ACCESSION NO: 1015288 **SUBFILE:** CRIS
PROJ NO: WNP00835 **AGENCY:** NIFA WN.P
PROJ TYPE: MCINTIRE-STENNIS **PROJ STATUS:** NEW
START: 27 FEB 2018 **TERM:** 31 JAN 2021

INVESTIGATOR: McIntyre, JE, .; Stark, JO, .

PERFORMING INSTITUTION:
WASHINGTON STATE UNIVERSITY
240 FRENCH ADMINISTRATION BLDG
PULLMAN, WASHINGTON 99164-0001

CONTROLLING TOXIC CHEMICAL POLLUTION OF URBAN AQUATIC ECOSYSTEMS

NON-TECHNICAL SUMMARY: Due in part to the success of the Clean Water Act controlling large point-sources of pollution, toxic chemicals in the U.S. enter urban water systems primarily through stormwater runoff. From a limited number of investigations, we know that urban road runoff is acutely toxic to a variety of fish and aquatic invertebrates (McIntyre et al. 2015, Kayhanian et al. 2008, Skinner et al. 1999, Bay et al. 2003), including adult coho salmon (Spromberg et al. 2016), as well as juveniles (McIntyre et al. 2015), and post-hatch embryos (McIntyre in prep). Overall, we have a very poor understanding of the vulnerability of aquatic wildlife to urban road runoff (e.g., Puget Sound; (Mackenzie & McIntyre 2017) in forested watersheds.Green stormwater infrastructure (GSI) encompasses an evolving set of technologies to mitigate the impacts that urban stormwater runoff has on physical and chemical habitats of aquatic ecosystems. Prominent among GSIs is the approach of 'bioretention' - methods for encouraging stormwater to infiltrate into the ground. By infiltrating rather than running off into waterways, bioretention has the potential to mimic the hydrology of undeveloped basins (DeBusk et al. 2011) and to filter contaminants present in urban stormwater (Ahiablame et al. 2012). Only recently has the biological effectiveness of GSI been explored. Bioretention can prevent acute mortality in adult coho salmon (Spromberg et al. 2016), in juvenile coho salmon and their prey (McIntyre et al. 2015), and can prevent sublethal toxicity in developing fish embryos (McIntyre et al. 2014), but much more work is needed to determine the most effective GSI implementation for protecting aquatic species and thus the forests that depend on them.In addition to a narrow understanding of the species that are sensitive to urban runoff in the Pacific Northwest (or elsewhere), there is little to no mechanistic understanding of the toxicity of urban runoff to aquatic animals. As a result, our toolbox for evaluating the biological effectiveness of GSI is extremely limited. A popular experimental model for exploring mechanisms of toxicity is the zebrafish (Danio rerio). Initial work into the mechanistic toxicology of urban runoff with zebrafish embryos has shown cardiovascular damage (McIntyre et al. 2014) related to exposure to aromatic hydrocarbons (McIntyre et al. 2016). Concomitant with visible cardiotoxicity, we identified a small set of cardiac-specific genes that were responsive to runoff exposure. Cardiotoxicity may be an important underlying pathophysiology in fishes exposed to urban stormwater runoff, but more studies are needed to elucidate the molecular initiating events that lead to toxicity in aquatic species exposed to urban runoff so that we can sustain forest ecosystems.In symptomatic coho salmon spawners, RNA sequencing has identified a number of

candidate genes that are upregulated. Primary among these are egr1 (early growth factor-1) in gill and heart, and c-jun and c-fos in gill, heart, and liver. All three genes are important to cell signaling pathways and are activated by conditions of inflammatory, oxidative, and other kinds of cellular stress. Much more research is needed to understand the role of these genes in the acute mortality syndrome of coho salmon exposed to urban runoff.In order to develop and implement GSI that will effectively protect sensitive species like coho salmon from extirpation in areas undergoing development, we need tools to assess GSI that are inexpensive to use, sensitive to urban runoff, relevant for the protection of at-risk species, and reliable in terms of reproducibility.

OBJECTIVES: Develop molecular biomarkers for exposure to urban stormwater runoff that are sensitive, relevant, and reliableUse molecular biomarkers to help determine the molecular initiative events that cause observed acute toxicity from urban runoff in relevant fishesTest the ability of green stormwater infrastructure technologies to prevent biomarker activation leading to acute toxicityGiven the potential deleterious effects of stormwater on important Puget Sound fishes, we propose developing tools to accurately assess the impacts of stormwater, and the ability of GSI to mitigate those effects. Specifically, we propose exploring the molecular initiating events that lead to the most sensitive and relevant impacts of urban runoff in coho salmon and zebrafish. Coho salmon are very sensitive to urban road runoff, dying after just a few hours of exposure in both the lab and the field. Zebrafish is a model fish species with rapid developmental timing that is useful for high throughput toxicology screening. Zebrafish can also be a useful surrogate for studying toxicity mechanisms in wild species-of-concern.Ideally, this work will result in reliable biomarkers of runoff exposure that are mechanistically linked to sensitive, relevant impacts in the field. Examples of questions that will be addressed include: Are multiple organ systems involved in acute toxicity from urban runoff? What are the molecular initiating events (MIE) that lead to observed impacts on affected organ systems? Are MIEs species-specific?Biomarkers are biochemical measurements used to assess an organism's response to acute or chronic exposure to contaminants. For example, induction of cyp1a transcription can be used to identify exposure of fish to PAHs (Whyte et al. 2000, Incardona et al. 2009, Incardona et al. 2015), or vitellogenin expression is used to identify male and immature fish exposed to estrogenic chemicals (Tyler et al. 1999). Ideally robust biomarkers of exposure should be specific to the contaminant of concern and indicative of the level and duration of exposure. However, the molecular markers identified in response to stormwater exposure so far (e.g. cyp1a, c-jun, c-fos) are genes commonly induced in response to stress. Moreover, with a complex mixture of contaminants such as stormwater, a suite of biomarkers may be necessary to form a "molecular fingerprint" of organisms' response to exposure.In order to identify a "fingerprint" of stormwater exposure, we propose to conduct high-throughput sequencing followed by RNA-Seq and pathway analyses. RNA-Seq analysis enables comparison of all the genes expressed in a given tissue without a priori knowledge of the molecular pathways involved. In this way, RNA-Seq is a useful tool for identifying novel biomarkers of stormwater exposure. In addition, pathway analyses can be used to identify signaling pathways and upstream molecular events involved in stormwater toxicity. These methods can improve our understanding of molecular mechanism of toxicity and prespawn mortality in salmon.

APPROACH: Runoff collectionUrban stormwater runoff is collected during rain events in stainless steel totes (240 gallon and/or 450 gallons) from an elevated urban highway in Seattle, WA at the facility of our collaborators at NOAA-NMFS Northwest Fisheries Science Center. Runoff can then be transported to Puyallup or other locations for use in toxicity testing within 72 h of collection. Runoff may alsobe frozen for later toxicity testing. We previously showed that frozen runoff does not cause a loss of toxicity in zebrafish embryo exposures (McIntyre et al. 2014).Green stormwater infrastructure treatment of runoffIn some experiments, runoff will be treated by GSI and co-exposed to test organisms with untreated runoff. The GSI used will be those for research projects funded with other grants and may include bioretention, bioswales, and green roofs.FacilitiesExperiments

will take place at a combination of the freshwater Fish Toxicology Laboratory at the WSU Research & Extension Center in Puyallup, the freshwater facilities at the NWFSC in Seattle, and the freshwater Suquamish Tribe Grovers Creek Salmon Hatchery in Indianola.The water quality and rearing capacity at the Fish Toxicology Lab need to be upgraded. The dechlorinated municipal city water used for rearing and as control water in all experiments contains dissolved zinc at concentrations that exceed limits recommended for the health of aquatic animals. Other water on the WSU campus does not contain these elevated zinc concentrations, therefore the problem is local to the fish lab and likely a result of old pipes that locally leach zinc. We will replace the existing water quality system with a reverse osmosis system that will continuously produce Type III purity water. The pure water will then be conditioned with salts to maintain a constant pH and concentration of micro- and macronutrients for healthy fish populations. This new water source will feed both the salmon rearing and zebrafish rearing facilities at the Fish Toxicology Lab.The rearing facilities for salmon will expanded to enable simultaneous rearing of more fish of different ages as well as the capacity to hold species other than coho salmon. Finally, the filtration system will be upgraded to one that needs less regular cleaning.Animals and ExposuresCoho salmon are reared at the Fish Toxicology Laboratory at the WSU Research & Extension Center in Puyallup. They can be used in toxicity testing beginning in the spring of their first year, through the spring of their third year. Exposures take place in glass or fiberglass tanks for up to 96 h following standard acute toxicity testing protocols (U.S. EPA 2002), including 3 replicates of 10 fish each maintained at 13 °C. Euthanized fish will be sampled for blood for hematology and other tissues for use in histology as well as transcriptomics. The Suquamish Tribe generously allows us use of their Grovers Creek facilities for experiments with adult coho salmon that have recently returned from the Pacific Ocean to spawn. Exposures with adult salmon take place in 800-L HDPE tanks for up to 24 h. Blood and tissues are similarly sampled from euthanized adult fish.Zebrafish are reared at both the Fish Toxicology Lab and the NWFSC. Embryos are exposed at 2-4 hours post fertilization (hpf) for up to 96 h. Four replicates of 15 embryos each are exposed in glass petri dishes containing 10 mL of rearing water (control) or runoff incubated at 28°C. At test termination, individuals are dechorionated (if necessary), embedded in 2% methylcellulose, and imaged live with a Nikon SMZ stereomicroscope. Digital photographs and are taken and morphometrics assessed with image analysis software, including embryo length, eye size, and pericardial area. From digital video, heart rates are counted and embryos are scored for presence/absence of cardiovascular defects such as arrhythmia, cardiac looping defects, and internal hemorrhaging. Groups of embryos are snap frozen in liquid nitrogen and later homogenized for molecular assays such as transcriptional changes in important genes such as biomarkers of contaminant exposure and cardiac injury.ExperimentsStudies will be conducted with each fish species to further explore the mechanistic toxicology of urban runoff. In concert with pathophysiological investigations supported by other funding sources, tissues will be sampled from individuals at various dilutions of runoff, exposure durations, and symptomatic stages. Total RNA will be isolated from whole embryos (zebrafish) or tissues of interest (salmon) from control and stormwater exposed fish and submitted for Illumina paired-end sequencing. Sequences will be aligned to existing reference genomes (zebrafish or salmon) or aligned de novo using the De novo RNA-Seq Assembly Pipeline (DRAP) and Trinity transcriptome assembler. For the de novo assembly, contiguous sequences (contigs) will be annotated using BLASTX (Altschul et al, 1990) againstthe NCBI non-redundant protein database (www.ncbi.nlm.nih.gov). Sequences with an e-value ≤ E-05 will be retained in the backbone.To examine differential transcript levels between control and exposed fish, RNA-Seq will be performed using RSEM (Li & Dewey 2011). Count estimates generated by RSEM will be analyzed for differentially expressed genes using DESeq (Anders & Huber 2010). Transcripts with an adjusted p-value ≤ 0.05 will be considered significantly altered. Clustering analysis will be performed on differentially expressed transcripts using the cluster::agnes package in R with the Spearman method (Maechler 2015). Pathway and network analyses will be conducted using

Ingenuity Pathway Analysis (IPA) software. Significantly altered pathways and biological functions will be determined using the Fisher Exact Test ($p \leq 0.05$). Following RNA-Seq and pathway analyses, targeted gene expression analysis will be conducted to confirm the utility of putative biomarkers. Confirmed biomarkers of stormwater exposure can then be used to determine the effectiveness of GSI to mitigate the toxicity of stormwater runoff.

KEYWORDS: urban stormwater runoff; aquatic toxicology; molecular biology; coho salmon; zebrafish; herring; biomarkers; molecular initiating event; green stormwater infrastructure; bioretention

---