ASSESSING THE TOXICOLOGICAL EFFECTS OF MOTOR OIL AND TIRE PARTICLES (SYNTHETIC STORMWATER) ON ZEBRAFISH, *DANIO RERIO*

by

Madeline Thompson

A Thesis
Submitted in partial fulfillment
Of the requirements for the degree
Master of Environmental Studies
The Evergreen State College
May 2024

This Thesis for the Master of Environmental Studies Degree

by

Madeline Thompson

has been approved for

The Evergreen State College

by

Carri LeRoy, Ph. D.

Member of Faculty

Date

ABSTRACT

Assessing the Toxicological Effects of Motor Oil and Tire Particles (Synthetic Stormwater) on Zebrafish, *Danio rerio*.

Madeline Thompson

Stormwater runoff resulting from urbanization poses numerous environmental concerns, notably increased water volumes and contaminant loads discharged into aquatic ecosystems. This influx of materials and pollutants, predominantly from roadway infrastructure, has been shown to adversely influence aquatic organisms. Utilizing zebrafish (*Danio rerio*) as a model organism, this thesis aims to investigate the effects of two synthetic stormwater components, namely used motor oil (UMO) and tire particles (TP), on the expression of the environmental stress gene, *cyp1a*. Addressing a critical gap in research focusing on sublethal effects on aquatic organisms, this study employed sublethal concentration ranges of UMO and TP to assess their effects on *cyp1a* gene expression in zebrafish. Notably, exposure to UMO concentrations ranging from 0.05% to 0.50% resulted in a significant dose-dependent increase in *cyp1a* gene expression, indicative of a biological response within the targeted range. Conversely, exposure to tire particle concentrations ranging from 1.0 g/L to 10 g/L did not exhibit significant differences across treatments, but were higher than control groups and the highest concentration resulted in complete mortality. This research unveiled a consistent upregulation of *cyp1a* across treatment groups for both experimental exposures. However, it is important to note that neither exposure accurately reflected the lower end of a sublethal concentration range. Despite this study's limitations in replicating sublethal environmental conditions for UMO and TP accumulation in stormwater, it offers valuable insights into dose-response relationships, identifying concentration ranges that elicit significant gene expression responses in a model organism.

Table of Contents

List of Figures

List of Tables

## Acknowledgements

I would like to thank Dr. Carri LeRoy, Dr. Jen McIntyre, Dr. Erin Martin, Dr. Amanda Gunn, Lauren Thompson, Tyler Thompson, Finley Thompson, Stephanie Blair (Ph. D), and everyone at the Fish Lab who played a role in my collaboration and development of this project.

Introduction

There are many environmental concerns associated with stormwater runoff due to urbanization. Heavy metals, polycyclic aromatic hydrocarbons (PAHs), and other emerging contaminants such as vehicular pollutants like tire wear particles (TWPs) can accumulate in nearby waterways (Trumbull and Bae, 2000 and Kriech and Osborn, 2022). One concern associated with stormwater is that it has increased in both volume and contaminant load due to the increased growth of roadway infrastructure, resulting in the leaching of materials and inputs of contaminants into aquatic environments (Kriech and Osborn, 2022). The accumulation of environmental contaminants into aquatic habitats has been demonstrated to have negative influences on aquatic organisms (Barbosa et al., 2012). Both the qualitative and quantitative traits of stormwater need further study at both national and regional levels to better inform site-specific, climatic, and local variables that may influence the toxicity of stormwater (Barbosa et al., 2012). By accurately measuring and monitoring urban aquatic ecosystems, we can understand how proper filtration systems can be applied to reduce the exposure of aquatic organisms to toxins mobilized by stormwater.

Improving the filtration of toxins and reducing the contaminants being released into nearby waterways are important strategies for freshwater ecosystem conservation. One method for filtration that is often utilized in urban environments is a bioretention system, which is used to improve water quality and reduce negative influences of stormwater runoff. Bioretention systems are designed as a landscape depression that treats on-site stormwater discharge primarily from impervious surfaces. This form of best management practice is growing in application and is engineered in urban areas to remove pollutants through a variety of physical, chemical, and biological processes (Vijayaraghavan et al., 2021). McIntyre et al. (2023) analyzed untreated and treated urban runoff in the early life stages of Pacific salmon and found that mortality and morphological development effects were reduced greatly when runoff was properly filtered using bioretention methods. These systems help to capture, filter, and treat stormwater runoff before it enters nearby waterways, thereby reducing the influx of pollutants and improving overall water quality. However, long-term research on these applications is lacking. Given the concerns about stormwater pollution and its effects on Puget Sound's sensitive marine environment, various municipalities, agencies, and organizations in the region have adopted bioretention systems as part of their stormwater management strategies (Rheaume and Ahearn, 2016 and Mahat et al., 2022). Numerous projects featuring bioretention systems have been implemented throughout the Puget Sound region, including residential, commercial, and public spaces (Rheaume and Ahearn, 2016). These projects often involve collaboration between local governments, environmental organizations, community groups, and private entities to design and construct bioretention facilities tailored to specific site conditions and stormwater management needs. Overall, bioretention systems play a crucial role in promoting sustainable stormwater management practices and protecting the health and integrity of aquatic ecosystems.

Reducing discharge from impervious surfaces such as roofs, driveways, sidewalks, and parking lots is another type of BMP. Green stormwater infrastructure (GSI) utilizes this approach by integrating soil-water-plant systems into permeable pavements (Environmental Protection Agency, 2023). Tire wear particles (TWPs) and their products, 6PPD-quinone and 6PPD, can be transported into aquatic ecosystems via stormwater and have been recently identified as acutely toxic chemicals to aquatic organisms (Jayakaran and Mitchell, 2024). Jayakaran and Mitchell (2024) studied permeable pavements by quantifying the performance of different types of pavements in the capture of TWPs and leachable tire chemicals. When examining 6PPDQ, concentrations were significantly reduced in the permeable pavement treatment (a reduction of 68%; Jayakaran and Mitchell, 2024). The use of GSI is growing, but further research is needed to identify the mechanisms by which these technologies can mediate stormwater toxicity. This research will allow for better applications of filtration systems so BMPs can be developed, reducing toxicity of stormwater discharge.

One method of assessing BMPs is through the use of toxicity studies. Many toxicity studies use zebrafish (*Danio rerio*) as a model organism to analyze the effects of pollutants on fish development, physiology, mortality, and stress. This is primarily due to their rapid and transparent early development and easy husbandry in lab settings (Schilling and Kimmel, 1997). Zebrafish have shown a variety of effects when exposed to stormwater contaminants such as TWPs, polycyclic aromatic hydrocarbons (PAHs), heavy metals, and other vehicular fluids that commonly occur in runoff (Zhang et al., 2023, Xie et al., 2023, Anderson et al., 2023, Pauka et al., 2011, and Du et al., 2022). When analyzing the effects of relevant stormwater pollutants on zebrafish there are several approaches to take. Stormwater could be replicated by: 1) collecting samples from a stormwater event, or 2) creating environmentally relevant reagents that mimic stormwater (synthetic stormwater). For studies that use synthetic stormwater, there are challenges such as creating concentrations of common runoff contaminants that include both lethal and sublethal effects on aquatic organisms. Due to the complexity of stormwater, there is a strong need to quantify and create environmentally relevant synthetic stormwater to fully understand how fish development, physiology, and mortality vary across concentrations (J. McIntyre, personal communication).

For this thesis, a comprehensive scholarly literature review was completed to examine the multifaceted topic of stormwater management. The review consisted of various stormwater aspects including the ramifications of global urbanization on stormwater dynamics, the characterization of contaminants within stormwater, prevalent management practices, contaminant types, and the effects on aquatic ecosystems. Through literature research, it became evident that there exists a notable gap in our understanding concerning the direct effects of specific components of stormwater contaminants, particularly those associated with vehicular runoff.

This thesis research addresses gaps by analyzing a specific environmental stress gene, the *cyp1A* gene, and its response to two types of synthetic stormwater. The synthetic stormwater components used in this study were used motor oil (UMO) and tire particles (TP). To address the gap in studies focusing on sublethal effects on aquatic organisms, this project addressed two stormwater components at sublethal concentrations. The toxicity of each synthetic stormwater component was analyzed by doing short-term exposures of zebrafish. To determine sublethal concentrations for the experimental exposures for qPCR, toxicity endpoints such as hatch rate, growth, heart development, and mortality were analyzed. Using the sublethal range allowed us to address the variation in gene expression across a concentration curve to determine the potential toxicity to aquatic organisms. The toxicity responses of zebrafish at both morphological and developmental toxic endpoints can be compared and used to determine the range of concern for these environmental pollutants. To our knowledge, this will be the first project to do RT-qPCR (reverse transcription-quantitative polymerase chain reaction) at sublethal concentrations for both of the synthetic stormwater components. Using each of the noted particle and fluid components of synthetic stormwater will continue previous research as well as provide new findings for areas that have not been well-studied. This project will address gaps in understanding of the direct influences of environmental contaminants on stress genes in the model aquatic organism, zebrafish.

Literature Review

Pollution in the form of stormwater runoff is a growing concern globally in areas of increased urbanization. There are a variety of environmental pollutants that can be sourced from urbanized areas that can enter nearby waterways. These environmental pollutants such as heavy metals, Polycyclic aromatic hydrocarbon (PAHS), and other organic materials have been shown to negatively influence aquatic ecosystems in a variety of ways. For this reason, there have been many studies assessing the toxicity of stormwater contaminants in urban environments. There are also many ways to minimize stormwater toxicity, but new contaminants are emerging. This makes studying the variety of potentially toxic and harmful stormwater components a vital component of improving best practices to reduce point-source and non-point source pollutants in aquatic ecosystems.

There are many concerns with regards to stormwater runoff that have increased our understanding of the role of urban pollutants in aquatic ecosystems. For this reason, a scholarly literature review was conducted to synthesize a comprehensive overview of stormwater, focusing specifically on its influences, prevalent management practices, specific stormwater contaminants, effects on aquatic organisms from known contaminants, and the intricacies surrounding synthetic stormwater studies.

Stormwater: Overview

As areas become more urbanized there is a steady increase in the pollutants being transported into aquatic ecosystems, as stormwater is the primary route for transport. This section will review the influences of global urbanization on stormwater runoff, the basic causes of stormwater runoff, the need to characterize stormwater pollutants, and methods of stormwater management.

Stormwater Influences of Globalization

Areas experiencing increased urbanization are typically also negatively influenced by an increase in stormwater runoff that results in the discharge of pollutants into surrounding waterways. Although multiple environmental challenges arise from global urbanization, urban stormwater runoff has a particularly strong negative influence on aquatic ecosystems (US EPA, 2017). There is increased awareness on the part of land management agencies of the importance of establishing best management practices (BMPs) regarding urban stormwater runoff that is primarily focused on decreasing its negative effects on aquatic ecosystems. Among its effects, stormwater runoff can lead to increased flooding, habitat alteration and loss, decreased aquatic biological diversity, pollution, and increased sedimentation and erosion (US Environmental Protection Agency (EPA), 2017). Stormwater runoff is managed under the National Pollutant Discharge Elimination Systems permit program that aims to reduce the amount of contaminants discharged, improve overall water quality, and improve aquatic ecological function (EPA, 2017). Land conversion due to an increase in urbanization has resulted in increased pollutant loading from hydrological alterations that result in increased stormwater runoff (EPA, 2017). These threats associated with global urbanization and urban stormwater make studying the ecological and biological impacts of stormwater extremely important.

Stormwater: Characterization of Contaminants

One of the major concerns associated with stormwater are the known environmental contaminants in the runoff that have cascading effects on aquatic ecosystem function. A large body of research characterizes the types of ecological consequences of stormwater runoff and the types of chemical pollutants that are present (EPA, 2023). However, BMPs for minimizing the ecological and environmental influences of urban stormwater are evolving as more information becomes available (Barbosa et al., 2012). Both qualitative and quantitative traits of stormwater need to be better characterized at both national and regional scales to better inform site-specific, climatic, and local variables that may drive toxicity in stormwater (Barbosa et al., 2012).

Stormwater varies in terms of pollutant concentrations and particle sizes, and it is essential to quantify solids, nutrients, metals, organic pollutants, and bacterial pathogens (Pamuru et al., 2022). Most of these components occur in higher concentrations where impervious surfaces dominate in highly urbanized areas (Pamuru et al., 2022). Stormwater passing over impervious surfaces may encounter other pollutants like particulate matter, nutrients, oil and grease, toxic organic compounds, metals, and other microorganisms, which are then also transported in runoff (Pamuru et al., 2022). The types of chemical pollutants present is heavily influenced by location, stormwater filtration systems, seasonality, and overall ecosystem dynamics, such as an increase in urbanization that results in a higher level of environmental disturbance (Pamuru et al., 2022).

Stormwater Management

As research progresses in understanding the complex nature of urban stormwater, the practice of using green stormwater infrastructure (GSI) has risen. This practice includes managing stormwater at the source to ensure it is captured, treated, and properly filtered with an additional goal of reducing flooding through the reduction of stormwater runoff volume entering nearby bodies of water (Bodus et al., 2023 and Clary et al., 2020). Due to these practices being relatively new, there are many contaminants that GSI methods have not included in designs for filtration or mitigation (Bodus et al., 2023). Bodus et al. (2023) determined that GSI without consideration of new contaminants, is subject to an increase in antibiotic resistance genes, microplastics, tire wear particles, per- and polyfluorinated substances (PFAS), and increased water temperatures.

The design considerations of GSI through better removal practices in filtration, sorption, and biogeochemical processes may reduce many of the risks associated with the accumulation of emerging contaminants such as tire wear particles and PFAS (Bodus et al., 2023). Both tire wear particles and PFAS are of great concern due to their volumes and their inability to break down in the environment (CDC, 2022). Despite the prevalence of both BMPs and GSI practices, further research is needed to better understand the effects emerging contaminants have, their ability to accumulate over time, and methods to effectively remove them or reduce their ecological impact (Bodus et al., 2023). Ensuring proper use of BMPs and identifying contaminants in stormwater runoff are critical for reducing the negative influence of stormwater overall.

Stormwater dynamics have been altered due to an influx of urban and vehicular pollutants which has influenced how mitigation strategies are implemented. Stormwater has been directly affected by the increased growth of roadway infrastructure that has resulted in leachate from materials into the environment (Kriech and Osborn, 2022). Permeable concrete (PC) systems are a tool used to improve runoff pollution by eliminating heavy metal (HM) occurrences and reducing the effects of HM leaching (Zhang et al., 2023). This management practice is influenced by site-specific parameters and is dependent on the quantity and quality of stormwater (Selbig et al., 2019). Selbig et al. (2019) asserted that urban stormwater systems using PC can remove the majority of pollutants but that there might be some unintended consequences that increase the presence of dissolved pollutants in that runoff. Therefore, continual monitoring and identification of the occurrence of urban/vehicular pollutants in nearby aquatic ecosystems is needed to reduce pollutant accumulation in these habitats.

Stormwater Contaminants

Stormwater contaminants may accumulate in aquatic ecosystems and can vary based on type, source, and concentration, all of which influence their potential toxicity. This section will cover the main types of stormwater contaminants in urban runoff: polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), per- and polyfluoroalkyl substances (PFAS), and tire wear particles, and 6PPD/6PPD-Q: N- (1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine, a tire additive that enhances tire longevity (with 6PPD-q: 6PPD-quinone, the transformation product; Department of Ecology, 2022).

Stormwater Contaminants: Overview

There are many traits and characteristics of stormwater runoff that influence its potential toxicity for aquatic environments. The primary measurements of stormwater runoff toxicity include the concentrations of each contaminant as well as contaminant particle size because size can influence the toxicity and overall behavior of each contaminant (Grant et al., 2003). Pollutants in stormwater are classified as either “dissolved” or “particulate” which is extremely important when determining their toxicity potential. Bioretention systems are designed to remove both dissolved pollutants and particulate matter from runoff through chemical, biological, and physical processes via sedimentation, filtration, sorption, reduction, vegetative uptake, and assimilation (Laurenson et al., 2013). Grant et al. (2003) in a review of stormwater components and their associated toxicity ratings state that particles (size distribution and concentration) influence chemical toxicity by increasing stress on aquatic organisms, shifting bioavailability, and altering exposure levels. Most BMPs treat the particulate fraction and not the absorbed particles that heavy metals and organic material occur in. Particulate matter (PM) also plays an important role in carrying and transporting other runoff pollutants which makes particles a critical aspect of consideration for BMPs regarding stormwater facilities (Xu et al., 2023). The main source of PM in urban environments is carried/transported via rainfall-runoff from impervious surfaces which is why it plays such an important role in current bioretention systems for reducing stormwater pollution. However, there is still a large gap in understanding how bioretention systems can be improved to address emergent contaminants.

Stormwater Contaminants: Polycyclic Aromatic Hydrocarbons: PAHs

A well-known class of environmental contaminant, polycyclic aromatic hydrocarbons (PAHs), is of great concern related to stormwater pollution. PAHs are a class of chemicals that occur in coal, crude oil, and gasoline and can be carried through the atmosphere by the burning of such products (CDC 2022). Stormwater runoff from roadways, gas stations, and other urban areas can contribute large concentrations of PAHs that can accumulate in surrounding waterways. Toxicity from PAHs varies due to emission type and the source of the PAHs (sources include stationary sources like power plants, chemical plants, industrial and manufacturing plants, mobile emissions, agricultural activities, and natural sources; Lee, 2010). The level of toxicity is based on where the PAHs are sourced from, the amount, and how they accumulate over time. PAH pollutants can be found in aquatic and terrestrial ecosystems but due to their higher hydrophobicity and low aqueous solubility, soil ecosystems have become a large sink for PAHs (Patel et al., 2020).

Most PAHs are classified as persistent organic pollutants that are derived from anthropogenic activities (incomplete combustion of organic matter) and their fate/occurrence in the environment is heavily dependent on their physical/chemical characteristics (Lee, 2010). A study assessing the relationship between urbanization and ecological risks of PAHs found that industry, transportation, and population greatly influenced PAH risks in urban areas (Han et al., 2021). When examining PAH characteristics they found that low molecular weight PAHs (emitted as gaseous phase) were correlated with industry sources and high molecular weight PAHs (emitted as particulate phase) were correlated with transportation sources (Han et al., 2021). This is important when considering which PAHs are more toxic to the environment and at what state they are accumulating in the environment. As urbanization increases, the ecological risks of PAHs rise, creating a global concern for improving stormwater infrastructure to minimize environmental consequences.

Stormwater Contaminants: Polychlorinated biphenyls (PCBs)

Another persistent organic pollutant found in stormwater are polychlorinated biphenyls (PCBs), which are industrial products that accumulate in the environment. Both dense urban areas and residential areas have reported high levels of PCBs because they are a class of chlorinated organic chemicals that are used for a variety of industrial and commercial purposes (Cao et al., 2019). The major issue with PCBs in the environment is that they do not easily degrade since they are lipophilic, which also makes them highly carcinogenic and harmful for animal and human health (Cao et al., 2019 and Othman et al., 2022). Although PCBs are no longer manufactured or used by industry in the U.S.A., they continue to be an issue in the environment as they are persistent and have accumulated in aquatic food webs (Encyclopedia of Toxicology, 2014). Additionally, there is global concern for mitigating the influences of PCBs on the environment. For this reason, PCBs in the environment are targeted by addressing the bioaccumulation and biomagnification of PCBs in the food chain (Othman et al., 2022). However, for stormwater management, it has been shown that bioretention systems need to target both the particulate and dissolved stages of PCBs to be effective at reducing the concentrations of PCBs in treated effluent (Cao et al., 2019). Because PCBs can be highly concentrated in both industrial and residential areas, it is important to understand their occurrence and the path they take when entering aquatic ecosystems via stormwater.

Stormwater Contaminants: Per- and Polyfluoroalkyl Substances: PFAS

Additional harmful components of anthropogenic stormwater are per- and polyfluoroalkyl substances (PFAS) which have been shown to have both environmental and human health effects

(National Biomonitoring Program, CDC 2022). These contaminants are defined as persistent organic pollutants and most traditional stormwater systems do not properly filter/remove these toxins (Banerjee, 2023; Baker and Knappe 2022). The toxicity data for PFAS are limited to only a handful of studies but they have been shown to alter immune health, thyroid function, and cause liver and kidney disease, cancer, and reproductive and developmental anomalies in humans (National Biomonitoring Program, CDC 2022). Heavy rainfall and flooding can increase the transportation and mobilization of PFAS in stormwater and their presence in urban ecosystems which is influenced by land use type (Banerjee 2023). Further research is needed to identify the effects of PFAS on multiple species and life stages to better support BMPs for this contaminant of emerging concern.

Stormwater Contaminants: 6PPD-Quinone

An environmental contaminant that is of growing concern in stormwater is 6PPD-quinone (6PPD-Q), which stands for the chemical N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine and is widely used to improve the durability of rubber in tires. 6PPD-Q can be found in water, dust, soil and air particles, and can accumulate in aquatic organisms (Hua and Wang, 2023). A review of this toxin by Nicomel and Li (2023) demonstrated that the main source of this chemical is in stormwater runoff via roadways and therefore it poses a significant threat to aquatic organisms. An analysis done by Chen et al. (2023) studied the presence and pathways of 6PPD-Q and identified key factors that can influence its abundance and distribution such as temperature, illumination, and storm events. When comparing concentrations of 6PPD-Q in dust samples from vehicles to indoor samples from other indoor environments, concentrations of 6PPD-Q were significantly higher in dust samples, suggesting that solar radiation or higher temperatures on roadways may accelerate the transformation of 6PPD-Q from the rubber materials (Chen et al., 2023). Storm events may further increase the distribution and transportation of 6PPD-Q in the environment, which can again influence its occurrence and accumulation in aquatic ecosystems (Chen et al., 2023). However, the toxicological effects of this compound still require further investigation so that we may better understand potential environmental hazzards.

Effects of Stormwater Exposure

There are a variety of stormwater contaminants that have been determined to be toxic to aquatic organisms like freshwater fish. Many studies examining developmental abnormalities and developmental pathways use a model organism such as the zebrafish (*Danio rerio*). When studying complex interactions such as genetic variation and environmental factors, zebrafish serve as a strong model organism (Raterman et al., 2020), primarily due to their rapid and transparent early development and easy husbandry in lab settings (Schilling and Kimmel, 1997). Due to variation in exposure time and pollutant concentrations, it is important to consider both the lethal and sublethal effects contaminants can have on aquatic organisms. This review will focus on studies exploring both the effects on physiological traits and gene expression in animals exposed to a variety of stormwater contaminants such as heavy metals, PAHs, PCBs, endocrine-disrupting chemicals (EDC)s, and 6PPD-Q.

Aquatic Organisms: Effects on Physiological Traits

Heavy Metals

There are many widely known lethal and sublethal effects of heavy metals on aquatic organisms that are exposed to urban stormwater (Levin, Howe, and Robertson, 2020). The toxins found in urban stormwater including heavy metals and PAHs, have been shown to have a direct effect on ecological processes at the individual, population, and community scales due to the immediate influences these contaminants have on the development of macroinvertebrates (Beasley et al., 2002). Altering the composition of the lowest members of the food chain and species diversity modifies the whole ecological structure of urban streams (Beasley et al., 2002). Increased anthropogenic inputs of heavy metals have been shown to have numerous toxic effects on fish systems such as the immune system and liver, olfactory systems, ion transport, and kidney function (Rand 1995, and Newman 2014). Toxic metals have also been shown to weaken fish immune systems resulting in immunosuppression that can lead to increased gene mutation and more susceptibility to pathogens and disease (Rand, 1995). Although all of these studies showcase the importance of analyzing the effects of complex urban runoff, there are still new contaminants being discovered each year. There are many novel and poorly characterized organic contaminants present in urban stormwater that need to be further studied to better assess the effects they may have on aquatic organisms (Du et al., 2017).

PAHs and Polyhalogenated Carbazoles (PHCZs)

In the environment, PAHs in runoff can pose a significant risk to aquatic organisms because they are bioavailable as opposed to undissolved heavy metals which may be less bioavailable because they can bind to organic matter (Al-Reasi et al., 2011). The PAHs found in urban stormwater runoff not only pose a significant threat to aquatic habitats but can also cause both functional and structural defects in developing fish hearts (McIntyre et al., 2016). A study assessing the effects of untreated runoff on the expression of cardiotoxicity genes in zebrafish found that the PAHs in a dissolved state in runoff were cardiotoxic (McIntyre et al., 2016). This study also showed that molecular markers were a more effective means of measuring contaminant exposure than any visible toxicity indicators (McIntyre et al., 2016). Another study that analyzed cardiotoxicity in zebrafish found that the expression of the *cyp1a* and *cyp1B1* genes were downregulated when exposed to polyhalogenated carbazoles (PHCZs) which induced both cardiotoxicity and behavioral changes in zebrafish (Du et al., 2022). McIntyre et al. (2016) examined zebrafish abnormalities when exposed to untreated runoff with high levels of PAHs and found that the fish displayed signs of toxicity such as delayed hatching, reduced growth, pericardial edema, microphthalmia (small eyes), and reduced swim bladder inflation. Overall, this study by McIntyre et al. (2016) supports previous and current findings on exposures to PAHs and cardiotoxicity outcomes.

Polychlorinated biphenyl (PCBs)

PCB exposure studies in fishes have mainly focused on the acute effects and toxicity associated with varying concentrations. There have been many studies done on the effects of PCB (in both dissolved and particulate states) exposure on zebrafish. Exposure to PCBs has been shown to cause deviations in morphological development as well as other developmental toxicity effects (Garner et al., 2013 and Timme-Laragy et al., 2007). For many developing fishes exposed to PCBs, the heart is a target for toxicity and can result in an array of cardiac deformities (Garner et al., 2013). Low concentration exposures to PCB in adult zebrafish have been also found to cause fat accumulation in liver tissues while higher concentration exposures resulted in inhibited growth rates (Li et al., 2019). One study examined adult zebrafish exposed to PCBs and found that PCBs at low concentrations interfere with lipid metabolism (Li et al., 2019). A study examining developing zebrafish embryos exposed to PCBs found that a PCB concentration of 20 μM did not affect gross morphology of developing embryos but did alter an array of liver development genes (Roy et al. 2019). Understanding how age and developmental stage influence toxicity is extremely important to consider when studying the toxicity of stormwater contaminants on aquatic ecosystems.

Endocrine-disrupting chemicals (EDCs)

Endocrine-disrupting chemicals (EDCs) are an emerging contaminant that is occurring in high levels in urban water ecosystems and can have a variety of effects on aquatic organisms. Stewart et al. (2023) examined the effects of EDCs on both hormone receptors and signaling in zebrafish.

Endocrine-disrupting chemicals can result in the alteration of estrogen signaling and affect craniofacial development which is an estrogen-dependent process in developing zebrafish (Stewart et al., 2023). Like previous studies exploring environmentally relevant concentrations of EDCs, Stewart et al. (2023) found that prolonged and chronic exposures resulted in craniofacial patterning in this aquatic model organism. They also determined that the developmental stage at which exposure occurs can greatly alter the craniofacial development and pathway of phenotypic outcomes (Stewart et al., 2023). This study is important because EDCs are another emerging contaminant that warrants further study in the future.

6PPD-Q

Another emerging contaminant of concern, 6PPD-Q, has been studied using zebrafish and has shown significant threats to aquatic organisms (Zhang et al., 2023). Many reviews on the topic of 6PPD and its by-product 6PPD-Q suggest that fish species are adversely affected. Even though this chemical has been present in both aquatic and urban ecosystems for a long time and has been detected for decades, its long-term ecological effects are still largely unknown (Hua et al., 2023). A study done by Zhang et al. (2023) examined 6PPD-Q and found that zebrafish exposed to this chemical experienced a variety of developmental toxicities and phenotypical abnormalities. Some notable phenotypical changes were convex eyeballs, the fusion of vessels, enlarged intestines, blood-coagulated guts, and activation of and over-expression of neutrophils and enteric neurons (Zhang et al., 2023). Exposures to these compounds have also resulted in an increase in induced malformations in zebrafish embryos (Zhang et al., 2023).

In western Washington, the presence of 6PPD-Q in stormwater and receiving water systems has been reported to cause acute mortality of coho salmon during their migration to urban creeks (Zoroufchi Benis et al., 2023). McIntyre et al. (2023) used Pacific salmon as an indicator species for analyzing treated and untreated stormwater runoff. When comparing untreated and treated urban runoff in the early life stages of Pacific salmon it was determined that survival and morphological development threats were reduced greatly when runoff was properly filtered using bioretention methods (McIntyre et al., 2023). An increase in the mortality of native fish species has been one of the main drivers for research on 6PPD-Q in western Washington.

Aquatic Organisms: Effects on Gene Expression:

To better understand phenotypic responses and developmental toxicities, researchers can use gene expression to explore nonlethal responses to toxic contaminants. As previously stated, the use of mechanistic developmental toxicity can help determine and confirm differential gene expression resulting from exposures to contaminants. The *cyp1a* gene is an environmental stress gene which is widely used for studying gene expression because its activity can be used to determine the presence and uptake of toxins. The expression of the *cyp1a* gene in zebrafish increases in response to contaminant levels and fish, when exposed to environmental contaminants, can benefit from downregulating the expression of *cyp1a* to reduce morphological effects (Williams et al., 2022). The aryl hydrocarbon receptor (AHR) pathway, which is a transcription factor that regulates gene expression, is associated with the activity of the *cyp1a* gene and is known to have broad biological functions such as responses to toxins/microbial pathogens, regulating immunity, stem cell maintenance, and cellular differentiation (Zhang et al., 2022 and Mulero-Navarro et al., 2016). Zhang et al. (2022) in a review stated the importance of the current advances in understanding AHR mechanisms and xenobiotic response elements (*cyp1a*) and that many studies have shown that toxic effects are predominately mediated through interactions with this pathway.

Polychlorinated biphenyls (PCBs) and cyp1a in zebrafish

For example, researchers have studied the effects of polychlorinated biphenyls (PCBs) on the embryotic development of zebrafish (Roy et al., 2019). One study used various concentrations of PCBs to determine the toxicity potential of the expression of the *cyp1a* gene, morphology (form and structure), and liver development in zebrafish embryos (Roy et al., 2019). It was determined that although morphology was not altered at low concentrations, there were significant effects on both liver development and transcription of xenobiotic (foreign chemicals/toxins) metabolism (*cyp1a*, gene expression) that reduced liver growth and increased hepatocyte formations (the dominant cell type in the liver, where formations are a result of the inability to carry out desired function in liver growth; Roy et al., 2019). At low concentrations, liver growth was reduced and the xenobiotic pathway, which metabolizes foreign chemicals and toxins, was induced meaning that the *cyp1a* gene was activated. Again, this gene is specific to metabolizing toxins in the body. This study shows that although morphological effects might require higher concentrations to display changes, such concentrations can start to alter the AHR pathway and hinder fishes' ability to modify the toxicity of compounds early on.

6PPD-Q and cyp1a in zebrafish

Zhang et al. (2023) also determined that 6PPD and 6PPD-Q resulted in specific toxic phenotypes that have distinct differential gene-associated expressions. Notably, 6PPD-Q activated the adaptive cellular response xenobiotics gene, *cyp1a*. This study is interesting to consider because it identified that non-lethal doses of both 6PPD and 6PPD-Q in the environment can still result in developmental toxicities (Zhang et al., 2023). Thus, it is important to consider that even at sublethal concentrations of 6PPD-Q there are various toxicity consequences for aquatic organisms. Further research is needed to determine just how the AHR pathway is affected under an array of environmental toxins to determine compound-specific effects and detailed preliminary mechanisms of toxicity. One method of doing this is by producing synthetic stormwater.

Synthetic Stormwater

This thesis will examine common fluids and particles associated with urban stormwater runoff. These metals and other trace elements can be highly toxic to fish. This section will review studies on both particle and fluid components of stormwater, specifically the components this thesis will examine. The components covered in this section are brake particles, exhaust particles, diesel exhaust particles, and tire particles as well as several fluid components including brake fluid, motor oil, and washer fluid (associated solvents such as dimethlysulfoxide, methanol, and dimethylformamide). For this thesis, tire particles and used motor oil will be used as individual components of synthetic urban stormwater runoff to test developmental toxicity in zebrafish by analyzing the transcriptional gene, *cyp1a*.

Synthetic Stormwater: Particle Components

Brake Particles

Vehicles contribute a large portion of metals and other deposited materials in urban runoff, which are often released when a vehicle brakes (Wiseman, 2017). The brakes of vehicles include heavy metals that can be turned into particles of heavy metals during braking. These heavy metal particles can accumulate in rivers, lakes, and oceans and have a negative influence on aquatic ecosystems. A review done by Arole et al. (2023) assessing the particles generated from the mechanical systems of vehicles found that the majority of brake debris was composed of traces of heavy metals (zinc, iron, copper, barium etc.) and organic compounds such as PAHs. These particles are transported into aquatic ecosystems and surrounding soils via runoff and are the most common cause of physiological damage in fish species and algae (Arole et al., 2023).

Because brake particles can accumulate in urban water ecosystems, it is important to understand the direct effects they have on a model organism to provide baseline information for toxicity.

Exhaust Particles

Exhaust particles are another major component of vehicle emissions that have been studied for their potential toxic influences on aquatic organisms. An environmental risk assessment was completed on different aquatic trophic levels exposed to vehicle exhaust particles to determine toxic effects at various concentrations (Pikula et al., 2021). This study focused on marine diatomic microalgae, the planktonic crustacean *Artemia salina*, and the sea urchin *Strongylocentrotus intermedius*, and their results demonstrated a serious negative effect on egg fertilization of the sea urchin and growth rate inhibition with exposures to high PAH concentrations (Pikula et al., 2021). Results from this study suggest that toxicity levels can vary from species to species and among exposure types (e.g., vehicle exhaust particles alone or in mixtures with PAHs). To understand urban aquatic ecosystems, the use of a model organism in an exhaust particle exposure study could provide baseline information for potential effects broadly to fishes.

The effects of exposure specifically to diesel exhaust particles on zebrafish are particularly well-documented and have been shown to have a variety of effects. Diesel particles (which are comprised of carbon and absorbed organic compounds such as PAHs, sulfate, nitrate, metals, and other trace elements) have been shown to contribute to both acute and chronic conditions when exposed at early life stages in various organs (heart, liver, gills, brains, eyes, etc.; Manjunatha et al., 2021). The developmental toxicity effects of diesel particulate matter on zebrafish have been shown to result in increased mortality, morphology variation, reduction in hatching rates, and cardiovascular impairments such as pericardial edemas and altered heart rates (Manjunatha et al., 2021). Other morphological effects noted from diesel particulate exposure are a reduction in motor neurons (in the trunk of zebrafish) and liver defects in zebrafish embryos (Manjunatha et al., 2021). Another study evaluating biological effects from diesel exhaust particulate exposure found that exposed zebrafish exhibited dysfunctional autophagy (a cellular recycling process that is important for cell survival and maintenance) and overall loss of neurons (Jami et al., 2023). This study used transcriptional expression analysis and determined that zebrafish exposed to diesel exhaust particles experienced several alterations to biological processes and pathway signaling (Jami et al., 2023). The largest induction of gene expression in their brains was in the *cyp1a* gene (over 30-fold compared to others) and the *cyp1a* induction was determined to be a compensatory protective mechanism (Jami et al., 2023). Analyzing the developmental toxicity effects of particle pollution from vehicles can be useful when determining which elements result in higher toxicity, thus there is a need for a robust investigation.

Tire Particles

Tire particles (TP) are another complex particulate contaminant released into the environment that has been shown to have a variety of effects on aquatic species. Of the particles generated from vehicles, TPs are the leading contributor of microplastic environmental pollution and have been shown to have ecotoxic effects across ecosystems and trophic levels (Kim et al., 2023). When assessing the effects of TP leachate on zebrafish toxicity, Kim et al. (2023) found that zebrafish embryos were severely affected, and TPs induced whole-body edema with growth reduction, delayed hatching, and developmental delays. These findings are similar to other studies that have determined that zebrafish exposed to TP-leachate often display toxicity in the form of developmental delays and cardiac edema (Chibwe et al., 2022 and Cunningham et al., 2022). The ubiquitous occurrence of tire particles in the environment makes assessing their toxicity and the potential role they play critical for understanding ways to mitigate urban stormwater runoff.

Synthetic Stormwater: Fluid Components

Brake Fluid

Brake fluid can harm the environment if it is not disposed of properly because it is a hazardous material that contains glycol ethers and heavy metals. The most widely used brake fluid is DOT3, which is highly corrosive and has been determined to be acutely toxic to fish, invertebrates, and aquatic algae (Klein, 2020). There have been studies done to analyze the ecotoxicological characteristics of common chemicals associated with brake fluid such as benzotriazoles that show both acute and chronic toxicity in aquatic organisms (Seeland et al., 2012). Benzotriazoles have also been shown to alter biological processes in zebrafish associated with genes involved in oxidative stress as well as causing delayed hatching (Liang et al., 2019). Another study exposed adult zebrafish to benzotriazoles to examine liver tissues to evaluate oxidative damage in hepatotoxicity (Hemalatha et al., 2020). This study showed that exposed adults experienced a variety of diseases within the liver tissues (lesions such as hypertrophy, cellular and nuclear enlargement, nuclear and cytoplasmic degradation, necrosis, etc.; Hemalatha et al., 2020). When using molecular analysis, these scientists determined that the transcription of the *cyp1a* gene was upregulated throughout the exposure duration (Hemalatha et al., 2020). These results suggest that oxidative stress, even at low doses of benzotriazole, can occur and over time can induce liver damage in zebrafish. When analyzing a variety of genes associated with different toxicological pathways in zebrafish, Fent et al. (2014) determined that the expression of the *cyp1a* gene experienced significant induction when exposed to benzotriazole. Although benzotriazole has been studied, the biological effects of brake fluid as a whole are still understudied in fishes and further research is needed to understand the direct effects it may have on developing embryos.

Motor Oil

Motor oil can accumulate in aquatic ecosystems in the form of used or unused oil entering through stormwater which can have adverse effects on fish. Synthetic motor oils have been manufactured by polymerizing short-chain hydrocarbons which makes them a complex environmental contaminant when discharged into aquatic systems. Sisman et al. (2016) investigated the toxicity of 10W-40 motor oil (widely used) on the early life stages of zebrafish and analyzed morphological endpoints. This study included both acute lethal and sublethal effects and overall determined that an increase in oil concentration resulted in higher embryonic malformation rates at early stages of development (Sisman et al., 2016). The acute lethal effects were associated with higher concentrations and lower concentrations demonstrated sublethal morphological effects such as head malformation, pericardial edemas, vertebra column defects and scoliosis, tail curvature, incomplete gastrulation, and weak pigmentation (Sisman et al., 2016). The embryonic development of zebrafish was noted to be greatly reduced, which resulted in delayed hatching and decreased body length (Sisman et al., 2016). Similarly, crude oil has been studied to understand the effects that conventional and unconventional crude oil have on zebrafish optomotor behavior and transgenerational differences in gene expression (Philibert et al., 2021). One study used different ratios of crude oil with unique volatile organic compounds and polycyclic aromatic compounds. The biological effects differed for each oil type (one oil decreased eye size, another altered behavioral responses, and each had their own effect on basal gene expression of the somatically exposed offspring; Philibert et al., 2021). These results indicate that chemical composition plays a role in determining the sublethal toxicity of motor oils when introduced to aquatic environments. However, relatively few studies have characterized the toxicity of motor oil which makes the need for further research critical for understanding the risk to freshwater aquatic environments.

Windshield Washer Fluid

Washer fluid, another poisonous substance that contains methanol, a toxic alcohol, has also been understudied and it may have unique effects on zebrafish. Due to the wide use of methanol, it can be found in stormwater and has become an environmental contaminant that has been shown to have negative effects on aquatic biota (Rico et al., 2006). Methanol is a neurotoxic compound and a study done by Rico et al. (2006) determined that acute exposures can result in inhibitory effects on enzymes that may contribute to neurodegenerative events. Another study examined the xenobiotic metabolism (the detoxification process that results in the expression of the *cyp1a* gene) in zebrafish when exposed to dimethlysulfoxide (DSMO) and methanol, both of which are widely used solvents (David et al., 2012). This study demonstrated that the metabolism of ethoxyresorufin (the fluorogenic substrate for, and competitive inhibitor of the cytochrome p450, CYP isoform *cyp1a*) was significantly reduced in both solvents (DSMO and methanol; David et al., 2012). These findings were supported by PCR results that showed a reduction in the expression of genes coding for drug-metabolizing enzymes, such as *cyp1a* (David et al., 2012). These results suggest that there are adverse solvent effects on aquatic organisms and should be further studied because it may not be the only way that gene expression is regulated.

Similarly, DSMO has also been shown to have effects on zebrafish behavior, acute toxicity, and developmental malformations in embryos (Christou et al., 2020 and Kim et al., 2021). Another study exposed zebrafish embryos to dimethylformamide (DMF) and DMSO to determine survival, development, and variation in expressed genes (Turner et al., 2012). Although survival and development did not experience any effects at concentrations up to 2.0 and 16.0 ml/L for DMF and DMSO, respectively, expressed genes differed significantly at concentrations well below the 0.1 ml/L solvent limit level (Turner et al., 2012). This study highlights the importance of using transcriptional biomarkers to identify sublethal effects and then apply those to observed morphological effects at higher concentrations (Turner et al., 2012). Again, analyzing genes associated with known toxicological pathways in zebrafish such as the *cyp1a* gene will allow for a better understanding of sublethal effects and their potential toxicity outcome in freshwater ecosystems.

Conclusions:

The above particle and fluid components are comprised of various chemical additives and heavy metals that can contaminate both water and soil sources and in turn, harm aquatic ecosystems. However, few studies, if any, have directly compared a particle component to a fluid component at sublethal conditions. To build off previous findings within the literature, I will investigate the sublethal effects of gene expression for tire particles and used motor oil on zebrafish. Using one particle and one fluid component as a source of synthetic stormwater will continue previous research as well as provide new findings for areas that have not been well-studied.

Methods

To address the research questions outlined above, zebrafish exposures to tire particles and used motor oil fluid were performed at the Puyallup Extension Campus of Washington State University (WSU) in Puyallup, WA. First, sublethal and lethal curves were generated for both tire particles (TP) and used motor oil (UMO) to determine the optimal concentrations for sublethal exposures. Zebrafish toxicity tests were conducted as 24–48-hour exposures for each treatment (TP and UMO) and a control in triplicate over the course of 12 weeks (January 2024-March 2024). Treatments at various levels for each contaminant (TP and UMO) were compared to each other and to controls. All of these procedures will be described in further detail below.

Chemicals and Reagents (developing sublethal concentrations)

Used motor oil was obtained from a nearby auto shop and set as a water-associated fraction (WAF). The WAF of the UMO was prepared according to a National Oceanic and Atmospheric Administration protocol (NOAA Technical Report, 2015). To summarize, the WAF was created by diluting motor oil with embryo medium (a salt-concentrated liquid designed for fish growth) to get a concentration of 1%. The oil and embryo medium (substituting reconstituted water) were placed in a bottle and slowly stirred at 20 rpm for a total of 20-24 hours at room temperature. After mixing the WAF, the solution was run through a separatory funnel and used as a 1% stock solution. Different concentrations (1%, 0.56%, 0.32%, 0.18%, and 0.10%) were prepared in flasks from the 1% stock solution and were used in the experiment to determine sublethal and lethal ranges. The WAF of the UMO concentrations was selected according to previously determined sublethal ranges from previous lab exposures (McIntyre et al., unpublished data). Since lethal effects were seen for the 1% concentration, a 0.50% concentration of stock was next created. Using the same protocol, final concentrations were diluted (0.5%, 0.28%, 0.16%, 0.09%, and 0.05%) to target the sublethal ranges.

Tire Particle Leachate Protocol

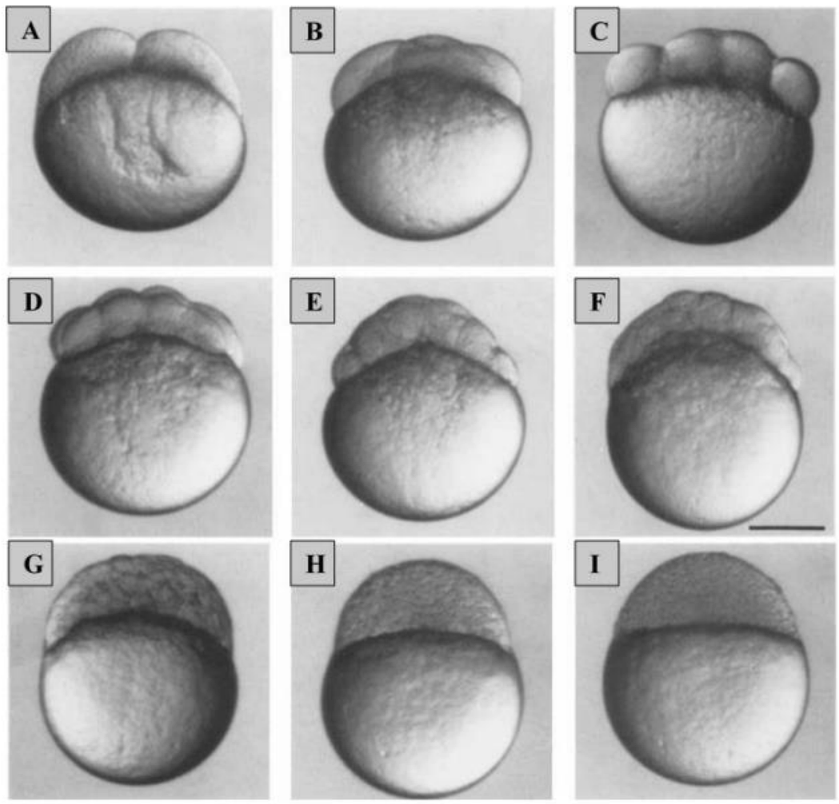
Tire particles were obtained from a commercial supplier and were sifted through a 500-micron filter. The sorted particles were then mixed with embryo medium for the following concentrations: 10g/L, 3.2g/L, 1g/L, 0.32g/L, and 0.10g/L. These concentrations were determined based on previous sublethal findings that determined a range that likely exceeds the solubility of some tire chemicals (McIntyre et al., unpublished data). The individual stocks of TP and embryo medium were placed in bottles and slowly stirred at 20 rpm for a total of 24 hours to create TP leachate. After 24 hours the solutions were stored in an incubator to reach the appropriate temperature and then the leachate containing the particles was used for the exposures.

Fish Source and Culture

The zebrafish were raised and collected in the EcoTox Lab at the Puyallup Extension Campus of WSU in Puyallup, WA. For egg collections, 2-3 females and males were selected for spawning 24 hours before the day of exposure. The morning before collection, the water was changed to ensure old eggs were disposed of and a freshwater change was supplied to the spawning fish. After the water change, the zebrafish were allowed to spawn for two to three hours. Eggs were collected, rinsed, and placed into petri dishes and held in the incubator at 28.5 °C until sorted. Eggs were sorted and selected based on the guidelines in Kimmel et al. (1995). From the guidelines, eggs were selected between 2-2.5 hours post-fertilization. Figure 1 was used as a reference for egg selection, with ideal eggs exhibiting traits closest to images D-H. Eggs were chosen that had even cell division and were symmetrical to ensure healthy eggs were used in the experiment. To create randomized egg selections, eggs were sorted into 12-well plates (three replicates for each treatment and control) until a total of 40 eggs were met for each individual well/treatment. The 12-well plates were covered and were placed in the incubator at 28.5 °C until exposures started.

Figure 1.

Zebrafish stages of embryo development



Note. Stages of zebrafish embryo development. Embryos were sorted for stages near or between 2-2.5 hours post fertilization.

Exposure Experiments: Zebrafish Toxicity Testing

To determine if there was a toxic response of aquatic organisms to either particle or fluid toxins, zebrafish embryos were exposed to UMO and TP. Treatments were set up 24 hours prior to exposure and placed in an incubator in the morning on the day of the exposure. The embryos were transferred from the 12-well plates into individual petri dishes for exposure. Treatment for each petri dish was determined by a computer-generated randomizer and labeled accordingly. For each treatment group, 15 mL of the exposure fluid was added to each dish and they were stored in the incubator for 24 hours. At 24 hours a water change was performed for each treatment and embryos were noted for mortality. The preparation and storage of samples was completed at 48 hours. Each treatment underwent assessments for live embryos, approximate developmental stage, and morphological abnormalities, with any deceased or severely deformed individuals being removed (Table 2). Deceased or severely deformed embryos were not used for qPCR. Samples were placed on styrofoam to keep them warm while checking embryo status. Embryos from each dish/replicate were transferred to a labeled conical 1.5 mL microcentrifuge tube. For each replicate, embryos were rinsed in embryo medium to remove excess treatment residue from the samples. After excess water was removed from the tubes, each tube was flash-frozen using an acetone and dry ice bath under a fume hood. Samples were kept in the -80 °C freezer until ribonucleic acid (RNA) extraction.

Molecular Indicator

For this experiment the cytochrome P450-1a (*cyp1a*) gene was used as an indicator of exposure to specific groups of contaminants. The *cyp1a* gene was selected because it encodes a key component of the aryl hydrocarbon receptor (AhR)-mediated polycyclic aromatic hydrocarbons (PAHs) metabolism pathway (McIntyre et al., 2016). This gene is expressed throughout the body during embryogenesis and is an ideal transcriptional biomarker. The reference gene WD and tetratricopeptide repeats-1 (*wdtc1*), were used to normalize gene expression levels to a gene that is expressed stably across the study groups (McIntyre et al., 2016). This reference gene is expressed evenly throughout zebrafish development and was used to get an accurate interpretation of Real-Time Quanitative Polymercase Chain Reaction (RT-qPCR) results.

qPCR Protocol and RNA Extractions

Total RNA was extracted from pools of flash-frozen larvae by mechanical homogenization (TissueLyser, Invitrogen, Inc.) in TRIzol Reagent (Invitrogen Inc.) and subsequently purified and DNase-treated with DirectZol spin-columns (Zymo, Inc.). Quantitative PCR (qPCR) reactions (20 μL) were run in triplicate using Fast SYBR Green chemistry (Applied Biosystems, Inc.), 10 nM primer, and 50 ng template on a Viia7 Real-Time qPCR Detection System (Applied Biosystems Inc.) under fast-cycling conditions (95 °C for 2 min, and then 40 cycles at 95 °C for 1 s and 60 °C for 20 s). For each experiment (UMO and TP) a total of 168 PCR samples were analyzed for both the *cyp1a* gene and the reference gene *wdtc1*. Information on primers is described in Table 1. Files were downloaded from the qPCR machine through the Applied Biosystems report and downloaded to the ThermoFisher Applied Biosystems connect. Curves were generated as the terminal step of all qPCR reactions to ensure single product amplification for each. The standard curves were then created for quality assurance purposes for each plate to determine their associated efficiencies (Figures 2, 3, 4, and 5). The standard curve analysis was conducted to minimize errors and maximize data quality to ensure that values were within the target concentration.

Table 1.

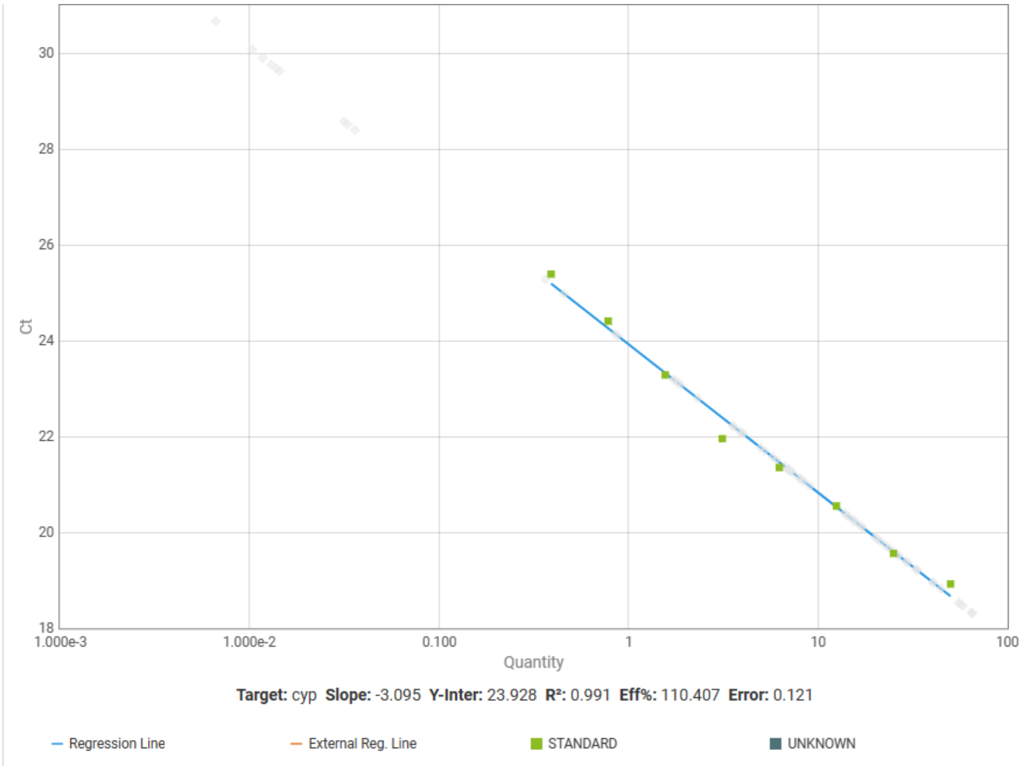
Gene Sequencing and Primers

Gene	Symbol	**Sequence (5'-3')**	**NCBI accession**	**Published primer**
Cytochrome P450, family 1, subfamily A	*Cyp1a*	F: GGGAAAGAGTCCCAAATATTCC R: CTCATATTAACCAGTCGCACCA	NM_131879.1	McIntyre et al., 2016a
WD and tetratricopeptide repeats 1	*Wdtc1*	F: GCAGCGCTCTTCTCCAAAAC R: CGACTCCTTCCGGCTGAAAT	NM_001130606.1	McIntyre et al., 2016a

Note. Gene sequencing and primers (Gene: *cyp1a* and reference gene, WD and tetratricopeptide repeats-1 (*wdtc1*).

Figure 2.

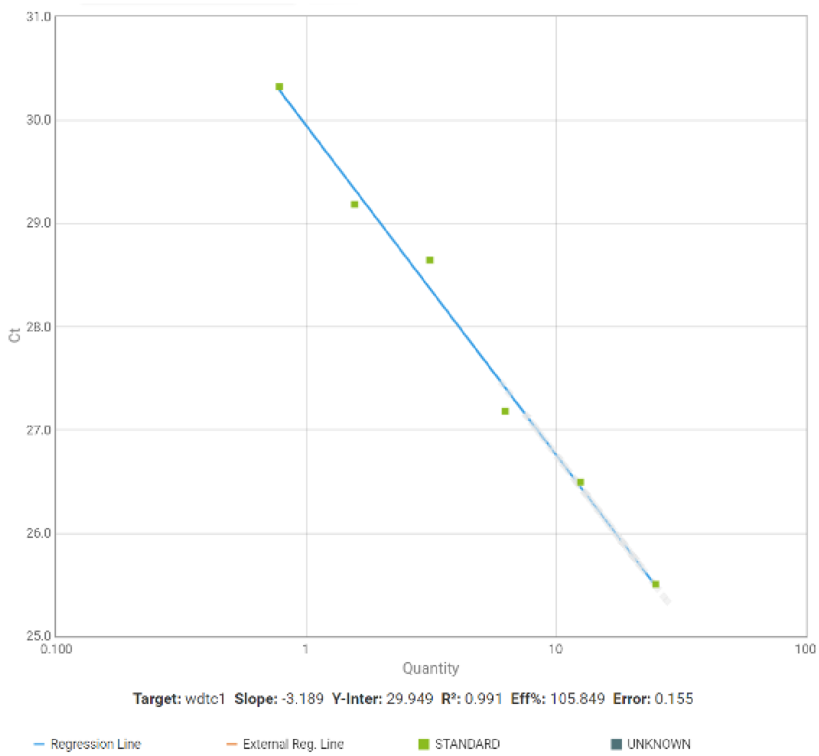
Used Motor Oil Standard curve, sample gene



Note. Used motor oil- Standard Curve for *cyp1a*: This was done to determine the efficiency of the PCR plate.

Figure 3.

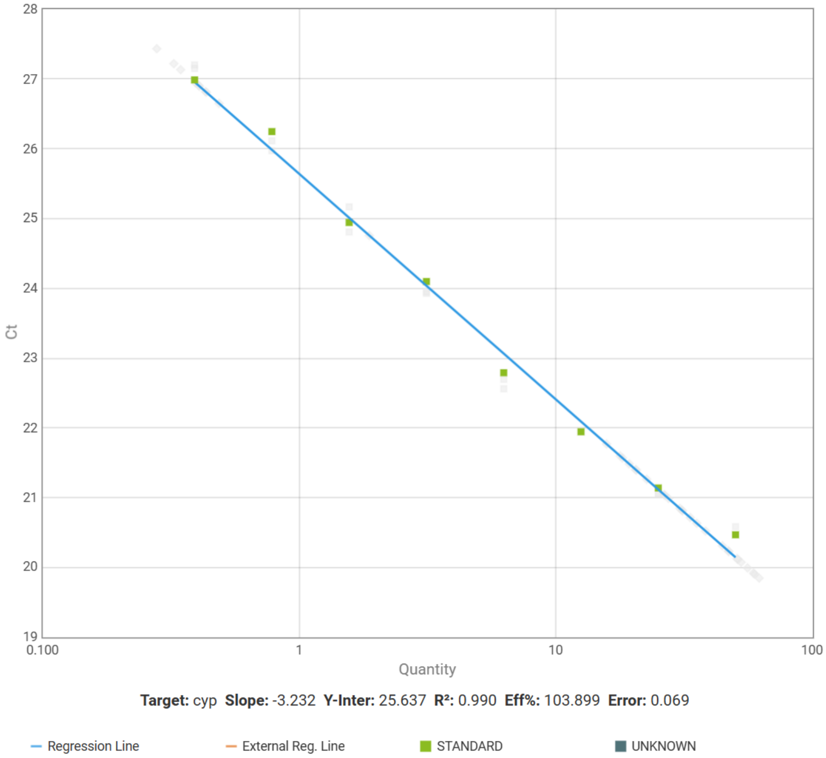
Used Motor Oil Standard curve, reference gene



Note. Used motor oil-Standard Curve for *wdtc1*: This was done to determine the efficiency of the PCR plate.

Figure 4.

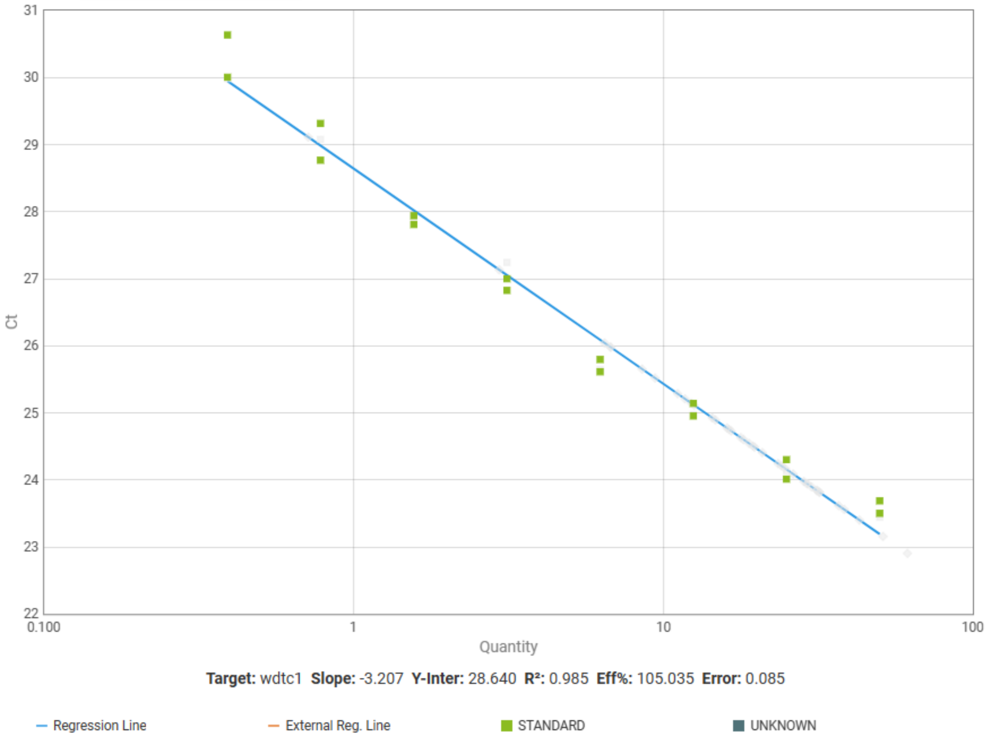
Tire Particle Standard curve, sample gene



Note. Tire particles-Standard Curve for *cyp1a*: This was done to determine the efficiency of the PCR plate.

Figure 5.

Tire Particle Standard curve, reference gene



Note. Tire particles-Standard Curve for *wdtc1*: This was done to determine the efficiency of the PCR plate.

Statistical Analysis

For the qPCR results the cycle threshold (CT) values were reported from Applied Biosystems 7500 and used to calculate normalized qPCR results. Gene transcription fold-changes were calculated using the Delta-DeltaCT (ddCT) method to determine gene expression of *cyp1a* for each treatment. Gene expression data (ddCT values) were assessed using a Levene's test to determine equality of variances across the control and treatment groups. A Shapiro-Wilk test was conducted for each treatment group to test for normality within the sampled populations. All assumptions were satisfied for the used motor oil (UMO) experiment; however, they were not met for the tire particle (TP) study, necessitating the utilization of a permutational analysis of variance instead. A one-way Analysis of Variance (ANOVA) with a Tukey-Kramer post hoc test were conducted to compare the means of all treatment groups for the UMO experiment. A one-way permutational ANOVA with a Tukey-Kramer post hoc test were conducted to compare the means of all treatment groups for the TP experiment. All statistical tests were performed in R (4.3.3). Statistically significant results were based on alpha < 0.05.

Results:

Used Motor Oil (UMO) Experimental Characteristics

Throughout the takedown process of the exposures to used motor oil, each treatment underwent assessment for mortality, hatch rate, and general morphological anomalies to identify and remove severely deformed or deceased individuals (see Table 2). While mortality rates across experimental groups remained consistent, the highest concentration exhibited the highest individual mortality count. Conversely, the control group displayed the highest hatch count, whereas no individuals hatched in the highest treatment. Furthermore, greater observations of pericardial edemas, pigmentation delays, yolk sac edemas, brain bleeds, and cardiac defects were observed with increasing concentrations of used motor oil.

Table 2.

Used Motor Oil Experimental Characteristics

Treatment: UMO	Average Mort	Hatch Count	Notes: 48 hours post fertilization
Control	3	14	48 hpf, no abnormalities
0.05%	3	10	42-48 hpf, mild pericardial edemas (PCE) present, pigment delays, bleeding near heart
0.09%	2	9	42 hpf, PCE present in all, more bleeding near the heart
0.16%	1	1	42 hpf, PCE severe, pigment delay, brain hemorrhaging
0.28%	4	1	40-42 hpf, PCE, un-looped hearts, Yoke sack edemas, brain bleeds
0.50%	4	0	30-35 hpf, severely deformed, spinal deformities, PCE, pigment delays, brain bleeds, un-looped hearts

Note. For each biological replicate, mortality, hatched counts, and general morphological abnormalities were noted at the time of experimental takedown (48 hours post fertilization (hpf)).

Tire Particle (TP) Experimental Characteristics

Throughout the takedown of the exposures to tire particles, each treatment underwent assessments for mortality, hatch rate, and general morphological characteristics (refer to Table 3). Mortality rates remained unaffected until concentrations exceeded 3.2 g/L, with the highest concentrations resulting in lethality. Conversely, no deaths were recorded in the control group, as well as in treatments at 0.01 g/L, 0.32 g/L, and 1 g/L. Hatch rates were highest in the control group and appeared unaffected until treatments surpassed 3.2 g/L, where no individuals hatched. Furthermore, greater observations of pericardial edemas, pigmentation delays, decreased movement, slower heart rate, body deformities, scoliosis, and overall malformations were observed with increasing concentrations of tire particles.

Table 3.

Tire Particle Experimental Characteristics

Treatment: TPs	Average Mort	Hatch Count	Notes: 48 hours post fertilization
Control	0	14	48 hpf, no abnormalities
0.01 g/L	0	2	48 hpf, slight pericardial edemas (PCE) in most individuals
0.32 g/L	0	2	42-48 hpf, PCE present, and pigment delayed,
1 g/L	0	13	42-48 hpf, PCE present, scoliosis, pigmentation delay, slower response/movement
3.2 g/L	10	0	40 hpf, PCE in all, slow heartbeat, lack of movement, scoliosis, and malformations
10 g/L	40	0	Each individual died during exposure, severely deformed and delayed

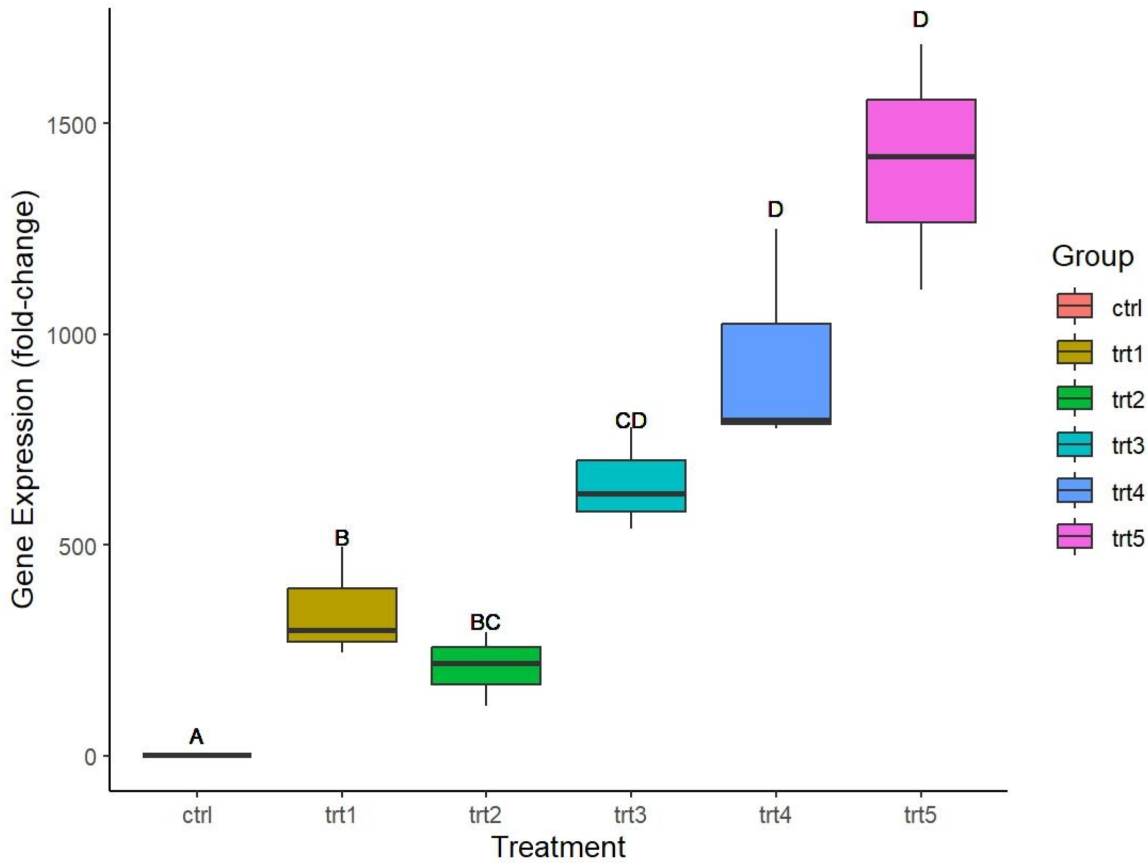
Note. For each biological replicate, mortality, hatched counts, and general morphological abnormalities were noted at the time of experimental takedown (48 hours post fertilization(hpf)).

Used Motor Oil (UMO) *cyp1a* Gene Expression

Gene expressions were measured in terms of fold-change. Gene expression of *cyp1a* varied significantly with different levels of used motor oil (UMO) ($F_{5,12} = 139.4$, $p < 0.05$; Figure 6). The higher UMO concentrations yielded significantly higher gene expression of *cyp1a* than lower UMO concentrations. Additionally, there is a significant difference in gene expression for each treatment when compared to the control ($p < 0.01$). In comparing treatments to one another, treatment 1 was significantly different from treatments 4 and 5 (p adjusted < 0.05). Similarly, treatment 2 was statistically different from treatments 3, 4, and 5 (p adjusted < 0.05). However, treatment 3 was only significantly different from treatment 2 (p adjusted < 0.05, all other p > 0.05). Overall, treatment 5 had the highest level of gene expression and treatment 2 had the lowest level of gene expression (Figure 6). Each treatment had a positive value indicating that UMO treatments increased *cyp1a* gene expression and that higher doses resulted in higher upregulation of *cyp1a* (Figure 6).

Figure 6.

Gene Expression for Used Motor Oil Treatments



Note. Mean gene expression of *cyp1a* (upregulation) across used motor oil treatments (n=3 pooled samples of 35-40 fish per pool per group, ANOVA with upper case letters denoting significant differences based on Tukey's post-hoc test, p<0.05). The treatment groups listed below are control (ctrl), 0.05% (trt1), 0.09% (trt2), 0.16% (trt3), 0.28% (trt4), and 0.50% (trt5).

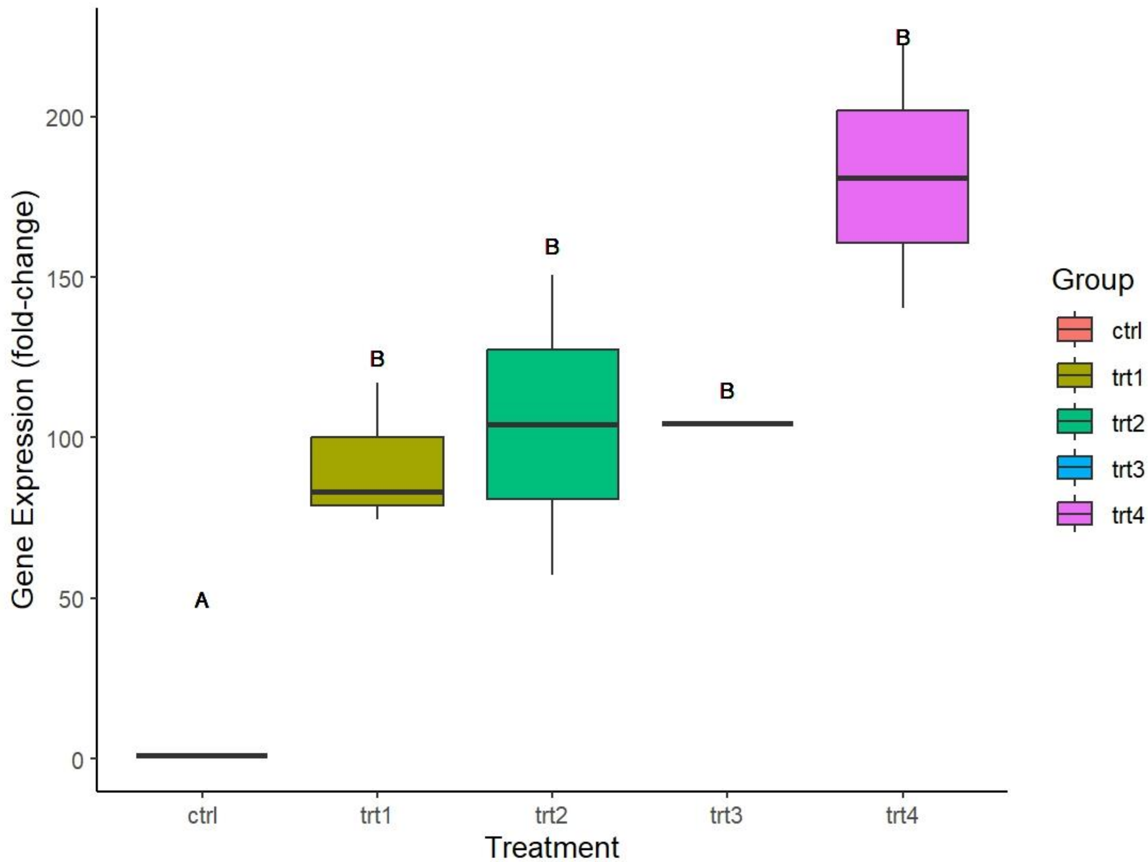
Tire Particle (TP) *cyp1a* Gene Expression

Cyp1a gene expression also varied significantly with different levels of tire particles (TP) ($F_{4,7}$ = 70.63, $p < 0.05$; Figure 7). All the treatments were significantly different from the control (p adjusted < 0.05). The treatments yielded a significantly higher fold-change for *cyp1a* gene expression than the control group. In comparing the treatments to one another there were no significant differences in gene expression among treatments (Figure 7; p adjusted > 0.05).

Although the treatments were significantly different than the control fold-changes, the concentration of TP used in this experiment did not demonstrate significantly different *cy1pa* expressions. Overall, treatment 4 had the highest level of gene expression and treatment 1 had the lowest level of gene expression (Figure 7). Each treatment had a positive value indicating that treatment increased *cyp1a* transcription resulting in upregulation of the gene (Figure 7).

Figure 7.

Gene Expression for Tire Particle Treatments



Note. Mean gene expression of *cyp1a* (upregulation) across tire particle treatments (n=3 pooled samples of 35-40 fish per pool per group, n=2 for treatment 2, and n=1 for treatment 3, permutational ANOVA with upper case letters denoting significant differences based on Tukey's post-hoc test, $p<0.05$). The treatments listed below are control (ctrl), 0.01 g/L (trt1), 0.32 g/L (trt2), 1.0 g/L (trt3), and 3.2 g/L (trt4).

Discussion

The primary objective of this thesis was to investigate the effects of two synthetic stormwater components on the expression of the environmental stress gene, *cyp1a*, in zebrafish within sublethal concentration ranges. Analysis revealed noteworthy findings regarding the effects of these components on gene expression levels. Notably, exposure to sublethal concentrations of used motor oil exhibited a significant dose-dependent increase in *cyp1a* gene expression, indicating a biological response within the targeted sublethal range. The observed overlap in average levels of gene expression across certain concentrations suggests a reasonable delineation of the sublethal range for this experimental exposure. Conversely, tire particle exposure exhibited minimal differences in gene expression across treatments which were equally expressed; however, all treatment groups exhibited significantly elevated levels of *cyp1a* expression compared to controls. In both cases the sublethal range was not well-designed and the lowest concentrations exhibited *cy1pa* expressions that were too high to be considered at an appropriate sublethal range. Nevertheless, these results collectively showcase the significant induction of gene expression by both synthetic stormwater components, highlighting the potential environmental effects of these pollutants on aquatic organisms.

The findings presented in this study will be contextualized and interpreted for the broader scientific community below. This discussion aims to explain the significance of our research outcomes, provide insights into the underlying mechanisms driving observed phenomena, and offer implications for future research directions in the field. By critically evaluating our findings in the context of existing literature and theoretical frameworks, we can discern key patterns, identify potential limitations, and underscore the novelty and significance of our contributions to the advancement of scientific understanding in the field of aquatic toxicology.

Used Motor Oil

In this experiment, our results suggest that an even narrower sublethal range could be delineated to precisely assess the influence of UMO on the expression of *cyp1a*. Initially, a 1% water-accommodated fraction (WAF) was chosen for the sublethal range; however, it became apparent that lethality was observed within each treatment, ranging from 1% to 0.10%. Consequently, a more refined concentration of 0.50% stock solution was employed. Despite this adjustment, sublethal effects remained predominant across all exposures but with varying levels of *cyp1a* gene expression observed within each exposure group.

Across all treatments of used motor oil, a consistent increase in the expression of the *cyp1a* gene was observed. Notably, the toxicity test revealed that the lowest concentration (0.05%) of the water-accommodated fraction (WAF) exhibited the lowest expression of the *cyp1a* gene. Surprisingly, however, this treatment still displayed elevated *cyp1a* gene expression compared to the control, indicative of potential toxicity throughout the exposure period and subsequent assessment. When establishing a sublethal range for gene expression analysis, it is essential to ensure that the lower end of the concentration range exhibits some degree of overlap or proximity to the control group. When examining treatment 1 (0.05%-lowest concentration), the fold-change was determined to be 345-fold higher than that of the control group. Given its significantly higher fold-change, this dose does not accurately reflect the lower sublethal range capable of inducing *cyp1a* expression, suggesting that a lower dosage would be more appropriate. Roy et al. (2019) conducted a study investigating the effects of PCB-11 concentrations at the lower end of the sublethal range on 96-hour post-fertilization (hpf) zebrafish embryos. Their findings indicated that concentrations of 0.2 μM and 2 μM PCB-11 did not significantly alter *cyp1a* transcription, whereas a concentration of 20 μM PCB-11 led to a significant 2.6-fold increase in *cyp1a* transcription. This narrower concentration range provides a more accurate representation of the concentrations that induce *cyp1a* transcription closest to control levels. Additionally, Roy et al. (2019) included assessments of gross morphology to facilitate comparison between gene expression and developmental abnormalities. This study lends support to the notion that incorporating lower concentrations may enable a more precise evaluation of the induction of *cyp1a* at levels comparable to non-exposed zebrafish.

Incorporating lower concentrations could be further enhanced by conducting concurrent morphological assessments, facilitating a comparative analysis of responses to contaminant exposures and offering insights into the correlation between gene expression and morphological abnormalities. While our zebrafish toxicity study solely documented morphological observations during the takedown process, valuable conclusions can still be drawn from these findings. Notable toxicity traits observed in the 0.05% treatment included slight pericardial edemas (PCE), mild manifestations of early scoliosis, but no discernible effects on overall development. Given the intricate composition of motor oil and the manifestation of morphological abnormalities even at the lowest concentrations, it is plausible to attribute the observed toxicity response in each treatment to contaminant exposure. Notably, the highest concentration of the WAF (0.50%) led to an escalation in spinal deformities, PCE, cardiac injuries (un-looped hearts), brain hemorrhages, delayed pigmentation, and overall developmental delays. Conversely, lower concentrations exhibited fewer morphological effects, suggesting a dose-dependent relationship between motor oil concentrations and toxicity outcomes. Sisman et al. (2016) conducted a morphological assessment on zebrafish embryos exposed to water-accommodated fractions (WAF) of motor oil at concentrations ranging from 1.25 to 40.0% (v/v). Their findings revealed that lower concentrations of motor oil exhibited sublethal effects, including tail curvature, weak pigmentation, head malformations, pericardial edemas, scoliosis, and incomplete gastrulation.

Conversely, higher concentrations were associated with acute toxicity, leading to lethality at elevated doses. Based on these findings from Sisman et al. (2016), this study was conducted across a spectrum that prioritized acute toxicity assessment and higher sublethal doses, revealing comparable morphological abnormalities to those observed within our experiment. For our study, the presence of contaminant exposure effects even at the lowest concentration underscores the potential need for a lower sublethal range to further refine toxicity assessments. Nevertheless, these findings offer valuable insights into establishing an appropriate sublethal range for motor oil exposure in zebrafish studies.

The insights gained from gene expression levels and observations made during the exposure period could be further enriched through replication of this study. A notable limitation of this study is that morphological assessments were only conducted at the time of exposure takedown and not systematically throughout a second exposure. Repeating the study under identical parameters while targeting a sublethal range for *cyp1a* expression would enable a comparative analysis of morphological anomalies alongside gene expression levels. Despite this limitation, this study provides valuable data on the relationship between motor oil concentrations and the expression levels of a crucial environmental stress gene.

Tire Particles:

This experiment suggests the potential for refining the sublethal range for tire particles in influencing the expression of *cyp1a*. Concentrations ranging from 10 g/L to 0.01 g/L were utilized; however, certain challenges within each treatment indicate the need for a more optimal sublethal range. Notably, at the highest concentration, all biological replicates experienced mortality within the 48-hour exposure period. Despite this, sublethal traits were still prominent within each exposure group, with minimal variation observed in *cyp1a* gene expression levels among treatments.

Each treatment within the tire particle exposure exhibited markedly higher levels of gene expression compared to the control groups. Despite the elevated expression levels, no significant differences were observed among the treatment groups. The toxicity test revealed that the lowest concentration (0.01 g/L) experienced the lowest average expression of the *cyp1a* gene, while the highest surviving concentration (3.2 g/L) demonstrated only a two-fold increase in expression. The 0.01 g/L treatment exhibited higher *cyp1a* expression than the control and exhibited signs of toxicity throughout the exposure period and subsequent takedown. When comparing treatment 1 to the control, treatment 1 exhibited a relative increase of 91-fold, indicating a substantial disparity between the control and the initial experimental treatment. While gene expression assessments for *cyp1a* using tire particles (TPs) and zebrafish remain scarce, other studies have examined gene expression due to polycyclic aromatic hydrocarbons (PAHs), which can leach from TPs (Xie et al., 2023). Notably, when examining concentrations of various PAHs at 100 μg/L, reported levels of *cyp1a* induction ranged from 1 to 70-fold over control groups. These findings suggest that our study could benefit from targeting a lower concentration range to delineate a more precise range of the induction of *cyp1a*.

Further refinement of our study could involve incorporating lower concentrations of tire particles (TPs) alongside concurrent morphological assessments. This approach would enable a comprehensive comparative analysis of responses to contaminant exposure, shedding light on the correlation between gene expression and morphological abnormalities. While our zebrafish toxicity study primarily focused on morphological observations conducted during the takedown process, these findings still offer valuable insights and can contribute to our understanding of the effects of TPs on zebrafish health. Notable toxicity traits observed at the lowest concentration included mild pericardial edema and early stages of blood pooling near the yolk sac, although overall developmental delays were not observed. Conversely, the highest concentration of tire particles (3.2 g/L) elicited significant morphological effects, including developmental delays, scoliosis, pericardial edema, and delayed heartbeat. Comparatively, lower concentrations displayed fewer morphological abnormalities, while higher concentrations were associated with significantly higher rates of abnormalities. Cunningham et al. (2022) investigated the effects of tire particles (TPs) and leachate exposures on zebrafish embryos, revealing both chemical and particle-specific toxicity. While the particle size and exposure types differed from those in our study, their findings indicated that concentrations of TPs or leachate ranging from 0 to 3.0 × 10^9 particles/ml and 0–100%, respectively, induced greater mortality and sublethal malformations in smaller particle exposures. Notably, TP leachates at concentrations of 80% or higher exhibited abnormalities such as malformed jaws, snouts, eyes, and yolk sac edemas. Moreover, their study demonstrated that toxicity was exacerbated when comparing microparticles (larger) to nanoparticles (smaller), resulting in higher mortality and developmental abnormalities such as pericardial edemas, albeit without altering hatching rates.

Drawing from the findings of Cunningham et al. (2022), our study similarly observed an array of TP exposures associated with both lethal (mortality) and sublethal effects (morphological abnormalities). While some similarities were noted between our research and theirs, these results underscore the critical importance of identifying differential toxicities across TP concentrations and the chemicals they leach. In our study, however, contaminant exposure effects did not significantly differ across TP exposures, and the lowest concentration still elicited notable effects, highlighting the necessity of further refining our sublethal range for a more precise toxicity assessment.

Despite the lack of variation in gene expression between the lowest and highest concentrations of TPs, both experienced indications of contaminant exposure in treatment. Therefore, a lower sublethal range could be targeted to refine the assessment of differences in both gene expression and morphological effects. Overall, these findings offer valuable insights into the effects of tire particles across a range of concentrations, providing foundational information for understanding toxicity exposures in zebrafish.

Further insights from the gene expression levels and observations made during the exposure period could be significantly augmented through replication of this study. Currently, comparative analysis is constrained to gene expression alone, with morphological assessments limited to observations made during the exposure takedown rather than a comprehensive morphometric exposure analysis. To address this limitation, future iterations of the study should incorporate a narrower subset of tire particle concentrations, including a higher concentration where particle dissolution in the liquid medium may be minimal. Additionally, adopting a more appropriate sublethal range would be beneficial. Furthermore, conducting toxicity exposures alongside gene expression assessments would enable a direct comparison between gene expression results and morphological abnormalities. Despite these limitations, this study provides valuable data on the range of tire particle exposures and their associated gene expression levels, shedding light on the responses of a highly important environmental stress gene.

Conclusion:

This research revealed a consistent upregulation of *cyp1a* gene expression across all treatments for both experimental exposures. The *cyp1a* cytochrome enzymes play pivotal roles in oxidative stress responses and xenobiotic metabolism in aquatic organisms (Stadint et al., 2020), thus supporting the hypothesis of *cyp1a* upregulation in response to chemical exposure. Notably, the highest concentrations of both used motor oil and tire particles corresponded to the highest levels of gene expression. It was anticipated that higher concentrations within the sublethal range, where the solubility of both chemicals (fluid and particle exposures) was achieved, would elicit elevated gene expression levels. While acknowledging that this study does not fully replicate environmental conditions for used motor oil or tire particle accumulation in stormwater, it nonetheless offers valuable insights into dose-response relationships, highlighting dose ranges that contribute significantly to elevated gene expression responses in a model organism.

Despite the concentrations used in this study not mirroring those typically found in the natural environment, the results shed light on the potential effects of these contaminants on aquatic ecosystems. The findings highlight the significant risks posed by the increasing presence of both tire particles and used motor oil in stormwater runoff. This research is crucial for understanding their direct effects on aquatic organisms and underscores the need for more comprehensive studies focusing on environmental levels. By evaluating the toxicological effects of these stormwater contaminants, we can gather valuable insights that can guide the design and implementation of bioretention systems and best management practices to mitigate their harmful effects. This knowledge can guide future research, providing a foundation for designing studies that more accurately reflect the environmental conditions associated with vehicular stormwater runoff, ultimately informing improved stormwater management practices.

References

Al-Reasi, H. A., Wood, C. M., & Smith, D. S. (2011). Physicochemical and spectroscopic properties of natural organic matter (NOM) from various sources and implications for ameliorative effects on metal toxicity to aquatic biota. *Aquatic Toxicology*, *103*(3), 179–190. https://doi.org/10.1016/j.aquatox.2011.02.015

Anderson, A. L., Dubanksy, B. D., Wilson, L. B., Tanguay, R. L., & Rice, C. D. (2022). Development and applications of a zebrafish (*Danio rerio*) *cyp1a*-targeted monoclonal antibody (CRC4) with reactivity across vertebrate taxa: Evidence for a conserved *cyp1a* Epitope. *Toxics*, *10*(7), 404. https://doi.org/10.3390/toxics10070404

Arole, K., Velhal, M., Tajedini, M., Xavier, P. G., Bardasz, E., Green, M. J., & Liang, H. (2023). Impacts of particles released from vehicles on environment and health. *Tribology International*, *184*, 108417. https://doi.org/10.1016/j.triboint.2023.108417

Baker, E. S., & Knappe, D. R. U. (2022). Per- and polyfluoroalkyl substances (PFAS)—contaminants of emerging concern. *Analytical and Bioanalytical Chemistry*, *414*(3), 1187–1188. https://doi.org/10.1007/s00216-021-03811-9

Banerjee, S. (2023). *Source, Distribution, and Removal of PFAS in Stormwater: Challenges & Opportunity* (Doctoral dissertation, University of California, Los Angeles).

Barbosa, A. E., Fernandes, J. N., & David, L. M. (2012). Key issues for sustainable urban stormwater management. *Water Research*, *46*(20), 6787–6798. https://doi.org/10.1016/j.watres.2012.05.029

Beasley, G., & Kneale, P. (2002). Reviewing the impact of metals and PAHs on macroinvertebrates in urban watercourses. *Progress in Physical Geography*, 26(2), 236-270.

Bodus, B., O'Malley, K., Dieter, G., Gunawardana, C., & McDonald, W. (2024). Review of emerging contaminants in green stormwater infrastructure: Antibiotic resistance genes, microplastics, tire wear particles, PFAS, and temperature. *Science of The Total Environment*, *906*, 167195. https://doi.org/10.1016/j.scitotenv.2023.167195

Center for Disease Control (2022). Polycyclic Aromatic Hydrocarbons (PAHs) Factsheet | National Biomonitoring Program https://www.cdc.gov/biomonitoring/PAHs_FactSheet.html

Center for Disease Control (2022). Per- and Polyfluorinated Substances (PFAS) Factsheet | National Biomonitoring Program https://www.cdc.gov/biomonitoring/PFAS_FactSheet.html

Chen, X., He, T., Yang, X., Gan, Y., Qing, X., Wang, J., & Huang, Y. (2023). Analysis, environmental occurrence, fate and potential toxicity of tire wear compounds 6PPD and 6PPD-quinone. *Journal of Hazardous Materials*, *452*, 131245. https://doi.org/10.1016/j.jhazmat.2023.131245

Chibwe, L., Parrott, J. L., Shires, K., Khan, H., Clarence, S., Lavalle, C., Sullivan, C., O'Brien, A. M., De Silva, A. O., Muir, D. C. G., & Rochman, C. M. (2022). A deep dive into the complex chemical mixture and toxicity of tire wear particle leachate in fathead minnow. *Environmental Toxicology and Chemistry*, *41*(5), 1144–1153. https://doi.org/10.1002/etc.5140

Christou, M., Kavaliauskis, A., Ropstad, E., & Fraser, T. W. K. (2020). DMSO effects larval zebrafish (*Danio rerio*) behavior, with additive and interaction effects when combined with positive controls. *Science of The Total Environment*, *709*, 134490. https://doi.org/10.1016/j.scitotenv.2019.134490

Clary, J., Jones, J., Leisenring, M., Hobson, P., & Strecker, E. (2020). *International stormwater BMP database: 2020 summary statistics. The Water Research Foundation: Alexandria, VA, USA.*

Cunningham, B., Harper, B., Brander, S., & Harper, S. (2022). Toxicity of micro and nano tire particles and leachate for model freshwater organisms. *Journal of Hazardous Materials*, *429*, 128319. https://doi.org/10.1016/j.jhazmat.2022.128319

David, R. M., Jones, H. S., Panter, G. H., Winter, M. J., Hutchinson, T. H., & Kevin Chipman, J. (2012). Interference with xenobiotic metabolic activity by the commonly used vehicle solvents dimethylsulfoxide and methanol in zebrafish (*Danio rerio*) larvae but not *Daphnia magna*. *Chemosphere*, *88*(8), 912–917. https://doi.org/10.1016/j.chemosphere.2012.03.018

Du, B., Lofton, J. M., Peter, K. T., Gipe, A. D., James, C. A., McIntyre, J. K., Scholz, N. L., Baker, J. E., & Kolodziej, E. P. (2017). Development of suspect and non-target screening methods for detection of organic contaminants in highway runoff and fish tissue with high-resolution time-of-flight mass spectrometry. *Environmental Science: Processes & Impacts*, *19*(9), 1185–1196. https://doi.org/10.1039/C7EM00243B

Du, Z., Hou, K., Zhou, T., Shi, B., Zhang, C., Zhu, L., Li, B., Wang, J., & Wang, J. (2022). Polyhalogenated carbazoles (PHCZs) induce cardiotoxicity and behavioral changes in zebrafish at early developmental stages. *Science of The Total Environment*, *841*, 156738. https://doi.org/10.1016/j.scitotenv.2022.156738

Fent, K., Chew, G., Li, J., & Gomez, E. (2014). Benzotriazole UV-stabilizers and benzotriazole: Antiandrogenic activity in vitro and activation of aryl hydrocarbon receptor pathway in zebrafish eleuthero-embryos. *Science of The Total Environment*, *482–483*, 125–136. https://doi.org/10.1016/j.scitotenv.2014.02.109

Grant, S. B., Rekhi, N. V., Pise, N. R., Reeves, R. L., Matsumoto, M., Wistrom, A., Kayhanian, M. (2003). A review of the contaminants and toxicity associated with particles in stormwater runoff. *Terminology*, 2(2), 1-173.

Han, G., Shi, Y., Lu, Y., Liu, C., Cui, H., & Zhang, M. (2021). Coupling relation between urbanization and ecological risk of PAHs on coastal terrestrial ecosystem around the Bohai and Yellow Sea. *Environmental Pollution*, *268*, 115680. https://doi.org/10.1016/j.envpol.2020.115680

Hemalatha, D., Rangasamy, B., Nataraj, B., Maharajan, K., Narayanasamy, A., & Ramesh, M. (2020). Transcriptional, biochemical and histological alterations in adult zebrafish (*Danio rerio*) exposed to benzotriazole ultraviolet stabilizer-328. *Science of The Total Environment*, *739*, 139851. https://doi.org/10.1016/j.scitotenv.2020.139851

Hua, X., & Wang, D. (2023). Tire-rubber related pollutant 6-PPD quinone: A review of its transformation, environmental distribution, bioavailability, and toxicity. *Journal of Hazardous Materials*, *459*, 132265. https://doi.org/10.1016/j.jhazmat.2023.132265

Hua, X., Feng, X., Liang, G., Chao, J., & Wang, D. (2023). Long-term exposure to tire-derived 6-PPD quinone causes intestinal toxicity by affecting functional state of intestinal barrier in *Caenorhabditis elegans*. *Science of The Total Environment*, *861*, 160591. https://doi.org/10.1016/j.scitotenv.2022.160591

Jami, M. S., Murata, H., Barnhill, L. M., Li, S., & Bronstein, J. M. (2023). Diesel exhaust exposure alters the expression of networks implicated in neurodegeneration in zebrafish brains. *Cell Biology and Toxicology*, *39*(3), 641–655. https://doi.org/10.1007/s10565-021-09618-9

Kim, K., & Lee, S.-E. (2021). Combined toxicity of dimethyl sulfoxide (DMSO) and vanadium towards zebrafish embryos (*Danio rerio*): Unexpected synergistic effect by DMSO. *Chemosphere*, *270*, 129405. https://doi.org/10.1016/j.chemosphere.2020.129405

Kim, L., Kim, H., Lee, T.-Y., & An, Y.-J. (2023). Chemical toxicity screening of tire particle leachates from vehicles and their effects on organisms across three trophic levels. *Marine Pollution Bulletin*, *192*, 114999. https://doi.org/10.1016/j.marpolbul.2023.114999

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., & Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Developmental Dynamics*, *203*(3), 253–310. https://doi.org/10.1002/aja.1002030302

Klein, J. (2020, December 16). *Brake Fluid: What is it and How Does it Work?* The Drive. https://www.thedrive.com/maintenance-repair/37284/brake-fluid

Kriech, A. J., & Osborn, L. V. (2022). Review of the impact of stormwater and leaching from pavements on the environment. *Journal of Environmental Management*, *319*, 115687. https://doi.org/10.1016/j.jenvman.2022.115687

Lee, B.-K. (2010). Sources, distribution and toxicity of polyaromatic hydrocarbons (PAHs) in particulate matter. In *Air Pollution*. IntechOpen. https://doi.org/10.5772/10045

Levin, P. S., Howe, E. R., & Robertson, J. C. (2020). Impacts of stormwater on coastal ecosystems: The need to match the scales of management objectives and solutions. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *375*(1814), 20190460. https://doi.org/10.1098/rstb.2019.0460

Liang, X., Adamovsky, O., Souders, C. L., & Martyniuk, C. J. (2019). Biological effects of the benzotriazole ultraviolet stabilizers UV-234 and UV-320 in early-staged zebrafish (*Danio rerio*). *Environmental Pollution*, *245*, 272–281. https://doi.org/10.1016/j.envpol.2018.10.130

Manjunatha, B., Deekshitha, B., Seo, E., Kim, J., & Lee, S. J. (2021). Developmental toxicity induced by particulate matter (PM2.5) in zebrafish (*Danio rerio*) model. *Aquatic Toxicology*, *238*, 105928. https://doi.org/10.1016/j.aquatox.2021.105928

McIntyre, J. K., Edmunds, R. C., Redig, M. G., Mudrock, E. M., Davis, J. W., Incardona, J. P., Stark, J. D., & Scholz, N. L. (2016). Confirmation of stormwater bioretention treatment effectiveness using molecular indicators of cardiovascular toxicity in developing fish. *Environmental Science & Technology*, *50*(3), 1561–1569. https://doi.org/10.1021/acs.est.5b04786

McIntyre, J. K., Spromberg, J., Cameron, J., Incardona, J. P., Davis, J. W., & Scholz, N. L. (2023). Bioretention filtration prevents acute mortality and reduces chronic toxicity for early life stage coho salmon (*Oncorhynchus kisutch*) episodically exposed to urban stormwater runoff. *Science of The Total Environment*, *902*, 165759. https://doi.org/10.1016/j.scitotenv.2023.165759

Mitchell, C. J., & Jayakaran, A. D. (2024). Mitigating tire wear particles and tire additive chemicals in stormwater with permeable pavements. *Science of the Total Environment*, 908, 168236.

Mulero-Navarro, S., & Fernandez-Salguero, P. M. (2016). New trends in aryl hydrocarbon receptor biology. *Frontiers in cell and developmental biology*, *4*, 45. https://www.frontiersin.org/articles/10.3389/fcell.2016.00045

Newman, M. C. N., Michael C. (2014). *Fundamentals of Ecotoxicology: The Science of Pollution, Fourth Edition* (4th ed.). CRC Press. https://doi.org/10.1201/b17658

Nicomel, N. R., & Li, L. (2023). Review of 6PPD-quinone environmental occurrence, fate, and toxicity in stormwater. *Ecocycles*, *9*(3), 33–46. https://doi.org/10.19040/ecocycles.v9i3.347

Oh, J., & Bartos, M. (2023). Model predictive control of stormwater basins coupled with real-time data assimilation enhances flood and pollution control under uncertainty. *Water Research*, *235*, 119825. https://doi.org/10.1016/j.watres.2023.119825

Pamuru, S. T., Forgione, E., Croft, K., Kjellerup, B. V., & Davis, A. P. (2022). Chemical characterization of urban stormwater: Traditional and emerging contaminants. *Science of The Total Environment*, *813*, 151887. https://doi.org/10.1016/j.scitotenv.2021.151887

Patel, A. B., Shaikh, S., Jain, K. R., Desai, C., & Madamwar, D. (2020). Polycyclic aromatic hydrocarbons: Sources, toxicity, and remediation approaches. *Frontiers in Microbiology*, 11, 562813. https://doi.org/10.3389/fmicb.2020.562813

Pauka, L. M., Maceno, M., Rossi, S. C., & Silva de Assis, H. C. (2011). Embryotoxicity and biotransformation responses in zebrafish exposed to water-soluble fraction of crude oil. *Bulletin of Environmental Contamination and Toxicology*, *86*(4), 389–393. https://doi.org/10.1007/s00128-011-0235-x

Philibert, D. A., Lyons, D. D., & Tierney, K. B. (2021). Comparing the effects of unconventional and conventional crude oil exposures on zebrafish and their progeny using behavioral and genetic markers. *Science of The Total Environment*, *770*, 144745. https://doi.org/10.1016/j.scitotenv.2020.144745

Pikula, K., Tretyakova, M., Zakharenko, A., Johari, S. A., Ugay, S., Chernyshev, V., Chaika, V., Kalenik, T., & Golokhvast, K. (2021). Environmental risk assessment of vehicle exhaust particles on aquatic organisms of different trophic levels. *Toxics*, *9*(10), 261. https://doi.org/10.3390/toxics9100261

Rand, G. M. (1995). Fundamentals of aquatic toxicology: Effects, environmental fate, and risk assessment (2nd ed.). Washington, D.C: Taylor & Francis.

Raterman, S. T., Metz, J. R., Wagener, F. A. D. T. G., & Von den Hoff, J. W. (2020). Zebrafish models of craniofacial malformations: Interactions of environmental factors. *Frontiers in Cell and Developmental Biology*, *8*. https://www.frontiersin.org/articles/10.3389/fcell.2020.600926

Rheaume, A., & Ahearn, D. (2016, Jan. 1). *A synthesis of bioretention performance in the Pacific Northwest.* Salish Sea Ecosystem 2016 Conference, University Archives, Heritage Resources, Western Libraries, Western Washington University, Vancouver, B.C. https://cedar.wwu.edu/ssec/2016ssec/fate_and_effects_of_pollutants/1

Rico, E. P., Rosemberg, D. B., Senger, M. R., de Bem Arizi, M., Bernardi, G. F., Dias, R. D., Bogo, M. R., & Bonan, C. D. (2006). Methanol alters ecto-nucleotidases and acetylcholinesterase in zebrafish brain. *Neurotoxicology and Teratology*, *28*(4), 489–496. https://doi.org/10.1016/j.ntt.2006.05.001

Roy, M. A., Sant, K. E., Venezia, O. L., Shipman, A. B., McCormick, S. D., Saktrakulkla, P., Hornbuckle, K. C., & Timme-Laragy, A. R. (2019). The emerging contaminant 3,3′-dichlorobiphenyl (PCB-11) impedes Ahr activation and *Cyp1a* activity to modify embryotoxicity of Ahr ligands in the zebrafish embryo model (*Danio rerio*). *Environmental Pollution*, *254*, 113027. https://doi.org/10.1016/j.envpol.2019.113027

Schilling, T. F., & Kimmel, C. B. (1997). Musculoskeletal patterning in the pharyngeal segments of the zebrafish embryo. *Development*, *124*(15), 2945–2960. https://doi.org/10.1242/dev.124.15.2945

Seeland, A., Oetken, M., Kiss, A., Fries, E., & Oehlmann, J. (2011). Acute and chronic toxicity of benzotriazoles to aquatic organisms. *Environmental Science and Pollution Research International*, *19*, 1781–1790. https://doi.org/10.1007/s11356-011-0705-z

Selbig, W. R., Buer, N., & Danz, M. E. (2019). Stormwater-quality performance of lined permeable pavement systems. *Journal of Environmental Management*, *251*, 109510. https://doi.org/10.1016/j.jenvman.2019.109510

Şişman, T., Atamanalp, M., Dane, H., & Adil, Ş. (2016). The toxicity of 10W-40 motor oil on zebrafish early life stages. *Turkish Journal of Fisheries and Aquatic Sciences*, 16(3), 597-604. https://www.trjfas.org/uploads/pdf_905.pdf

Stading, R., Chu, C., Couroucli, X., Lingappan, K., & Moorthy, B. (2020). Molecular role of cytochrome P4501A enzymes inoxidative stress. *Current Opinion in Toxicology*, *20–21*, 77–84. https://doi.org/10.1016/j.cotox.2020.07.001

Stewart, M. K., Hoehne, L., Dudczig, S., Mattiske, D. M., Pask, A. J., & Jusuf, P. R. (2023). Exposure to an environmentally relevant concentration of 17α-ethinylestradiol disrupts craniofacial development of juvenile zebrafish. *Ecotoxicology and Environmental Safety*, *251*, 114541. https://doi.org/10.1016/j.ecoenv.2023.114541

Turner, C., Sawle, A., Fenske, M., & Cossins, A. (2012). Implications of the solvent vehicles dimethylformamide and dimethylsulfoxide for establishing transcriptomic endpoints in the zebrafish embryo toxicity test. *Environmental Toxicology and Chemistry*, *31*(3), 593–604. https://doi.org/10.1002/etc.1718

Vijayaraghavan, K., Biswal, B. K., Adam, M. G., Soh, S. H., Tsen-Tieng, D. L., Davis, A. P., Chew, S. H., Tan, P. Y., Babovic, V., & Balasubramanian, R. (2021). Bioretention systems for stormwater management: Recent advances and future prospects. *Journal of Environmental Management*, *292*, 112766. https://doi.org/10.1016/j.jenvman.2021.112766

Williams, R., Taggart, T., Ganger, K., Koetsier, T., Johnson, S., & Dinchman, A. (2022). *Cyp1a* expression in freshwater fish of western New York as an indicator of pollution levels. *Ecology and Evolution*, *12*(1), e8526. https://doi.org/10.1002/ece3.8526

Wiseman, C. (2018). Ecology Publications & Forms. Better Brakes Enforcement Study 2017. Washington State Department of Ecology. https://apps.ecology.wa.gov/publications/SummaryPages/1804003.html

Xie, S., Feng, Y., Zhou, A., Lu, Z., & JixingZou. (2023). Comparative analysis of two new zebrafish models: The *cyp1a* low-expression line and *cyp1a* knockout line under PAHs exposure. *Gene*, *869*, 147391. https://doi.org/10.1016/j.gene.2023.147391

Xie, S., Yang, B., Li, S., Ge, L., Li, M., Chen, Q., Qing, X., & Zou, J. (2023). Generation and application of a novel transgenic zebrafish line Tg(GAcyp1a:eGFP/Luc) as an in vivo assay to sensitive and specific monitoring of DLCs in the environment. *Ecotoxicology and Environmental Safety*, *264*, 115471. https://doi.org/10.1016/j.ecoenv.2023.115471

Xu, Y., Li, H., Zhang, X., Bai, X., Wu, L., Tan, C., & Zhang, Z. (2023). Removal, migration, and distribution of naphthalene in bioretention facilities: The influences of particulate matter. *Environmental Science and Pollution Research*, *30*(16), 46940–46949. https://doi.org/10.1007/s11356-023-25330-z

Zhang, S.-Y., Gan, X., Shen, B., Jiang, J., Shen, H., Lei, Y., Liang, Q., Bai, C., Huang, C., Wu, W., Guo, Y., Song, Y., & Chen, J. (2023). 6PPD and its metabolite 6PPDQ induce different developmental toxicities and phenotypes in embryonic zebrafish. *Journal of Hazardous Materials*, *455*, 131601. https://doi.org/10.1016/j.jhazmat.2023.131601

Zhang, W., Xie, H. Q., Li, Y., Zhou, M., Zhou, Z., Wang, R., Hahn, M. E., & Zhao, B. (2022). The aryl hydrocarbon receptor: A predominant mediator for the toxicity of emerging dioxin-like compounds. *Journal of Hazardous Materials*, *426*, 128084. https://doi.org/10.1016/j.jhazmat.2021.128084

Zhang, Z., Qi, H., Chen, H., Zhang, X., Tan, C., Bai, X., Gong, Y., & Li, H. (2023). The control efficiency and mechanism of heavy metals by permeable pavement system in runoff based on enhanced infiltration materials. *Journal of Environmental Management*, *329*, 117042. https://doi.org/10.1016/j.jenvman.2022.117042

Zheng, J.-L., Zhu, Q.-L., Hu, X.-C., Parsons, D., Lawson, R., & Hogstrand, C. (2022). Transgenerational effects of zinc in zebrafish following early life stage exposure. *Science of The Total Environment*, *828*, 154443. https://doi.org/10.1016/j.scitotenv.2022.154443

Zoroufchi Benis, K., Behnami, A., Minaei, S., Brinkmann, M., McPhedran, K. N., & Soltan, J. (2023). Environmental occurrence and toxicity of 6PPD quinone, an emerging tire rubber-derived chemical: A Review. *Environmental Science & Technology Letters*, *10*(10), 815–823. https://doi.org/10.1021/acs.estlett.3c00521