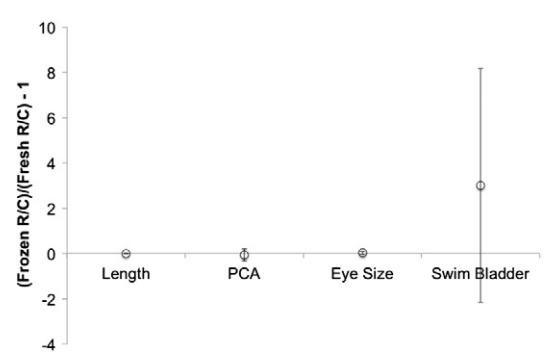


**Fig. 3.** Mean difference between the relative response of zebrafish to frozen and fresh runoff (R) versus controls (C) for four sublethal metrics; embryo length, pericardial area (PCA), eye area, and proportion of individuals with inflated swim bladders at 96 hpf. Error bars are ± one standard error of the mean.

significant effect on cardiac function (proportion normal: $F(3,12) = 21.22$, $p < 0.001$; atrial regurgitation: $F(3,12) = 5.93$, $p = 0.020$; circulatory stasis: $F(3,12) = 32.25$, $p < 0.001$). More than half (54%) of the embryos in untreated runoff had atrial regurgitation, as compared to just 4% of controls (Dunnett post-hoc: $p < 0.001$). By contrast, cardiac function in embryos exposed to treated stormwater was indistinguishable from controls (Fig. 4). Although more individuals in treated runoff showed atrial regurgitation (28% in Plants; 27% in No Plants) compared with controls (4%), this difference was not statistically significant (Dunnett post hoc: $p_{Plants} = 0.164$; $p_{NoPlants} = 0.194$). The most severe functional abnormality (circulatory stasis) was observed in 29% of embryos in untreated runoff, but was absent in embryos exposed to either treated runoff or control water.

In addition to circulation defects, there was a significant effect of treatment on zebrafish length ($F(3,12) = 42.338$, $p < 0.001$), eye area ($F(3,12) = 132.713$, $p < 0.001$), pericardial area ($F(3,12) = 19.032$, $p = 0.001$), and swim bladder inflation ($F(3,12) = 66.63$, $p < 0.001$) at 96 hpf. Zebrafish exposed to untreated runoff were significantly smaller, with smaller eyes, pronounced pericardial edema, and a complete lack of swim bladder inflation compared with controls (Dunnett post-hoc, p all $\leq 0.001$). Bioretention treatment of runoff restored zebrafish development to nearly normal, with embryos in treated effluent that were comparable to controls in terms of length ($p_{Plants} = 0.895$, $p_{NoPlants} = 0.715$), pericardial area ($p_{Plants} = 1.0$, $p_{NoPlants} = 0.754$), and swim bladder inflation ($p_{Plants} = 1.0$, $p_{NoPlants} = 0.941$). A modest but significant effect on eye size remained in embryos exposed to treated runoff (5–7% smaller than controls) ($p_{Plants} = 0.038$, $p_{NoPlants} = 0.013$), although the effect was greatly reduced compared to embryos exposed to untreated runoff (32% smaller than controls; Fig. 5).

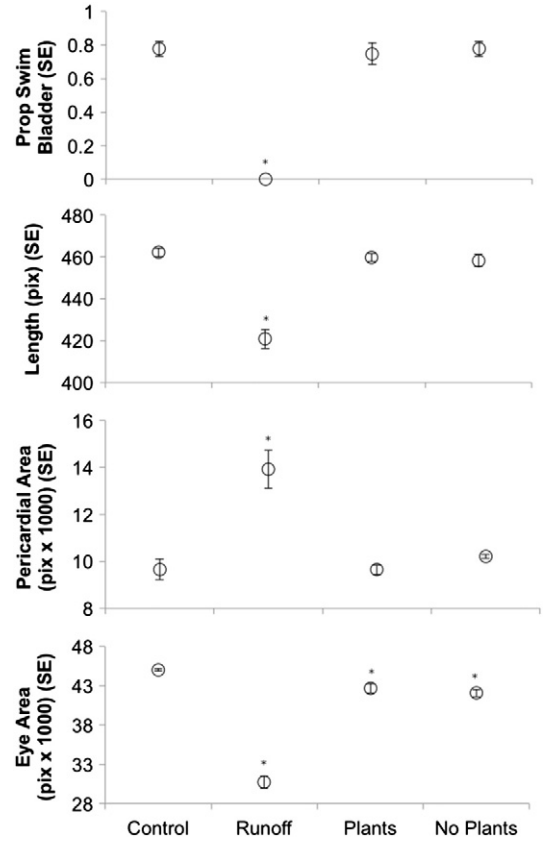


**Fig. 5.** Sublethal effects in zebrafish exposed for 96 h to control water, untreated highway runoff, or treated runoff (with or without plants). Asterisks indicate significant difference from control. Error bars are ± one standard error of the mean of three replicates.

## 4. Discussion

We have shown that urban stormwater runoff across multiple storm events is toxic to developing zebrafish embryos, and that soil infiltration reverses nearly all of the characteristically harmful effects. This form of bioretention is therefore a promising GSI technique for removing contaminants from stormwater and reducing associated impacts on aquatic species. Our experimental soil columns were simple to construct and relatively inexpensive, and the approach should be scalable to treat runoff in different types of locations in the built environment, particularly at sites with limited available space for installing and maintaining water quality improvement technologies.

Delayed hatching and small eyes were consistently the most sensitive indicators of baseline runoff toxicity across all storms, with effects that were significant even when the stormwater was diluted 90–95% (Oct 2011 event). Untreated runoff also caused pericardial edema within treatment groups, albeit with high variability among individual embryos. Less common were more severe occurrences of heart defects and vascular abnormalities. We did not explore the physiological or behavioral consequences of these observed developmental defects at later life stages. However, disruptions of cardiac function during the critical period of embryonic morphogenesis have been shown to cause permanent changes in heart shape, reducing swimming performance at later life stages and presumably increasing the likelihood of delayed mortality (Hicken et al., 2011; Incardona et al., 2014).

The observed developmental abnormalities in zebrafish embryos and larvae correspond closely to those previously documented for larval medaka (*Oryzias latipes*) and inland silverside (*Menidia beryllina*) over longer exposures to runoff from urban and mixed use landscapes (Skinner et al., 1999). This suggests common forms of urban runoff toxicity to early life stages across fish species and geographic regions, over decadal timescales. The soil column treatments assessed here can effectively prevent the severe health effects visible in translucent fish embryos using conventional light microscopy. However, the development of slightly smaller eyes and an indication of atrial regurgitation (albeit not significant) in zebrafish exposed to the treated effluent suggest that some chemicals may have passed through the columns at concentrations near a threshold for these and possibly other subtle effects. More sensitive methods will be needed to better characterize this sublethal toxicity, and determine whether it poses a risk to fish at more dilute exposure concentrations.

Despite high concentrations of dissolved metals in runoff, we found no evidence of structural toxicity to the larval lateral line. Among metals, copper is the most neurotoxic, with an EC50 of 11.5 μg/L for zebrafish mechanosensory neurons (Linbo et al., 2006). Dissolved copper concentrations in the current runoff samples were much higher than this (23–300 μg/L). However, copper complexes with DOC, protecting fish sensory neurons from neurodegenerative toxicity (Kennedy et al., 2012; Linbo et al., 2009; McIntyre et al., 2008). The DOC content of the untreated runoff samples assessed in the current study was very high (27–400 mg/L), suggesting that binding (complexation) removed most or all of the metals

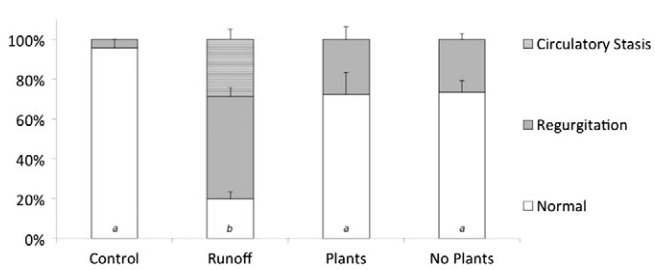


**Fig. 4.** Cardiac dysfunction in zebrafish exposed to control water, untreated runoff, and runoff treated by bioretention with or without plants. Only untreated runoff ('*b*') caused increased levels of circulatory stasis and atrial regurgitation compared with controls ('*a*'). Error bars are ± one standard error of the mean.

from the bioavailable phase. The exception was a single storm with zinc and DOC levels that were unusually high and low, respectively (Jan 2012, 2210 μg/L Zn and 27 mg/L DOC). Although not a focus for the current study, zinc is known to interfere with the proteolytic enzymes involved in hatching (Fraysse et al., 2006; Jezierska et al., 2009), and this was the only storm to cause a significant failure to hatch by 96 hpf. Metals, when bioavailable, may also cause pericardial edema in developing zebrafish (Fraysse et al., 2006).

In contrast to metals, PAH levels in tested stormwater samples (2–23 μg/L) were high enough to affect the developing fish heart (Fallahtafti et al., 2012; Incardona et al., 2012; Jung et al., 2013). Most of the gross morphological defects observed here are attributable to the enriched fraction of cardiotoxic three-ring (tricyclic) PAHs in highway runoff (dibenzothiophenes, phenanthrenes, and fluorenes). These tricyclics are known to disrupt cardiogenesis in a dose-dependent manner (Incardona et al., 2004, 2005; Jung et al., 2013). Previous studies using genetic knockdown methods to silence the embryonic zebrafish heart have shown that craniofacial and eye defects, as well as lack of swim bladder inflation, are downstream consequences of heart failure and circulatory loss (Incardona et al., 2004; Winata et al., 2010). Thus, our observations of enlarged hearts, unlooped or improperly looped hearts, atrial and atrio-ventricular regurgitation, circulatory stasis, small eyes, and lack of swim bladder inflation are all consistent with cardiotoxicity caused by three-ring PAHs, with possible contributions from the higher molecular weight four- and five-ring PAHs as well (Goodale et al., 2013; Huang et al., 2012; Incardona et al., 2006, 2011; Zhang et al., 2012). Notably, the effects we observed here are very similar to those reported previously in zebrafish embryos exposed to crude oil-derived PAHs (Carls et al., 2008; Incardona et al., 2005; Jung et al., 2013). At a coarse level, therefore, the effects of PAHs on fish early life stages appear consistent irrespective of whether they originate from crude oil or refined petroleum that was subsequently burned in a combustion engine and deposited on urban roadways as motor vehicle exhaust (Bui et al., 2012).

The transport and storage of field-collected stormwater samples can be problematic, as PAHs and other hydrophobic contaminants will adhere to plastic containers and degrade over time at ambient temperatures or 4 °C. To avoid underestimating the toxic potential of runoff, samples should be tested within 24 h of collection. If this is not possible, we found that freezing — at 20 °C in glass jars was a reliable storage method, in terms of reproducing toxicity to zebrafish embryos two months after the date of sample collection. Although there may have been subtle changes in the chemical composition of the water samples over time, these were not sufficient to alter the basic stormwater injury phenotype.

Although our experimental design included soil column treatments with and without plants, we saw no differences between the two in terms of protecting zebrafish early development. At the time of the Sep 2012 storm, the overlaying sedge plantings were relatively young and likely had not yet established an extensive root structure. The influence of vegetation may become more clear in future studies with more mature plantings.

Finally, we chose zebrafish for this study because the species is widely available, easy to maintain, with translucent embryos that develop rapidly. The formation of the heart and other organs that are known targets for the toxic components of stormwater takes place on a timescale of hours. These features have made zebrafish an increasingly popular model for toxicological studies (de Esch et al., 2012; Fraysse et al., 2006). Using only conventional videomicroscopy, we were able to document a range of sublethal effects, including reduced growth, delayed hatch, small eyes, abnormal swim bladder inflation, pericardial edema, and other cardiovascular abnormalities. Similar methods can be used to monitor the effectiveness of other GSI technologies, in both the lab and the field. Zebrafish also offer a sophisticated and expanding suite of genetic and molecular tools that can be used to identify specific mechanisms of toxicity (Hill et al., 2005; Scholz et al., 2008). In the future, it is likely that these tools (e.g., probes for targeted gene expression) will complement and extend the simple morphological metrics used here.

Available online are a table describing the individual PAHs analyzed for this study (Table S1), a table summarizing total metal concentrations in runoff samples (Table S2), and a figure of the distribution of PAHs for each runoff sample (Fig. S1), and a figure showing typical cardiovascular abnormalities (Fig. S2). Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.scitotenv.2014.08.066.

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