AN ABSTRACT OF THE DISSERTATION OF

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Title: Utilization of the Zebrafish Model for Investigating Nitrated Polycyclic Aromatic Hydrocarbon Developmental Toxicity

Abstract approved:

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Polycyclic aromatic hydrocarbons (PAHs) are among the most widely known and studied environmental contaminants, originating from a range of natural and anthropogenic sources. PAHs are known to occur in the environment as complex mixtures, containing both unsubstituted PAHs, as well as a range of PAH derivatives. Among the less-studied of these derivative PAH classes are nitrated PAHs (NPAHs). NPAHs are known to form from atmospheric reactions with PAHs and can be found in the environment in a variety of matrices. Many NPAHs are known to be mutagenic, in some cases more so than the corresponding unsubstituted PAH. Less is known about the toxicity of NPAHs in whole-animal systems and for non-cancer endpoints, in particular with regard to the developmental toxicity and metabolism across a wide number of NPAH compounds, in a consistent model system.

One of the major challenges in studying PAHs, and related compounds, is the high hydrophobicity and low water solubility of these compounds, which can result in losses due to partitioning of the analytes out of the aqueous phase and on to the walls of the container or exposure vessel. Numerous *in vitro* and *in vivo* models utilize plastic plates as exposure vessels, including the use of polystyrene 96-well plates for developmental toxicity testing in the developing zebrafish (*Danio rerio*) model. We directly measured the losses which occur due to sorption to the polystyrene plates during zebrafish testing for a set of PAHs and NPAHs. Sorptive losses in some instances were greater than fifty percent, in particular for the lower of the two exposure concentrations tested. These sorptive losses decrease the concentration of chemical available to the zebrafish embryos, and therefore impact the interpretation of dose-response toxicity data. In an attempt to create a predictive model for sorptive losses, the measured sorption was modeled against the log K_{ow}, molecular weight, and subcooled liquid solubilities of the corresponding compounds. The correlations between subcooled liquid solubility and PAH sorption was statistically significant ($p<0.05$) for both concentrations tested, as well as molecular weight at the higher concentrations tested. However, none of the correlations were statistically significant for NPAH sorption, indicating a need for increased research in this area.

We utilized the developing zebrafish model to investigate the developmental toxicity, and potential contributing mechanisms of action, of a suite of 27 NPAHs, as well as 10 heterocyclic PAHs (HPAHs) and 2 amino-PAHs (potential

metabolites of NPAHs). Results from the toxicity screen indicate that NPAHs and HPAHs have a wide range of bioactivities in the developing zebrafish, from non-toxic at the concentrations tested to acutely toxic at sub-micromolar exposure concentrations. Activation of the aryl hydrocarbon receptor (AHR) pathway was investigated using a transgenic reporter zebrafish line and morpholino oligonucleotide knockdown to isolate specific isoforms of the AHR. The compounds investigated induced *cyp1a* expression in five distinct tissues (liver, vasculature, skin, yolk, and neuromast), which we determined to be due to different isoforms of the AHR. A subset of NPAHs was also selected for further mechanistic analysis via qPCR, and genes related to xenobiotic metabolism, cardiac stress, and oxidative stress were investigated. Each NPAH resulted in a unique profile of differentially regulated genes, indicating several potential contributing mechanisms of action. Combined, the results indicate that NPAHs and HPAHs are diverse in their bioactivities towards developing zebrafish.

To further investigate metabolism as a contributing factor in the developmental toxicity of NPAHs, we explored the use of a transgenic nitroreductase-expressing zebrafish line for developmental toxicity testing of NPAHs, as well as the applicability of this transgenic line for high-throughput toxicity screening for hepatotoxicity. Humans, and other vertebrate model systems, have endogenous nitroreductase activity, which is responsible for the reduction of nitro functional groups to amino functional groups, while zebrafish do not. Published protocols utilized this transgenic line for tissue-specific cell ablation for a specific exposure

scenario. We expanded upon the published protocols for the utilization of this transgenic line for tissue-specific cell ablation, as well as explored potential uses beyond cell ablation. Nitroreduction of a NPAH to the corresponding amino-PAH would have resulted in a shift in the toxicity profile. Unfortunately, no such changes were observed, despite validation of the nitroreductase functionality of the transgenic line, suggesting that the nitroreductase does not have a suitably high affinity for NPAHs to allow for this use. We also exposed the transgenic embryos to compounds known to undergo hepatic metabolism (benzo[a]pyrene and retene) or known hepatotoxins (flutamide, acetaminophen), with and without hepatic ablation, to demonstrate the utility of this transgenic line in isolating the role of the liver in observed toxicity. Overall, the uses for this transgenic line expand beyond the intended and published protocol, but would be further expanded with the development of a similar line with utilized a higher-affinity nitroreductase enzyme.

Together, these studies show the overall utility and challenges of using the zebrafish model for the investigation of NPAHs and similar hydrophobic or nitro-containing compounds.

Utilization of the Zebrafish Model for Investigating Nitrated Polycyclic Aromatic Hydrocarbon Developmental Toxicity

by
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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Anna C. Chlebowski, Author

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CONTRIBUTION OF AUTHORS

In all chapters, Dr. Staci Simonich and Dr. Robert Tanguay contributed to intellectual formulation, writing, and, where appropriate, study design.

Chapter 2: Prepared by Anna Chlebowski with editorial comments provided by Robert Tanguay and Staci Simonich. Anna Chlebowski developed and performed extraction techniques, GC/MS analysis, and modeling.

Chapter 3: Prepared by Anna Chlebowski with editorial comments provided by Gloria Garcia, Jane La Du, William Bisson, Lisa Truong, Staci Simonich and Robert Tanguay. Gloria Garcia assisted with qPCR primer validation and data analysis. Jane La Du assisted with morpholino oligonucleotide injections. AHR docking predictions and modeling were done by William Bisson.

Chapter 4: Prepared by Anna Chlebowski with editorial comments from Jane La Du, Lisa Truong, Staci Simonich and Robert Tanguay. Jane La Du established the homozygous spawning population of transgenic zebrafish embryos. All exposures and analysis were done by Anna Chlebowski.

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DEDICATION

This dissertation is dedicated to my father, Patrick Chlebowski, who showed me the importance of always trying my best to be my best, and to live life while I am fortunate enough to have it.

This dissertation is also dedicated to Pig. Miss you, little buddy.

Utilization of the Zebrafish Model for Investigating Nitrated Polycyclic Aromatic Hydrocarbon Developmental Toxicity

CHAPTER 1 -- INTRODUCTION

PAHs in the Environment and Exposure Pathways

As human civilization has grown over the past few hundred years, the increasing use of fossil fuels for human endeavors, and the resulting rise in industrial activities, has led to increased levels of many classes of environmental pollutants. Among these are polycyclic aromatic hydrocarbons, or PAHs. PAHs consist of two or more fused benzene rings, and originate from combustion (pyrogenic) processes as well as fossil fuels (petrogenic) sources (Howsam and Jones, 1998; Manzetti, 2013). Sixteen PAHs are recognized by the US Environmental Protection Agency (US EPA) as Priority Pollutants (Figure 1.1) (US EPA, 2014).

PAHs have several natural sources to the environment, including as a result of biomass burning, volcanic activity, and natural sources of oil and fossil fuels (Baek et al., 1991; Freeman and Cattell, 1990; Usenko et al., 2007). However, in the environment as a whole, human activity is the major contributing source of PAHs (Zhang and Tao, 2009). As a result of vehicular combustion, and increased use of biomass burning for heat or cooking, cities and areas with higher vehicle traffic have increased levels of PAHs (Nielsen et al., 1996; Pedersen et al., 2005; Polidori et al., 2010; Rogge et al., 1993). Industrial sites, such as manufactured gas plants (MGP) and wood creosoting sites can also be highly contaminated with PAHs (Hyötyläinen and Olkari, 1999; Meyer et al., 1999). Different sources and processes result in the production of unique fingerprint signatures of PAHs produced, which can be used for source apportionment (Howsam and Jones, 1998; Khalili et al., 1995; Tobiszewski and Namieśnik, 2012; Yunker et al., 2002).

PAHs have been detected in a range of environmental matrices, including soils and sediments (Ozaki et al., 2009; Van Metre and Mahler, 2010; Yunker et al., 2002), particulate matter in the atmosphere (Ciganek et al., 2004; Jyethi et al., 2014; Ramírez et al., 2011; Schauer et al., 2003), and snow pack (Boom and Marsalek, 1988; Kukučka et al., 2010; Walsh et al., 2015). PAHs can be transported long distance through the atmosphere (Jaffe et al., 1999; Lafontaine et al., 2015), and the presence of PAHs in remote locations, distant from human activity, indicates the long-range transport potential of these compounds in the atmosphere.

Dietary exposure to PAHs can occur through consumption of a range of food products, in particular when the food preparation process includes grilling or smoking (Larsson et al., 1983; Maga, 1986; Menzie et al., 1992; Ramesh et al., 2004; Wretling et al., 2010). This results in a low to moderate risk for consumers (Alomirah et al., 2011; Veyrand et al., 2013), with a higher risk in populations where grilled or smoked foods are a significant component of their diet (Akpambang et al., 2009; Essumang et al., 2012; Forsberg et al., 2012). Cigarette smoking can also be a significant source of PAH exposure on the individual scale, both as a result of direct inhalation (Hecht, 2003) and through settled household dust (Hoh et al., 2012). Environmental tobacco smoke is classified as a human carcinogen and is an indoor air pollutant (Hackshaw et al., 1997; Lloyd and Denton, 2005; Satcher et al., 2000), the effects of which are not explained by nicotine alone (Massarsky et al., 2015).

PAH Derivatives in the Environment and Exposure Pathways

In the environment, PAHs occur as components of complex mixtures. These mixtures contain both a range of unsubstituted PAHs, as well as a range of PAH derivatives. These derivatives can

form from the same processes as PAHs, as well as from secondary reactions, including atmospheric reactions and photolysis (Jariyasopit et al., 2013, 2014; Miller and Olejnik, 2001; Niu et al., 2007; Sabaté et al., 2001; Zimmermann et al., 2013). PAH derivatives include hydroxylated PAHs (OHPAHs), oxygenated PAHs (OPAHs), nitrated PAHs (NPAHs), and heterocyclic PAHs (HPAHs), example structures are shown in Figure 1.2. The first three groups possess a functional group substituted on the outside of the ring system, replacing one or more hydrogen atoms. Heterocyclic PAHs, in contrast, have the heteroatom, typically nitrogen, oxygen, or sulfur, substituted in place of one or more carbons within the ring system. Each of these derivative classes has physical-chemical properties and environmental fates divergent from the corresponding unsubstituted PAHs, due to the addition of the various functional groups. These functional groups will also alter the ways in which these compounds interact with biological systems.

Nitrated PAHs can be formed from combustion processes alongside unsubstituted PAHs (Shen et al., 2013), but are primarily formed as a result of secondary reactions between PAHs and nitrogen oxide (NO_x) radicals in the environment. These reactions occur primarily on particulate matter in the atmosphere as a result of vehicular combustion, especially diesel, (Arey et al., 1986; Atkinson et al., 1990; Atkinson and Arey, 2007; Carrara et al., 2010; Cecinato et al., 2001; Cochran et al., 2016; Esteve et al., 2006; Kamens et al., 1990; Reisen and Arey, 2005) as well as on natural mineral dust particles (Kameda et al., 2016). The NPAH formation reaction is initiated when a OH (or NO_3) radical attacks an aromatic carbon, forming a radical hydroxyPAH adduct (or radical NO_3-PAH adduct). Ortho-addition of NO_2 creates a nitroaromatic intermediate product, followed by loss of water (or HNO_3) to yield the final NPAH, as illustrated in Figure

1.3. In addition to vehicular combustion, (Holly A Bamford et al., 2003; Hu et al., 2013; Schuetzle et al., 1982; Yang et al., 2010) NPAHs have been detected in metropolitan and industrial air pollution, although at concentrations one to two orders of magnitude lower that the corresponding unsubstituted PAHs (Albinet et al., 2007; Dimashki et al., 2000; Hattori et al., 2007; Hayakawa et al., 1995; Lafontaine et al., 2015; Taga et al., 2005). NPAHs have also been detected in soils (Niederer, 1998) and waterways (Ohe and Nukaya, 1996), where bioaccumulation into mussels and oysters has been observed (Uno et al., 2011).

NPAHs are also detected alongside PAHs on foodstuffs, including grilled and smoked food products as a result of cooking and processing, in particular meats (Chen et al., 2014; Schlemitz and Pfannhauser, 1996), and homes with indoor combustion-based cooking methods had higher levels of NPAHs than homes with electric cooking methods (Chen et al., 2016). NPAHs are also detected on vegetables and leafy greens as a result of atmospheric deposition (Wickström et al., 1986).

Heterocyclic PAHs are found alongside unsubstituted PAHs in petroleum and fossil fuel products and contaminated sites, in particular as a result of coal tar and related product contamination (Blum et al., 2011; Chibwe et al., 2015; Lundstedt et al., 2003; Mahler et al., 2014; Meyer et al., 1999; Titaley et al., 2016). Heterocyclic PAHs have also been detected in particulate matter, most likely due to coal combustion processes (Bandowe et al., 2016; Bandowe and Nkansah, 2016; Wei et al., 2015). Detection of nitrogen-containing heterocyclic PAHs (azaarenes) in recently-deposited lake sediments was attributed to street dust from vehicular combustion, as opposed to natural sources (Wakeham, 1979). Similar to PAHs and NPAHs,

heterocyclic PAHs, in particular those containing nitrogen, have also been detected in grilled and smoked meat products (Janoszka et al., 2004; Joe Jr et al., 1985).

Health Effects of PAHs

Unsubstituted PAHs are some of the most well-known mutagenic and carcinogenic compounds (McCann et al., 1975), and many are classified as known, probable, or possible human carcinogens (Table 1.1) (WHO, 2015). The higher molecular weight PAHs (six or more fused benzene rings) tend to be the most mutagenic. In some cases these compounds have benzo[a]pyrene equivalency factors an order of magnitude or more greater than benzo[a]pyrene, listed in Table 1.2 (Collins et al., 1998; RI DEM, 2008; US EPA National Center for Environmental Assessment and Jones, n.d.). Human exposure to air pollution and fine particulate matter have been associated with genetic damage, including cardiopulmonary disease and lung cancer mortality (Lewtas, 2007). Air pollution from coal combustion, diesel fuel, and wood, which contains high levels of PAHs, is positively correlated with several types of cancer, including respiratory, digestive tract, urogenital, reproductive, blood, and skin cancers, with air pollution estimated to account for 5% of male and 3% of female cancer deaths occurring from 1970-1994 (Armstrong et al., 2004; Grant, 2009). Gene expression analysis in coke-oven workers comparing high and low exposure groups (indicated by urinary 1-hydroxypyrene levels) showed differential expression of 26 genes, with functions including apoptosis, chromosome stability and DNA repair, tumor suppression, immune function, reproductive function, and neuronal cell function (Wu et al., 2011).

In addition to the well-established link between PAHs and lung cancer, PAHs are known respiratory irritants, particularly from inhalation of PAH-containing particulate matter into the lungs (Kim et al., 2011). Children exposed to parental tobacco smoke during pregnancy displayed alterations in arterial structure and function, with effects visible at 5 years of age (Geerts et al., 2012). In preschool-aged children, increasing concentrations of PAHs and air pollution were associated with increased rates of bronchitis (Hertz-Picciotto et al., 2007) as well as wheeze in asthmatic children (Gale et al., 2012) and exposure to PAHs may increase the risk of peripheral arterial disease, independent of smoking status (Xu et al., 2013). Occupational exposure to PAHs can also result in alterations in cardiac autonomic function and reductions in hearth rate variability (Lee et al., 2011), and has been linked with mortality from ischemic heart disease (Burstyn et al., 2005). In animal model systems, exposure to benzo[a]pyrene of rats *in utero* led to cardiovascular changes suggestive of dysfunction later in life (Jules et al., 2012), and exposure to benzo[a]pyrene influenced cardiac development and expression of cardiac-related genes in zebrafish (*Danio rerio*) embryos (Huang et al., 2012a).

PAHs are also known developmental toxicants in humans and laboratory animal studies, and PAH exposure to a developing embryo or fetus can result in a range of mental and physical challenges. Exposure to environmental tobacco smoke is significantly correlated with smaller head circumference and a reduction in birth weight, as well as decreased cognitive development scores (Duarte-Salles et al., 2012; Perera et al., 2005; Rauh et al., 2004). PAH-DNA adducts measured in umbilical cord blood showed a correlation with decreased birth length, weight, and head circumference in newborns cohorts in Poland (Perera et al., 1998) and China (Tang et al., 2006). Children born to women exposed to higher levels of traffic-related pollution and

particulate matter were smaller, (Wilhelm et al., 2012), potentially related to reduced vascularization of the fetoplacental arterial tree, leading to reduced blood flow (Rennie et al., 2011). Prenatal exposure to airborne PAHs caused a decrease in cognitive development in children by 5 years of age, an estimated decrease in 3.8-4.67 IQ points, and the potential for implications in school performance (Edwards et al., 2010; Perera et al., 2009). Elevated levels of prenatal exposure to PAHs in a Chinese population was correlated with an increased risk for neural tube defects, anencephaly, and spina bifida (Ren et al., 2011). In mice, lactational exposure to benzo[a]pyrene caused adverse neurobiological and behavioral changes during postnatal development and until young adulthood (Bouayed et al., 2009). Benzo[a]pyrene is also a known neurotoxicant in rats, resulting in neurobehavioral impairments in the hippocampus following subacute exposure (Cheng et al., 2013).

Exposure to PAHs has also been reported to have negative effects on wildlife and ecosystem health, in particular for organisms exposed to contaminated sediments. Atlantic killifish (*Fundulus heteroclitus*) at the Atlantic Wood Industries Superfund Site, which has high levels of PAH contamination, showed increased DNA damage compared to fish from a reference site, at levels consistent with the PAH levels found in the sediment (Jung et al., 2011). Exposure to a complex environmental PAH mixture caused both short and long-term behavioral effects in naïve fish, as well as altered gene expression and metabolic response (Brown et al., 2016; Jung et al., 2011). However, killifish from a population living in an exposed site did not display behavioral abnormalities (Brown et al., 2016) or changes in gene expression (Wills et al., 2010) as a result of PAH exposure. Produced water, a by-product of offshore drilling operations, is known to contain PAHs, and is toxic to a range of marine and freshwater organisms, with marine

algae, bivalve mollusk larvae, and crustaceans among the most sensitive, and fish generally being among the least sensitive species (Neff, 2002). Documented effects on aquatic organisms include oxidative stress, effects on the immune system, endocrine regulation or dysregulation and disruption, and development (Hylland, 2006). Oil products, such as those released from the Deepwater Horizon or Exxon Valdez spills, also induced cardiotoxicity in numerous species of fish (Hicken et al., 2011; Incardona et al., 2014, 2013; Jung et al., 2013).

Health Effects of NPAHs and HPAHs

For PAH derivatives, limited data exists about the impacts of exposure, with a majority of the data focused on mutagenicity. Many NPAHs are documented mutagens (Fu and Herreno-Saenz, 1999; Kovacic and Somanathan, 2014; Rosenkranz and Mermelstein, 1983), several are listed as probable or possible human carcinogens (Table 1.1), and benzo[a]pyrene equivalency factors have been determined for a limited number of NPAHs (Table 1.2). Some NPAHS have also been shown to have tumor-promoting activity in cultured mouse fibroblast cells (Misaki et al., 2016), are tumorigenic in the newborn mouse assay (Wislocki et al., 1986), are capable of transforming normal human fibroblasts to an invasive state (Howard et al., 1983), or are known carcinogens in animal models (Ohgaki et al., 1982).

Limited data exists for non-mutagenic endpoints as a result of NPAH exposure (Kovacic and Somanathan, 2014). 1-nitronaphthalene has been documented to cause pulmonary and liver toxicity in rats following intraperitoneal injection (Johnson et al., 1984; Sauer et al., 1997). 1-nitropyrene is known to have acute, genetic, and respiratory toxicity to rats (Chan, 1996; Marshall et al., 1982). Several NPAHs have also been shown to be acutely toxic to diatoms,

crustaceans, and fish (Onduka et al., 2012). Both 1-nitronaphthalene and 1-nitropyrene negatively impacted the hatchability of *F. heteroclitus* eggs, but at concentrations three orders of magnitude higher than what has been detected in the environment (Onduka et al., 2015). Injection of rats with 9-nitrophenanthrene caused altered gene expression indicative of hepatotoxicity (Yoshikawa et al., 1988). While limited vertebrate toxicity data is available for a limited number of compounds, practical considerations have thus far limited the ability of a wide number of NPAHs to be evaluated in non-bacterial or cell-culture models.

Similarly, heterocyclic PAHs are less well-studied than unsubstituted PAHs. Several are known to have estrogenic effects using the estrogen receptor CALUX assay, with estradiol equivalence factors comparable to other xenoestrogens, such as bisphenol A (Brinkmann et al., 2014). Nitrogen-containing heterocyclic PAHs are known to induce oxidative stress and have acute toxicity in terrestrial plants (Pašková et al., 2006) and midge (*Chironomus riparius*) larvae (Bleeker et al., 1996). Several heterocyclic PAHs were determined to have ecologically relevant toxicities in algae and *Daphnia magna* (Eastmond et al., 1984; Eisentraeger et al., 2008).

The Aryl Hydrocarbon Receptor and PAH Metabolism

Unsubstituted PAHs are known to operate through several mechanisms of action, with metabolism, predominantly in the liver, playing a predominant role (Ramesh et al., 2004). Among the most-studied mechanistic pathways for PAHs is the aryl hydrocarbon receptor (AHR) pathway, which is also shared by other hydrophobic contaminants, including dioxins and polychlorobiphenyls (PCBs) (Kafafi et al., 1993; Teraoka et al., 2003). The AHR is a member of the bHLH/PAS family of heterodimeric transcriptional regulators (basic-region helix-loop-

helix/Period[PER]- aryl hydrocarbon receptor nuclear translocator [ARNT]-single minded [SIM]), and has known roles in many processes, including carcinogenesis, development, and the stress response to hypoxia, among others (Kewley et al., 2004; Shimizu et al., 2000; Swanson, 2002).

Prior to ligand binding, the AHR resides primarily in the cytosol. Following binding of a PAH or similar molecule, AHR dimerizes with the aryl hydrocarbon nuclear translator (ARNT) and translocates to the nucleus. The complex can then interact with xenobiotic response elements (XRE), resulting in altered expression of downstream genes. This ultimately results in induction or up-regulation of many genes, including those for proteins involved in xenobiotic metabolism, ideally detoxification (Baird et al., 2005; Puga et al., 2009; Whitlock Jr, 1999), as well as downregulation of some genes, such as the transcription factor *Sox9b*, which plays an essential role in cartilage development (Andreasen et al., 2006; Xiong et al., 2008).

Among the genes often upregulated as a result of AHR pathway activation are the Cytochrome P450s (CYP), specifically the CYP1 family, which is responsible for the oxidation of xenobiotics and AHR ligands, such as PAHs (Guengerich, 2001; Hrycay and Bandiera, 2010; Nebert et al., 2004). CYP1A and CYP1B are substrate-inducible mono-oxygenases with broad and overlapping substrate specificity, but variable metabolic efficiencies with specific compounds (Scornaienchi et al., 2010). CYP metabolism of a PAH begins with the addition of an epoxide group, which can then be converted to a dihydrodiol by epoxide hydrolase. Additional metabolism of this product by CYPs is possible, forming a diol epoxide. The diol epoxides, which can be one of two pairs of enantiomers, as shown in Figure 1.4, can covalently bind to the exocyclic amino groups of purines via a *cis* or *trans* addition (Baird et al., 2005; Harvey, 1996;

Ramesh et al., 2004; Shimada and Fujii-Kuriyama, 2004). These DNA adducts, if not repaired, can lead to mutations and eventually contribute to the development of cancer (Nebert et al., 2004).

Following this Phase I metabolism by CYP enzymes, PAH derivatives can undergo Phase II metabolism by various other enzymes, including glutathione S-transferases (GST), glucuronosyl transferase, epoxide hydrolases, UDP-glucuronosyltransferase 1a6 (Ugt1a6), NAD(P)H:quinone oxydoreductase 1 (Nqo1), and aldehyde dehydrogenases, in order to increase the hydrophobicity and promote excretion (Baird et al., 2005; Puga et al., 2009). Metabolism of PAHs occurs primarily in the liver (Ramesh et al., 2004), although metabolism in other tissues, including the intestine as a result of microbiota, also occurs (Van de Wiele et al., 2004).

The AHR is involved in pathways other than the CYP metabolism pathway described, and these other pathways can also play important roles in the toxicity of AHR ligands. The "AHR gene battery", which also requires the nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor, includes CYP1A1, Nqo1, UGT1a6, and Gsta1 (Yeager et al., 2009). The AHR/ARNT complex can also interact with other transcription factors, resulting in a wide range of downstream effects (Beischlag et al., 2008).Activation of the AHR pathway by certain ligands can result in changes to pathways related to cell cycle regulation, nitrogen-activated protein kinase cascades, immediate-early gene induction, crosstalk with the RB/E2F axis, and mobilization of calcium stores (Puga et al., 2009). AHR also has roles in the proper functioning of the immune, hepatic, cardiovascular, vascular, and reproductive systems, as well as cellular functions including proliferation and cell cycle, morphology, adhesion, and migration (Mulero-Navarro and Fernandez-Salguero, 2016).

NPAH Metabolism

Like PAHs, NPAHs can induce and undergo oxidative metabolism by the CYP family of enzymes (Asokan et al., 1986; Iwanari et al., 2002; Jung et al., 2001), forming products similar to that of unsubstituted PAHs. Reduction at the nitro functional group (nitroreduction) is an additional and parallel metabolic pathway which can occur simultaneously to oxidation (Chou et al., 1983; Peterson et al., 1979), producing a wide range of potential metabolites, many of which are capable of binding to DNA or inducing mutations (Ball et al., 1984; Châtel et al., 2014; Purohit and Basu, 2000). Many NPAHs are strong mutagens (Campbell et al., 1981; Debnath et al., 1992; Rosenkranz and Mermelstein, 1983), in some cases orders of magnitude higher than the corresponding unsubstituted PAHs. In contrast to unsubstituted PAHs, NPAHs can be direct-acting, and do not require metabolic activation in the Ames *Salmonella* mutagenicity assay in order to be mutagenic (Campbell et al., 1981; Fu and Herreno-Saenz, 1999).

Nitroreduction, catalyzed by nitroreductases, is a multi-step pathway, proceeding through nitroso and hydroxylamino intermediates before final reduction to the corresponding amino compound, as shown in Figure 1.5. Previous data has implicated the intermediate hydroxyamino compound as the mutagenic or toxic compound, rather than the fully-reduced amino or the fully-oxidized nitro compound (El-Bayoumy and Hecht, 1982; Fu et al., 1988; Möller, 1994). The mutagenicity of NPAHs in *Salmonella* strains expressing nitroreductases is higher than in non-expressing strains (Einistö et al., 1991; McCoy et al., 1981; Watanabe et al., 1989), demonstrating that nitroreduction can be an important component of NPAHs toxicity, and warrants consideration in toxicity and risk evaluations (Purohit and Basu, 2000).

Nitroreductase activity is found in humans and other mammals, present in the liver, lungs and nasal tissue (Bond, 1983; Nachtman and Wei, 1982), as well as in the intestine due to the presence of microbiota (Cerniglia et al., 1988; El-Bayoumy et al., 1983; Howard et al., 1983). Nitroreductases have also been explored as remedial tools for clean-up of sites contaminated with nitrated explosives (Hannink et al., 2001), as well as for tissue-specific cell ablation studies in transgenic animals (Clark et al., 1997; Curado et al., 2008; Pisharath and Parsons, 2009). Heterocyclic PAHs can undergo similar metabolic reactions to other polycyclic aromatic compounds, such as activation of the AHR and subsequent metabolism by the CYP family of enzymes (Barron et al., 2004; Brack and Schirmer, 2003; Jung et al., 2001). The tentatively identified metabolites are in some instances also predicted to be toxic, however, like other PAH derivatives, the lack of analytical standards hinders the identification and further investigation of these compounds (Brinkmann et al., 2014).

Zebrafish as a Model Organism

Zebrafish (*Danio rerio*) are a vertebrate model well-suited to high-throughput developmental toxicity testing (Bugel et al., 2014; Garcia et al., 2016; Hill et al., 2005; Yang et al., 2009), and has also been used for environmental risk assessment (Scholz et al., 2008) and as a model for human disease (Lieschke and Currie, 2007). Zebrafish are relatively easy to cultivate in a laboratory setting, are amenable to cell-culture techniques, and a high fecundity rate allows for production of large numbers of eggs from a single spawning pair. Embryos develop externally, are transparent, and develop rapidly, with a heartbeat by 24 hours post fertilization, metabolic competence within 72 hours post fertilization, and are free-feeding by 120 hours post fertilization

(Kimmel et al., 1995; Elke A. Ober et al., 2003). Zebrafish have a high genetic homology to humans, where 70% of human genes have a zebrafish orthologue, including 82% of disease-associated genes, and the genome has been fully sequenced (Howe et al., 2013). Zebrafish have been previously used for the investigation of toxicity and mechanisms of action of unsubstituted and oxygenated PAHs (Goodale et al., 2013a; Knecht et al., 2013, 2016) as well as PAH-containing complex mixtures (Mesquita et al., 2016; Philibert et al., 2016; Sogbanmu et al., 2016; Wincent et al., 2015), and have indicated several distinct contributing mechanisms of action for these classes of compounds.

The AHR pathway in particular has been well-studied in zebrafish. In contrast to humans, which have only one isoform of the AHR, zebrafish have three isoforms (AHR1A, 1B, and 2), each with different tissue expression patterns and ligand affinities. The AHR1 and AHR2 paralogs in zebrafish (and other bony fish) arose from a gene duplication event, and AHR2 is functionally similar to AAHR in humans (Hahn et al., n.d.). The zebrafish AHR1A, primarily expressed in the liver, has been associated with PAH toxicity (Garner et al., 2013). Zebrafish AHR2 is expressed in the skin and vasculature (Andreasen et al., 2002), among other tissues, as is AHR1B, although with different ligand affinities (Karchner et al., 2005). *In silico* models for the active sites of all three zebrafish as well as the human AHR have been developed, with a fairly high predictive success rate (Bisson et al., 2009; Perkins et al., 2014). Induction of CYP1A expression following ligand binding to the AHR is a well-established pathway in zebrafish, and has been studied extensively as an essential component of toxicity for 2,3,7,8-tetracholordibenzo-*p*-dioxin (Antkiewicz et al., 2006; Prasch et al., 2003; Teraoka et al., 2003), as well as some PAHs (Billiard et al., 2006; Van Tiem and Di Giulio, 2011). While

zebrafish have a greater number of CYP enzymes than humans, especially in the CYP1 family, the similarity in exon sequences between the human and zebrafish genes is high, with similar xenobiotic-metabolizing functionality (Goldstone et al., 2010; Scornaienchi et al., 2010) and upstream xenobiotic response elements, indicating inducibility by the AHR (Jönsson et al., 2007). However, there are also some instances where toxicity can be AHR2 dependent, but CYP1A independent, such as retene-induced cardiotoxicity (Scott et al., 2011), indicating that, at least for some compounds, the mechanism of toxicity can be complex.

Zebrafish are well-suited for genetic manipulation, for a variety of purposes (Garcia et al., 2016). Temporary or transient knockdown using morpholino oligonucleotides has been commonly used for a range of genes, including the AHRs (Goodale et al., 2012; Knecht et al., 2013). Transgenic lines are also popular for a variety of other purposes. Fluorescent proteins can be expressed using a specific promoter sequence, allowing for a visual representation of gene expression, such as *cyp1a* (Kim et al., 2013) and *cyp191b* (Petersen et al., 2013) as indicators of metabolic potential, or *fli1* as an indicator of vasculature (Lawson and Weinstein, 2002). Generation of transgenic lines with specific genes, such as AHR, removed allows for further elucidation of functions of proteins or genes of interest (Goodale et al., 2012). Other uses for transgenic lines have also been developed, including the use of nitroreductase for tissue-specific cell ablation (Curado et al., 2007), calcium channels for visualization of nerve activity (Fosque et al., 2015), and a range of heart mutants used for studying human-relevant heart function and disease (Sehnert and Stainier, 2002). The continued development of gene-editing systems, such as CRISPR/Cas9 (Hruscha et al., 2013; Hwang et al., 2013), will allow for the further creation and use of transgenic zebrafish, with an expanding range of potential uses.

The use of zebrafish, being an aquatic model, for hydrophobic compounds such as PAHs and PAH derivatives, poses several challenges (Truong et al., 2016). First, compounds under investigation must be introduced into an aqueous environment for the zebrafish exposure, which is challenging for many PAHs and PAH derivatives, some of which have very low water solubility. Most PAHs and derivatives can be made soluble with the use of a co-solvent, such as dimethyl sulfoxide (DMSO), but may precipitate out of solution when the DMSO solution is added to the aqueous media. These issues can be addressed by increased mixing, although this can itself have other impacts on the developing embryos if done too vigorously (Truong et al., 2016). Once the compound has been solubilized in the aqueous media, the compounds may volatilize (depending on the vapor pressure), or may sorb to the walls of the exposure vessel (depending on the material of the container and the hydrophobicity of the compound in question) may occur (Riedl and Altenburger, 2007). The final concentration of these hydrophobic analytes, and the dose which the embryo actually receives, have been studied for only a very limited number of compounds (Goodale et al., 2013), and leads to a large degree of uncertainty in this type of testing. Further understanding of the exposure and dosimetry paradigm will be important for better understanding and interpreting the resulting of toxicity evaluations, in particular for hydrophobic analytes such as PAHs and PAH derivatives. While various methods, including passive dosing, have been investigated to compensate for losses due to sorption or volatilization (Mayer et al., 1999; K. E. Smith et al., 2009; Vergauwen et al., 2015), a quantitative measure of the exposure concentration and dose remains needed.

Thesis Objectives

The overarching goal of this project was to utilize the zebrafish model to determine the bioactivities and potential mechanisms of action for nitrated PAHs, while also defining the challenges in studying this group of compounds in an aquatic system. In chapter 2, we sought to better understand and quantify the sorptive losses of PAHs and NPAHs which occur to the polystyrene plates during zebrafish developmental toxicity testing. The sorptive losses were measured using gas chromatography/mass spectrometry (GC/MS), and were determined to be upwards of 50% for some PAHs and NPAHs. Using this data, we then attempted development of a model for predicting the sorptive losses, and observed some correlations between sorptive losses measured for PAHs and their physical-chemical properties, although no such relationship was observed for NPAHs.

In chapter 3, we utilized the developing zebrafish model to investigate the developmental toxicity of NPAHs and HPAHs. A range of toxicity endpoints and effective exposure concentrations was observed, with the observed toxicities varying from non-toxic to acutely toxic, at the concentrations tested. Activation of the AHR was investigated as a potential mechanism of toxicity by using a transgenic zebrafish line which expresses green fluorescent protein (GFP) as an indicator of CYP1A expression, as well as immunohistochemistry (IHC) for CYP1A. Five unique CYP1A expression patterns were observed, which were determined to be due to different isoforms of the AHR using morpholino oligonucleotide knockdown. Additional mechanisms of action, including cardiac stress and oxidative stress, were investigated for a subset of NPAHs using qPCR, and were determined to contribute to the mechanisms of action of at least some NPAHs.

In chapter 4, we characterized and investigated alternative uses in high-throughput screening for a nitroreductase-expressing transgenic zebrafish line, which had been developed for use in tissue-specific cell ablation. We first investigated the ablation potential under scenarios beyond the published protocols, and established a timeline for hepatocyte ablation and recovery, as well as a wider range of embryo ages at which ablation can occur. We also sought to demonstrate alternative uses for this transgenic line, in the toxicity testing of NPAHs as well as the study of hepatotoxic compounds. While the affinity of the nitroreductase does not appear to be high enough to make this transgenic line useful in the investigation of NPAHs, the use of this line for the investigation of hepatotoxins established the expanded utility of this transgenic line in the high-throughput screening if hepatotoxins, and the development of a similar line with a higher-affinity nitroreductase would have great potential for a range of uses.

Together, these studies demonstrate the utility and challenges of using the zebrafish model in the investigation of NPAHs. We demonstrated that some NPAHs are developmentally toxic in zebrafish, potentially through multiple mechanisms of action. We also demonstrated a major challenge with the testing of hydrophobic molecules in aquatic systems, in accurately describing or predicting the concentration to which the embryos are actually exposed. We also highlighted the importance of nitroreductase as an area for expansion in NPAH research, as well as the potential for expanded use of established transgenic animal models. These studies not only demonstrate the power of the zebrafish model, but the continued need for research in emerging environmental contaminants, such as NPAHs.

References

Akpambang, V.O.E., Purcaro, G., Lajide, L., Amoo, I.A., Conte, L.S., Moret, S., 2009. Determination of polycyclic aromatic hydrocarbons (PAHs) in commonly consumed Nigerian smoked/grilled fish and meat. Food Addit. Contam. Part A 26, 1096–1103. doi:10.1080/02652030902855406

Albinet, A., Leoz-Garziandia, E., Budzinski, H., ViIlenave, E., 2007. Polycyclic aromatic hydrocarbons (PAHs), nitrated PAHs and oxygenated PAHs in ambient air of the Marseilles area (South of France): Concentrations and sources. Sci. Total Environ. 384, 280–292. doi:10.1016/j.scitotenv.2007.04.028

Alomirah, H., Al-Zenki, S., Al-Hooti, S., Zaghloul, S., Sawaya, W., Ahmed, N., Kannan, K., 2011. Concentrations and dietary exposure to polycyclic aromatic hydrocarbons (PAHs) from grilled and smoked foods. Food Control 22, 2028–2035. doi:10.1016/j.foodcont.2011.05.024

Andreasen, E.A., Mathew, L.K., Tanguay, R.L., 2006. Regenerative Growth Is Impacted by TCDD: Gene Expression Analysis Reveals Extracellular Matrix Modulation. Toxicol. Sci. 92, 254–269. doi:10.1093/toxsci/kfj118

Andreasen, E.A., Spitsbergen, J.M., Tanguay, R.L., Stegeman, J.J., Heideman, W., Peterson, R.E., 2002. Tissue-Specific Expression of AHR2, ARNT2, and CYP1A in Zebrafish Embryos and Larvae: Effects of Developmental Stage and 2,3,7,8-Tetrachlorodibenzo-p-dioxin Exposure. Toxicol. Sci. 68, 403–419. doi:10.1093/toxsci/68.2.403

Antkiewicz, D.S., Peterson, R.E., Heideman, W., 2006. Blocking Expression of AHR2 and ARNT1 in Zebrafish Larvae Protects Against Cardiac Toxicity of 2,3,7,8-Tetrachlorodibenzo-p-dioxin. Toxicol. Sci. 94, 175–182. doi:10.1093/toxsci/kfl093

Arey, J., Zielinska, B., Atkinson, R., Winer, A.M., Ramdahl, T., Pitts, J.N., 1986. The formation of nitro-PAH from the gas-phase reactions of fluoranthene and pyrene with the OH radical in the presence of NO x. Atmospheric Environ. 1967 20, 2339–2345.

Armstrong, B., Hutchinson, E., Unwin, J., Fletcher, T., 2004. Lung Cancer Risk after Exposure to Polycyclic Aromatic Hydrocarbons: A Review and Meta-Analysis. Environ. Health Perspect. 112, 970–978.

Asokan, P., Das, M., Bik, D.P., Howard, P.C., McCoy, G.D., Rosenkranz, H.S., Bickers, D.R., Mukhtar, H., 1986. Comparative effects of topically applied nitrated arenes and their nonnitrated parent arenes on cutaneous and hepatic drug and carcinogen metabolism in neonatal rats. Toxicol. Appl. Pharmacol. 86, 33–43.

Atkinson, R., Arey, J., 2007. Mechanisms of the Gas-Phase Reactions of Aromatic Hydrocarbons and Pahs with Oh and No 3 Radicals. Polycycl. Aromat. Compd. 27, 15–40. doi:10.1080/10406630601134243

Atkinson, R., Arey, J., Zielinska, B., Aschmann, S.M., 1990. Kinetics and nitro-products of the gas-phase OH and NO3 radical-initiated reactions of naphthalene-d8, Fluoranthene-d10, and pyrene. Int. J. Chem. Kinet. 22, 999–1014.

Baek, S.O., Field, R.A., Goldstone, M.E., Kirk, P.W., Lester, J.N., Perry, R., 1991. A review of atmospheric polycyclic aromatic hydrocarbons: Sources, fate and behavior. Water. Air. Soil Pollut. 60, 279–300. doi:10.1007/BF00282628

Baird, W.M., Hooven, L.A., Mahadevan, B., 2005. Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. Environ. Mol. Mutagen. 45, 106–114. doi:10.1002/em.20095

Ball, L.M., Kohan, M.J., Claxton, L.D., Lewtas, J., 1984. Mutagenicity of derivatives and metabolites of 1-nitropyrene: activation by rat liver S9 and bacterial enzymes. Mutat. Res. Toxicol. 138, 113–125. doi:10.1016/0165-1218(84)90033-8

Bamford, H.A., Bezabeh, D.Z., Schantz, M.M., Wise, S.A., Baker, J.E., 2003. Determination and comparison of nitrated-polycyclic aromatic hydrocarbons measured in air and diesel particulate reference materials. Chemosphere 50, 575–587. doi:10.1016/S0045-6535(02)00667-7

Bandowe, B.A.M., Meusel, H., Huang, R., Hoffmann, T., Cao, J., Ho, K., 2016. Azaarenes in fine particulate matter from the atmosphere of a Chinese megacity. Environ. Sci. Pollut. Res. 1–12. doi:10.1007/s11356-016-6740-z

Bandowe, B.A.M., Nkansah, M.A., 2016. Occurrence, distribution and health risk from polycyclic aromatic compounds (PAHs, oxygenated-PAHs and azaarenes) in street dust from a major West African Metropolis. Sci. Total Environ. 553, 439–449. doi:10.1016/j.scitotenv.2016.02.142

Barron, M.G., Heintz, R., Rice, S.D., 2004. Relative potency of PAHs and heterocycles as aryl hydrocarbon receptor agonists in fish. Mar. Environ. Res., Twelfth International Symposium on Pollutant Responses in Marine Organisms 58, 95–100. doi:10.1016/j.marenvres.2004.03.001

Beischlag, T.V., Morales, J.L., Hollingshead, B.D., Perdew, G.H., 2008. The Aryl Hydrocarbon Receptor Complex and the Control of Gene Expression. Crit. Rev. Eukaryot. Gene Expr. 18. doi:10.1615/CritRevEukarGeneExpr.v18.i3.20

Billiard, S.M., Timme-Laragy, A.R., Wassenberg, D.M., Cockman, C., Giulio, R.T.D., 2006. The Role of the Aryl Hydrocarbon Receptor Pathway in Mediating Synergistic Developmental Toxicity of Polycyclic Aromatic Hydrocarbons to Zebrafish. Toxicol. Sci. 92, 526–536. doi:10.1093/toxsci/kfl011

Bisson, W.H., Koch, D.C., O'Donnell, E.F., Khalil, S.M., Kerkvliet, N.I., Tanguay, R.L., Abagyan, R., Kolluri, S.K., 2009. Modeling of the aryl hydrocarbon receptor (AhR) ligand binding domain and its utility in virtual ligand screening to predict new AhR ligands. J. Med. Chem. 52, 5635–5641.

Bleeker, E.A., Buckert-de Jong, M.C., van der Geest, H.G., Kraak, M.H., 1996. Toxicity of nitrogencontaining polycyclic aromatic hydrocarbons (NPAH) to the midge Chironomus riparus (Diptera). Proc Exp Appl. Entomol 7, 197–202.

Blum, P., Sagner, A., Tiehm, A., Martus, P., Wendel, T., Grathwohl, P., 2011. Importance of heterocylic aromatic compounds in monitored natural attenuation for coal tar contaminated aquifers: A review. J. Contam. Hydrol. 126, 181–194. doi:10.1016/j.jconhyd.2011.08.004

Bond, J.A., 1983. Bioactivation and biotransformation of 1-nitropyrene in liver, lung and nasal tissue of rats. Mutat. Res. Toxicol. 124, 315–324.

Boom, A., Marsalek, J., 1988. Accumulation of polycyclic aromatic hydrocarbons (PAHs) in an urban snowpack. Sci. Total Environ. 74, 133–148. doi:10.1016/0048-9697(88)90134-9

Bouayed, J., Desor, F., Rammal, H., Kiemer, A.K., Tybl, E., Schroeder, H., Rychen, G., Soulimani, R., 2009. Effects of lactational exposure to benzo[α]pyrene (B[α]P) on postnatal neurodevelopment, neuronal receptor gene expression and behaviour in mice. Toxicology 259, 97–106. doi:10.1016/j.tox.2009.02.010

Brack, W., Schirmer, K., 2003. Effect-Directed Identification of Oxygen and Sulfur Heterocycles as Major Polycyclic Aromatic Cytochrome P4501A-Inducers in a Contaminated Sediment. Environ. Sci. Technol. 37, 3062–3070. doi:10.1021/es020248j

Brinkmann, M., Maletz, S., Krauss, M., Bluhm, K., Schiwy, S., Kuckelkorn, J., Tiehm, A., Brack, W., Hollert, H., 2014. Heterocyclic Aromatic Hydrocarbons Show Estrogenic Activity upon Metabolization in a Recombinant Transactivation Assay. Environ. Sci. Technol. 48, 5892–5901. doi:10.1021/es405731j

Brown, D.R., Bailey, J.M., Oliveri, A.N., Levin, E.D., Di Giulio, R.T., 2016. Developmental exposure to a complex PAH mixture causes persistent behavioral effects in naive Fundulus heteroclitus (killifish) but not in a population of PAH-adapted killifish. Neurotoxicol. Teratol. 53, 55–63. doi:10.1016/j.ntt.2015.10.007

Bugel, S.M., Tanguay, R.L., Planchart, A., 2014. Zebrafish: A Marvel of High-Throughput Biology for 21st Century Toxicology. Curr. Environ. Health Rep. 1, 341–352. doi:10.1007/s40572-014-0029-5

Burstyn, I., Kromhout, H., Partanen, T., Svane, O., Lang\a ard, S., Ahrens, W., Kauppinen, T., Stücker, I., Shaham, J., Heederik, D., others, 2005. Polycyclic aromatic hydrocarbons and fatal ischemic heart disease. Epidemiology 16, 744–750.

Campbell, J., Crumplin, G., Garner, J.V., Garner, R.C., Martin, C.N., Rutter, A., 1981. Nitrated polycyclic aromatic hydrocarbons: potent bacterial mutagens and stimulators of DNA repair synthesis in cultured human cells. Carcinogenesis 2, 559–565.

Carrara, M., Wolf, J.-C., Niessner, R., 2010. Nitro-PAH formation studied by interacting artificially PAH-coated soot aerosol with NO2 in the temperature range of 295–523 K. Atmos. Environ. 44, 3878–3885. doi:10.1016/j.atmosenv.2010.07.032

Cecinato, A., Mabilia, R., Brachetti, A., Filippo, P.D., Liberti, A., 2001. Nitrated-Pah in Urban Air of Italy as Indicators of Motor Vehicle Emission and Light-Induced Reactions. Anal. Lett. 34, 927–936. doi:10.1081/AL-100103603

Cerniglia, C.E., Lambert, K.J., White, G.L., Heflich, R.H., Franklin, W., Fifer, E.K., Beland, F.A., 1988. Metabolism of 1, 8-dinitropyrene by human, rhesus monkey, and rat intestinal microflora. Toxic. Assess. 3, 147–159.

Chan, P., 1996. NTP technical report on the toxicity studies of 1-Nitropyrene (CAS No. 5522-43-0) Administered by Inhalation to F344/N Rats. Toxic. Rep. Ser. 34, 1–D2.

Châtel, A., Faucet-Marquis, V., Pfohl-Leszkowicz, A., Gourlay-Francé, C., Vincent-Hubert, F., 2014. DNA adduct formation and induction of detoxification mechanisms in Dreissena polymorpha exposed to nitro-PAHs. Mutagenesis 29, 457–465. doi:10.1093/mutage/geu040

Chen, Y., Du, W., Shen, G., Zhuo, S., Zhu, X., Shen, H., Huang, Y., Su, S., Lin, N., Pei, L., Zheng, X., Wu, J., Duan, Y., Wang, X., Liu, W., Wong, M., Tao, S., 2016. Household air pollution and personal exposure to nitrated and oxygenated polycyclic aromatics (PAHs) in rural households: Influence of household cooking energies. Indoor Air n/a-n/a. doi:10.1111/ina.12300

Chen, Y., Shen, G., Su, S., Shen, H., Huang, Y., Li, T., Li, W., Zhang, Y., Lu, Y., Chen, H., others, 2014. Contamination and distribution of parent, nitrated, and oxygenated polycyclic aromatic hydrocarbons in smoked meat. Environ. Sci. Pollut. Res. 21, 11521–11530.

Cheng, S., Xia, Y., He, J., Liu, X., Chen, X., Ding, Y., Wang, Y., Peng, B., Tu, B., 2013. Neurotoxic effect of subacute benzo(a)pyrene exposure on gene and protein expression in Sprague-Dawley rats. Environ. Toxicol. Pharmacol. 36, 648–658. doi:10.1016/j.etap.2013.06.008

Chibwe, L., Geier, M.C., Nakamura, J., Tanguay, R.L., Aitken, M.D., Simonich, S.L.M., 2015. Aerobic Bioremediation of PAH Contaminated Soil Results in Increased Genotoxicity and Developmental Toxicity. Environ. Sci. Technol. 49, 13889–13898. doi:10.1021/acs.est.5b00499

Chou, M.W., Evans, F.E., Yang, S.K., Fu, P.P., 1983. Evidence for a 2, 3-epoxide as an intermediate in the microsomal metabolism of 6-nitrobenzo [a] pyrene. Carcinogenesis 4, 699–702.

Ciganek, M., Neca, J., Adamec, V., Janosek, J., Machala, M., 2004. A combined chemical and bioassay analysis of traffic-emitted polycyclic aromatic hydrocarbons. Sci. Total Environ., Highway and Urban Pollution 334–335, 141–148. doi:10.1016/j.scitotenv.2004.04.034

Clark, A.J., Iwobi, M., Cui, W., Crompton, M., Harold, G., Hobbs, S., Kamalati, T., Knox, R., Neil, C., Yull, F., others, 1997. Selective cell ablation in transgenic mice expressing E. coli nitroreductase. Gene Ther. 4, 101–110.

Cochran, R.E., Jeong, H., Haddadi, S., Fisseha Derseh, R., Gowan, A., Beránek, J., Kubátová, A., 2016. Identification of products formed during the heterogeneous nitration and ozonation of polycyclic aromatic hydrocarbons. Atmos. Environ. 128, 92–103. doi:10.1016/j.atmosenv.2015.12.036

Collins, J.F., Brown, J.P., Alexeeff, G.V., Salmon, A.G., 1998. Potency Equivalency Factors for Some Polycyclic Aromatic Hydrocarbons and Polycyclic Aromatic Hydrocarbon Derivatives. Regul. Toxicol. Pharmacol. 28, 45–54. doi:10.1006/rtph.1998.1235

Curado, S., Anderson, R.M., Jungblut, B., Mumm, J., Schroeter, E., Stainier, D.Y., 2007. Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies. Dev. Dyn. 236, 1025–1035.

Curado, S., Stainier, D.Y., Anderson, R.M., 2008. Nitroreductase-mediated cell/tissue ablation in zebrafish: a spatially and temporally controlled ablation method with applications in developmental and regeneration studies. Nat. Protoc. 3, 948–954.

Debnath, A.K., Lopez Compadre, R.L., Shusterman, A.J., Hansch, C., 1992. Quantitative structure-activity relationship investigation of the role of hydrophobicity in regulating mutagenicity in the Ames test: 2. Mutagenicity of aromatic and heteroaromatic nitro compounds in Salmonella typhimurium TA100. Environ. Mol. Mutagen. 19, 53–70.

Dimashki, M., Harrad, S., Harrison, R.M., 2000. Measurements of nitro-PAH in the atmospheres of two cities. Atmos. Environ. 34, 2459–2469. doi:10.1016/S1352-2310(99)00417-3

Duarte-Salles, T., Mendez, M.A., Morales, E., Bustamante, M., Rodríguez-Vicente, A., Kogevinas, M., Sunyer, J., 2012. Dietary benzo(a)pyrene and fetal growth: Effect

modification by vitamin C intake and glutathione S-transferase P1 polymorphism. Environ. Int. 45, 1–8. doi:10.1016/j.envint.2012.04.002

Eastmond, D.A., Booth, G.M., Lee, M.L., 1984. Toxicity, accumulation, and elimination of polycyclic aromatic sulfur heterocycles inDaphnia magna. Arch. Environ. Contam. Toxicol. 13, 105–111. doi:10.1007/BF01055652

Edwards, S.C., Jedrychowski, W., Butscher, M., Camann, D., Kieltyka, A., Mroz, E., Flak, E., Li, Z., Wang, S., Rauh, V., others, 2010. Prenatal exposure to airborne polycyclic aromatic hydrocarbons and children's intelligence at 5 years of age in a prospective cohort study in Poland. Environ. Health Perspect. 118, 1326.

Einistö, P., Watanabe, M., Motoi Ishidate, J., Nohmi, T., 1991. Mutagenicity of 30 chemicals in Salmonella typhimurium strains possessing different nitroreductase or O-acetyltransferase activities. Mutat. Res. Toxicol. 259, 95–102. doi:10.1016/0165-1218(91)90113-Z

Eisentraeger, A., Brinkmann, C., Hollert, H., Sagner, A., Tiehm, A., Neuwoehner, J., 2008. Heterocyclic compounds: Toxic effects using algae, daphnids, and the Salmonella/microsome test taking methodical quantitative aspects into account. Environ. Toxicol. Chem. 27, 1590–1596. doi:10.1897/07-201.1

El-Bayoumy, K., Hecht, S.S., 1982. Identification of Mutagenic Metabolites Formed by C-Hydroxylation and Nitroreduction of 5-Nitroacenaphthene in Rat Liver. Cancer Res. 42, 1243–1248.

El-Bayoumy, K., Sharma, C., Louis, Y.M., Reddy, B., Hecht, S.S., 1983. The role of intestinal microflora in the metabolic reduction of 1-nitropyrene to 1-aminopyrene in conventional and germfree rats and in humans. Cancer Lett. 19, 311–316.

Essumang, D.K., Dodoo, D.K., Adjei, J.K., 2012. Polycyclic aromatic hydrocarbon (PAH) contamination in smoke-cured fish products. J. Food Compos. Anal. 27, 128–138. doi:10.1016/j.jfca.2012.04.007

Esteve, W., Budzinski, H., Villenave, E., 2006. Relative rate constants for the heterogeneous reactions of NO2 and OH radicals with polycyclic aromatic hydrocarbons adsorbed on carbonaceous particles. Part 2: PAHs adsorbed on diesel particulate exhaust SRM 1650a. Atmos. Environ. 40, 201–211. doi:10.1016/j.atmosenv.2005.07.053

Forsberg, N.D., Stone, D., Harding, A., Harper, B., Harris, S., Matzke, M.M., Cardenas, A., Waters, K.M., Anderson, K.A., 2012. Effect of Native American Fish Smoking Methods on Dietary Exposure to Polycyclic Aromatic Hydrocarbons and Possible Risks to Human Health. J. Agric. Food Chem. 60, 6899–6906. doi:10.1021/jf300978m

Fosque, B.F., Sun, Y., Dana, H., Yang, C.-T., Ohyama, T., Tadross, M.R., Patel, R., Zlatic, M., Kim, D.S., Ahrens, M.B., Jayaraman, V., Looger, L.L., Schreiter, E.R., 2015. Labeling of active neural circuits in vivo with designed calcium integrators. Science 347, 755–760. doi:10.1126/science.1260922

Freeman, D.J., Cattell, F.C., 1990. Woodburning as a source of atmospheric polycyclic aromatic hydrocarbons. Environ. Sci. Technol. 24, 1581–1585.

Fu, P.P., Cerniglia, C.E., Richardson, K.E., Heflich, R.H., 1988. Nitroreduction of 6-nitrobenzo [a] pyrene: a potential activation pathway in humans. Mutat. Res. Lett. 209, 123–129.

Fu, P.P., Herreno-Saenz, D., 1999. Nitro-polycyclic aromatic hydrocarbons: A class of genotoxic environmental pollutants. J. Environ. Sci. Health Part C 17, 1–43.

Gale, S.L., Noth, E.M., Mann, J., Balmes, J., Hammond, S.K., Tager, I.B., 2012. Polycyclic aromatic hydrocarbon exposure and wheeze in a cohort of children with asthma in Fresno, CA. J. Expo. Sci. Environ. Epidemiol. 22, 386–392. doi:10.1038/jes.2012.29

Garcia, G.R., Noyes, P.D., Tanguay, R.L., 2016. Advancements in zebrafish applications for 21st century toxicology. Pharmacol. Ther. 161, 11–21. doi:10.1016/j.pharmthera.2016.03.009

Garner, L.V.T., Brown, D.R., Di Giulio, R.T., 2013. Knockdown of AHR1A but not AHR1B exacerbates PAH and PCB-126 toxicity in zebrafish (Danio rerio) embryos. Aquat. Toxicol. Amst. Neth. 142–143, 336–346. doi:10.1016/j.aquatox.2013.09.007

Geerts, C.C., Bots, M.L., Ent, C.K. van der, Grobbee, D.E., Uiterwaal, C.S.P.M., 2012. Parental Smoking and Vascular Damage in Their 5-year-old Children. Pediatrics 129, 45–54. doi:10.1542/peds.2011-0249

Goldstone, J.V., McArthur, A.G., Kubota, A., Zanette, J., Parente, T., Jönsson, M.E., Nelson, D.R., Stegeman, J.J., 2010. Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. BMC Genomics 11, 643. doi:10.1186/1471-2164-11-643

Goodale, B.C., Du, J.K.L., Bisson, W.H., Janszen, D.B., Waters, K.M., Tanguay, R.L., 2012. AHR2 Mutant Reveals Functional Diversity of Aryl Hydrocarbon Receptors in Zebrafish. PLOS ONE 7, e29346. doi:10.1371/journal.pone.0029346

Goodale, B.C., Tilton, S.C., Corvi, M.M., Wilson, G.R., Janszen, D.B., Anderson, K.A., Waters, K.M., Tanguay, R.L., 2013. Structurally distinct polycyclic aromatic hydrocarbons induce differential transcriptional responses in developing zebrafish. Toxicol. Appl. Pharmacol. 272, 656–670.

Grant, W.B., 2009. Air Pollution in Relation to U.S. Cancer Mortality Rates: An Ecological Study; Likely Role of Carbonaceous Aerosols and Polycyclic Aromatic Hydrocarbons. Anticancer Res. 29, 3537–3545.

Guengerich, F.P., 2001. Common and Uncommon Cytochrome P450 Reactions Related to Metabolism and Chemical Toxicity. Chem. Res. Toxicol. 14, 611–650. doi:10.1021/tx0002583

Hackshaw, A.K., Law, M.R., Wald, N.J., 1997. The accumulated evidence on lung cancer and environmental tobacco smoke. Bmj 315, 980–988.

Hahn, M.E., Karchner, S.I., Merson, R.R., n.d. Diversity as Opportunity: Insights from 600 Million Years of AHR Evolution. Curr. Opin. Toxicol. doi:10.1016/j.cotox.2017.02.003

Hannink, N., Rosser, S.J., French, C.E., Basran, A., Murray, J.A.H., Nicklin, S., Bruce, N.C., 2001. Phytodetoxification of TNT by transgenic plants expressing a bacterial nitroreductase. Nat. Biotechnol. 19, 1168–1172. doi:10.1038/nbt1201-1168

Harvey, R.G., 1996. Mechanisms Of Carcinogenesis of Polycyclic Aromatic Hydrocarbons. Polycycl. Aromat. Compd. 9, 1–23. doi:10.1080/10406639608031196

Hattori, T., Tang, N., Tamura, K., Hokoda, A., Yang, X., Igarashi, K., Ohno, M., Okada, Y., Kameda, T., Toriba, A., Hayakawa, K., 2007. Particulate Polycyclic Aromatic Hydrocarbons and Their Nitrated Derivatives in Three Cities in Liaoning Province, China. Environ. Forensics 8, 165–172. doi:10.1080/15275920601180701

Hayakawa, K., Kawaguchi, Y., Murahashi, T., Miyazaki, M., 1995. Distribution of nitropyrenes and mutagenicity in airborne particulates collected with an Andersen sampler. Mutat. Res. Lett. 348, 57–61. doi:10.1016/0165-7992(95)00046-1

Hecht, S.S., 2003. Tobacco carcinogens, their biomarkers and tobacco-induced cancer. Nat. Rev. Cancer 3, 733–744. doi:10.1038/nrc1190

Hertz-Picciotto, I., Baker, R.J., Yap, P.-S., Dostá, M., Joad, J.P., Lipsett, M., Greenfield, T., Herr, C.E.W., Beneš, I., Shumway, R.H., Pinkerton, K.E., Šrám, R., 2007. Early Childhood Lower Respiratory Illness and Air Pollution. Environ. Health Perspect. 115, 1510–1518.

Hicken, C.E., Linbo, T.L., Baldwin, D.H., Willis, M.L., Myers, M.S., Holland, L., Larsen, M., Stekoll, M.S., Rice, S.D., Collier, T.K., Scholz, N.L., Incardona, J.P., 2011. Sublethal exposure to crude oil during embryonic development alters cardiac morphology and reduces aerobic capacity in adult fish. Proc. Natl. Acad. Sci. 108, 7086–7090. doi:10.1073/pnas.1019031108

Hill, A.J., Teraoka, H., Heideman, W., Peterson, R.E., 2005. Zebrafish as a Model Vertebrate for Investigating Chemical Toxicity. Toxicol. Sci. 86, 6–19. doi:10.1093/toxsci/kfi110

Hoh, E., Hunt, R.N., Quintana, P.J., Zakarian, J.M., Chatfield, D.A., Wittry, B.C., Rodriguez, E., Matt, G.E., 2012. Environmental tobacco smoke as a source of polycyclic aromatic hydrocarbons in settled household dust. Environ. Sci. Technol. 46, 4174–4183.

Howard, P.C., Beland, F.A., Cerniglia, C.E., 1983a. Reduction of the carcinogen 1-nitropyrene to 1-aminopyrene by rat intestinal bacteria. Carcinogenesis 4, 985–990. doi:10.1093/carcin/4.8.985

Howard, P.C., Gerrard, J.A., Milo, G.E., Peter, P.F., Beland, F.A., Kadlubar, F.F., 1983b. Transformation of normal human skin fibroblasts by 1-nitropyrene and 6-nitrobenzo [a] pyrene. Carcinogenesis 4, 353–355. doi:10.1093/carcin/4.3.353

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S., McLaren, K., Matthews, L., others, 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498–503.

Howsam, M., Jones, K.C., 1998. Sources of PAHs in the Environment, in: Neilson, D.A.H. (Ed.), PAHs and Related Compounds, The Handbook of Environmental Chemistry. Springer Berlin Heidelberg, pp. 137–174. doi:10.1007/978-3-540-49697-7_4

Hruscha, A., Krawitz, P., Rechenberg, A., Heinrich, V., Hecht, J., Haass, C., Schmid, B., 2013. Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish. Development 140, 4982–4987. doi:10.1242/dev.099085

Hrycay, E.G., Bandiera, S.M., 2010. Cytochrome P450 Enzymes, in: Pharmaceutical Sciences Encyclopedia. John Wiley & Sons, Inc. doi:10.1002/9780470571224.pse048

Hu, S., Herner, J.D., Robertson, W., Kobayashi, R., Chang, M.-C.O., Huang, S., Zielinska, B., Kado, N., Collins, J.F., Rieger, P., others, 2013. Emissions of polycyclic aromatic hydrocarbons (PAHs) and nitro-PAHs from heavy-duty diesel vehicles with DPF and SCR. J. Air Waste Manag. Assoc. 63, 984–996.

Huang, L., Wang, C., Zhang, Y., Li, J., Zhong, Y., Zhou, Y., Chen, Y., Zuo, Z., 2012. Benzo[a]pyrene exposure influences the cardiac development and the expression of cardiovascular relative genes in zebrafish (Danio rerio) embryos. Chemosphere 87, 369–375. doi:10.1016/j.chemosphere.2011.12.026

Hwang, W.Y., Fu, Y., Reyon, D., Maeder, M.L., Tsai, S.Q., Sander, J.D., Peterson, R.T., Yeh, J.-R.J., Joung, J.K., 2013. Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat. Biotechnol. 31, 227–229. doi:10.1038/nbt.2501

Hylland, K., 2006. Polycyclic Aromatic Hydrocarbon (PAH) Ecotoxicology in Marine Ecosystems. J. Toxicol. Environ. Health A 69, 109–123. doi:10.1080/15287390500259327

Hyötyläinen, T., Olkari, A., 1999. The toxicity and concentrations of PAHs in creosote-contaminated lake sediment. Chemosphere 38, 1135–1144. doi:10.1016/S0045-6535(98)00362-2

Incardona, J.P., Gardner, L.D., Linbo, T.L., Brown, T.L., Esbaugh, A.J., Mager, E.M., Stieglitz, J.D., French, B.L., Labenia, J.S., Laetz, C.A., Tagal, M., Sloan, C.A., Elizur, A., Benetti, D.D., Grosell, M., Block, B.A., Scholz, N.L., 2014. Deepwater Horizon crude oil impacts the developing hearts of large predatory pelagic fish. Proc. Natl. Acad. Sci. 111, E1510–E1518. doi:10.1073/pnas.1320950111

Incardona, J.P., Swarts, T.L., Edmunds, R.C., Linbo, T.L., Aquilina-Beck, A., Sloan, C.A., Gardner, L.D., Block, B.A., Scholz, N.L., 2013. Exxon Valdez to Deepwater Horizon: Comparable toxicity of both crude oils to fish early life stages. Aquat. Toxicol. 142–143, 303–316. doi:10.1016/j.aquatox.2013.08.011

Iwanari, M., Nakajima, M., Kizu, R., Hayakawa, K., Yokoi, T., 2002. Induction of CYP1A1, CYP1A2, and CYP1B1 mRNAs by nitropolycyclic aromatic hydrocarbons in various human tissue-derived cells: chemical-, cytochrome P450 isoform-, and cell-specific differences. Arch. Toxicol. 76, 287–298. doi:10.1007/s00204-002-0340-z

Jaffe, D., Anderson, T., Covert, D., Kotchenruther, R., Trost, B., Danielson, J., Simpson, W., Berntsen, T., Karlsdottir, S., Blake, D., Harris, J., Carmichael, G., Uno, I., 1999. Transport of Asian air pollution to North America. Geophys. Res. Lett. 26, 711–714. doi:10.1029/1999GL900100

Janoszka, B., Warzecha, L., Blaszczyk, U., Bodzek, D., 2004. Organic compounds formed in thermally treated high-protein food. Part II: Azaarenes. Acta Chromatogr. 129–141.

Jariyasopit, N., McIntosh, M., Zimmermann, K., Arey, J., Atkinson, R., Cheong, P.H.-Y., Carter, R.G., Yu, T.-W., Dashwood, R.H., Massey Simonich, S.L., 2013. Novel nitro-PAH formation from heterogeneous reactions of PAHs with NO2, NO3/N2O5, and OH radicals: prediction, laboratory studies, and mutagenicity. Environ. Sci. Technol. 48, 412–419.

Jariyasopit, N., Zimmermann, K., Schrlau, J., Arey, J., Atkinson, R., Yu, T.-W., Dashwood, R.H., Tao, S., Simonich, S.L.M., 2014. Heterogeneous Reactions of Particulate Matter-Bound PAHs and NPAHs with NO3/N2O5, OH Radicals, and O3 under Simulated Long-Range Atmospheric Transport Conditions: Reactivity and Mutagenicity. Environ. Sci. Technol. 48, 10155–10164.

Joe Jr, F.L., Salemme, J., Fazio, T., 1985. Liquid chromatographic determination of basic nitrogen-containing polynuclear aromatic hydrocarbons in smoked foods. J.-Assoc. Off. Anal. Chem. 69, 218–222.

Johnson, D.E., Riley, M.G., Cornish, H.H., 1984. Acute target organ toxicity of 1-nitronaphthalene in the rat. J. Appl. Toxicol. 4, 253–257.

Jönsson, M.E., Orrego, R., Woodin, B.R., Goldstone, J.V., Stegeman, J.J., 2007. Basal and 3,3′,4,4′,5-pentachlorobiphenyl-induced expression of cytochrome P450 1A, 1B and 1C genes in zebrafish. Toxicol. Appl. Pharmacol. 221, 29–41. doi:10.1016/j.taap.2007.02.017

Jules, G.E., Pratap, S., Ramesh, A., Hood, D.B., 2012. In utero exposure to benzo(a)pyrene predisposes offspring to cardiovascular dysfunction in later-life. Toxicology 295, 56–67. doi:10.1016/j.tox.2012.01.017

Jung, D., Matson, C.W., Collins, L.B., Laban, G., Stapleton, H.M., Bickham, J.W., Swenberg, J.A., Giulio, R.T.D., 2011. Genotoxicity in Atlantic killifish (Fundulus heteroclitus) from a PAH-contaminated Superfund site on the Elizabeth River, Virginia. Ecotoxicology 20, 1890–1899. doi:10.1007/s10646-011-0727-9

Jung, D.K.J., Klaus, T., Fent, K., 2001. Cytochrome P450 induction by nitrated polycyclic aromatic hydrocarbons, azaarenes, and binary mixtures in fish hepatoma cell line PLHC-1. Environ. Toxicol. Chem. 20, 149–159. doi:10.1002/etc.5620200117

Jung, J.-H., Hicken, C.E., Boyd, D., Anulacion, B.F., Carls, M.G., Shim, W.J., Incardona, J.P., 2013. Geologically distinct crude oils cause a common cardiotoxicity syndrome in developing zebrafish. Chemosphere 91, 1146–1155. doi:10.1016/j.chemosphere.2013.01.019

Jyethi, D.S., Khillare, P.S., Sarkar, S., 2014. Risk assessment of inhalation exposure to polycyclic aromatic hydrocarbons in school children. Environ. Sci. Pollut. Res. 21, 366–378. doi:10.1007/s11356-013-1912-6

Kafafi, S.A., Afeefy, H.Y., Ali, A.H., Said, H.K., Kafafi, A.G., 1993. Binding of polychlorinated biphenyls to the aryl hydrocarbon receptor. Environ. Health Perspect. 101, 422–428.

Kameda, T., Azumi, E., Fukushima, A., Tang, N., Matsuki, A., Kamiya, Y., Toriba, A., Hayakawa, K., 2016. Mineral dust aerosols promote the formation of toxic nitropolycyclic aromatic compounds. Sci. Rep. 6. doi:10.1038/srep24427

Kamens, R.M., Guo, J., Guo, Z., McDow, S.R., 1990. Polynuclear aromatic hydrocarbon degradation by heterogeneous reactions with N2O5 on atmospheric particles. Atmospheric Environ. Part Gen. Top. 24, 1161–1173. doi:10.1016/0960-1686(90)90081-W

Karchner, S.I., Franks, D.G., Hahn, M.E., 2005. AHR1B, a new functional aryl hydrocarbon receptor in zebrafish: tandem arrangement of ahr1b and ahr2 genes. Biochem. J. 392, 153–161. doi:10.1042/BJ20050713

Kewley, R.J., Whitelaw, M.L., Chapman-Smith, A., 2004. The mammalian basic helix–loop–helix/PAS family of transcriptional regulators. Int. J. Biochem. Cell Biol. 36, 189–204. doi:10.1016/S1357-2725(03)00211-5

Khalili, N.R., Scheff, P.A., Holsen, T.M., 1995. PAH source fingerprints for coke ovens, diesel and, gasoline engines, highway tunnels, and wood combustion emissions. Atmos. Environ. 29, 533–542. doi:10.1016/1352-2310(94)00275-P

Kim, K.-H., Jahan, S.A., Kabir, E., 2011. A review of diseases associated with household air pollution due to the use of biomass fuels. J. Hazard. Mater. 192, 425–431. doi:10.1016/j.jhazmat.2011.05.087

Kim, K.-H., Park, H.-J., Kim, J.H., Kim, S., Williams, D.R., Kim, M.-K., Jung, Y.D., Teraoka, H., Park, H.-C., Choy, H.E., Shin, B.A., Choi, S.-Y., 2013. Cyp1a reporter zebrafish reveals target tissues for dioxin. Aquat. Toxicol. Amst. Neth. 134–135, 57–65. doi:10.1016/j.aquatox.2013.03.010

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310. doi:10.1002/aja.1002030302

Knecht, A.L., Goodale, B.C., Truong, L., Simonich, M.T., Swanson, A.J., Matzke, M.M., Anderson, K.A., Waters, K.M., Tanguay, R.L., 2013. Comparative developmental toxicity of environmentally relevant oxygenated PAHs. Toxicol. Appl. Pharmacol. 271, 266–275.

Knecht, A.L., Truong, L., Simonich, M.T., Tanguay, R.L., 2016. Developmental benzo[a]pyrene (B[a]P) exposure impacts larval behavior and impairs adult learning in zebrafish. Neurotoxicol. Teratol. doi:10.1016/j.ntt.2016.10.006

Kovacic, P., Somanathan, R., 2014. Nitroaromatic compounds: Environmental toxicity, carcinogenicity, mutagenicity, therapy and mechanism. J. Appl. Toxicol. 34, 810–824.

Kukučka, P., Lammel, G., Dvorská, A., Klánová, J., Möller, A., Fries, E., 2010. Contamination of Antarctic snow by polycyclic aromatic hydrocarbons dominated by combustion sources in the polar region. Environ. Chem. 7, 504–513. doi:10.1071/EN10066

Lafontaine, S., Schrlau, J., Butler, J., Jia, Y., Harper, B., Harris, S., Bramer, L.M., Waters, K.M., Harding, A., Simonich, S.L.M., 2015. Relative Influence of Trans-Pacific and Regional Atmospheric Transport of PAHs in the Pacific Northwest, U.S. Environ. Sci. Technol. 49, 13807–13816. doi:10.1021/acs.est.5b00800

Larsson, B.K., Sahlberg, G.P., Eriksson, A.T., Busk, L.A., 1983. Polycyclic aromatic hydrocarbons in grilled food. J. Agric. Food Chem. 31, 867–873.

Lawson, N.D., Weinstein, B.M., 2002. In Vivo Imaging of Embryonic Vascular Development Using Transgenic Zebrafish. Dev. Biol. 248, 307–318. doi:10.1006/dbio.2002.0711

Lee, M.-S., Magari, S., Christiani, D.C., 2011. Cardiac autonomic dysfunction from occupational exposure to polycyclic aromatic hydrocarbons. Occup. Environ. Med. 68, 474–478. doi:10.1136/oem.2010.055681

Lewtas, J., 2007. Air pollution combustion emissions: Characterization of causative agents and mechanisms associated with cancer, reproductive, and cardiovascular effects. Mutat. Res. Mutat. Res., The Sources and Potential Hazards of Mutagens in Complex Environmental Matrices - Part II 636, 95–133. doi:10.1016/j.mrrev.2007.08.003

Lieschke, G.J., Currie, P.D., 2007. Animal models of human disease: zebrafish swim into view. Nat. Rev. Genet. 8, 353–367. doi:10.1038/nrg2091

Lloyd, A.C., Denton, J.E., 2005. Proposed Identification of Environmental Tobacco Smoke as a Toxic Air Contaminant. Part B Health Eff. Calif. Environ. Prot. Agency Off. Environ. Health Hazard Assess. Httpwww Arb Ca Govregactets2006ets2006 Htm Accès Plus Récent Au Lien 29 Juillet 2009.

Lundstedt, S., Haglund, P., Öberg, L., 2003. Degradation and formation of polycyclic aromatic compounds during bioslurry treatment of an aged gasworks soil. Environ. Toxicol. Chem. 22, 1413–1420. doi:10.1002/etc.5620220701

Maga, J.A., 1986. Polycyclic aromatic hydrocarbon (PAH) composition of mesquite (Prosopis fuliflora) smoke and grilled beef. J. Agric. Food Chem. 34, 249–251.

Mahler, B.J., Van Metre, P.C., Foreman, W.T., 2014. Concentrations of polycyclic aromatic hydrocarbons (PAHs) and azaarenes in runoff from coal-tar- and asphalt-sealcoated pavement. Environ. Pollut. 188, 81–87. doi:10.1016/j.envpol.2014.01.008

Manzetti, S., 2013. Polycyclic Aromatic Hydrocarbons in the Environment: Environmental Fate and Transformation. Polycycl. Aromat. Compd. 33, 311–330. doi:10.1080/10406638.2013.781042

Marshall, T.C., Royer, R.E., Li, A.P., Kusewitt, D.F., Brooks, A.L., 1982. Acute and genetic toxicity of 1-nitropyrene and its fate after single oral doses to rats. J. Toxicol. Environ. Health Part Curr. Issues 10, 373–384.

Massarsky, A., Jayasundara, N., Bailey, J.M., Oliveri, A.N., Levin, E.D., Prasad, G.L., Di Giulio, R.T., 2015. Teratogenic, bioenergetic, and behavioral effects of exposure to total particulate matter on early development of zebrafish (Danio rerio) are not mimicked by nicotine. Neurotoxicol. Teratol. 51, 77–88. doi:10.1016/j.ntt.2015.09.006

Mayer, P., Wernsing, J., Tolls, J., de Maagd, P.G.-J., Sijm, D.T., 1999. Establishing and controlling dissolved concentrations of hydrophobic organics by partitioning from a solid phase. Environ. Sci. Technol. 33, 2284–2290.

McCann, J., Choi, E., Yamasaki, E., Ames, B.N., 1975. Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. 72, 5135–5139.

McCoy, E.C., Rosenkranz, H.S., Mermelstein, R., 1981. Evidence for the existence of a family of bacterial nitroreductases capable of activating nitrated polycyclics to mutagens. Environ. Mutagen. 3, 421–427. doi:10.1002/em.2860030403

Menzie, C.A., Potocki, B.B., Santodonato, J., 1992. Exposure to carcinogenic PAHs in the environment. Environ. Sci. Technol. 26, 1278–1284. doi:10.1021/es00031a002

Mesquita, S.R., Dachs, J., van Drooge, B.L., Castro-Jiménez, J., Navarro-Martín, L., Barata, C., Vieira, N., Guimarães, L., Piña, B., 2016. Toxicity assessment of atmospheric particulate matter in the Mediterranean and Black Seas open waters. Sci. Total Environ. 545–546, 163–170. doi:10.1016/j.scitotenv.2015.12.055

Meyer, S., Cartellieri, S., Steinhart, H., 1999. Simultaneous Determination of PAHs, Hetero-PAHs (N, S, O), and Their Degradation Products in Creosote-Contaminated Soils. Method Development, Validation, and Application to Hazardous Waste Sites. Anal. Chem. 71, 4023–4029. doi:10.1021/ac990136j

Miller, J.S., Olejnik, D., 2001. Photolysis of polycyclic aromatic hydrocarbons in water. Water Res. 35, 233–243. doi:10.1016/S0043-1354(00)00230-X

Misaki, K., Takamura-Enya, T., Ogawa, H., Takamori, K., Yanagida, M., 2016. Tumour-promoting activity of polycyclic aromatic hydrocarbons and their oxygenated or nitrated derivatives. Mutagenesis 31, 205–213. doi:10.1093/mutage/gev076

Möller, L., 1994. In vivo metabolism and genotoxic effects of nitrated polycyclic aromatic hydrocarbons. Environ. Health Perspect. 102, 139–146.

Mulero-Navarro, S., Fernandez-Salguero, P.M., 2016. New Trends in Aryl Hydrocarbon Receptor Biology. Signaling 45. doi:10.3389/fcell.2016.00045

Nachtman, J.P., Wei, E.T., 1982. Evidence for enzymatic reduction of 1-nitropyrene by rat liver fractions. Cell. Mol. Life Sci. 38, 837–838.

Nebert, D.W., Dalton, T.P., Okey, A.B., Gonzalez, F.J., 2004. Role of Aryl Hydrocarbon Receptor-mediated Induction of the CYP1 Enzymes in Environmental Toxicity and Cancer. J. Biol. Chem. 279, 23847–23850. doi:10.1074/jbc.R400004200

Neff, J.M., 2002. Bioaccumulation in Marine Organisms: Effect of Contaminants from Oil Well Produced Water. Elsevier.

Niederer, M., 1998. Determination of polycyclic aromatic hydrocarbons and substitutes (Nitro-, Oxy-PAHs) in urban soil and airborne particulate by GC-MS and NCI-MS/MS. Environ. Sci. Pollut. Res. 5, 209. doi:10.1007/BF02986403

Nielsen, T., Jørgensen, H.E., Larsen, J.C., Poulsen, M., 1996. City air pollution of polycyclic aromatic hydrocarbons and other mutagens: occurrence, sources and health effects. Sci. Total Environ., Highway and Urban Pollution 189, 41–49. doi:10.1016/0048-9697(96)05189-3

Niu, J., Sun, P., Schramm, K.-W., 2007. Photolysis of polycyclic aromatic hydrocarbons associated with fly ash particles under simulated sunlight irradiation. J. Photochem. Photobiol. Chem. 186, 93–98. doi:10.1016/j.jphotochem.2006.07.016

Ober, E.A., Field, H.A., Stainier, D.Y., 2003. From endoderm formation to liver and pancreas development in zebrafish. Mech. Dev. 120, 5–18.

Ohe, T., Nukaya, H., 1996. Genotoxic activity of 1-nitropyrene in water from the Yodo River, Japan. Sci. Total Environ. 181, 7–12. doi:10.1016/0048-9697(95)04952-5

Ohgaki, H., Matsukura, N., Morino, K., Kawachi, T., Sugimura, T., Morita, K., Tokiwa, H., Hirota, T., 1982. Carcinogenicity in rats of the mutagenic compounds 1-nitropyrene and 3-nitrofluoranthene. Cancer Lett. 15, 1–7. doi:10.1016/0304-3835(82)90068-4

Onduka, T., Ojima, D., Ito, K., Mochida, K., Koyama, J., Fujii, K., 2015. Reproductive toxicity of 1-nitronaphthalene and 1-nitropyrene exposure in the mummichog, Fundulus heteroclitus. Ecotoxicology 24, 648–656. doi:10.1007/s10646-014-1412-6

Onduka, T., Ojima, D., Kakuno, A., Ito, K., Koyama, J., Fujii, K., 2012. Nitrated polycyclic aromatic hydrocarbons in the marine environment: acute toxicities for organisms at three trophic levels. Jpn. J. Environ. Toxicol. 15, 1–10. doi:10.11403/jset.15.1

Ozaki, N., Takemoto, N., Kindaichi, T., 2009. Nitro-PAHs and PAHs in Atmospheric Particulate Matters and Sea Sediments in Hiroshima Bay Area, Japan. Water. Air. Soil Pollut. 207, 263–271. doi:10.1007/s11270-009-0134-5

Pašková, V., Hilscherová, K., Feldmannová, M., Bláha, L., 2006. Toxic effects and oxidative stress in higher plants exposed to polycyclic aromatic hydrocarbons and their N-heterocyclic derivatives. Environ. Toxicol. Chem. 25, 3238–3245. doi:10.1897/06-162R.1

Pedersen, D.U., Durant, J.L., Taghizadeh, K., Hemond, H.F., Lafleur, A.L., Cass, G.R., 2005. Human cell mutagens in respirable airborne particles from the northeastern United States. 2. Quantification of mutagens and other organic compounds. Environ. Sci. Technol. 39, 9547–9560.

Perera, F.P., Li, Z., Whyatt, R., Hoepner, L., Wang, S., Camann, D., Rauh, V., 2009. Prenatal Airborne Polycyclic Aromatic Hydrocarbon Exposure and Child IQ at Age 5 Years. Pediatrics 124, e195–e202. doi:10.1542/peds.2008-3506

Perera, F.P., Rauh, V., Whyatt, R.M., Tang, D., Tsai, W.Y., Bernert, J.T., Tu, Y.H., Andrews, H., Barr, D.B., Camann, D.E., Diaz, D., Dietrich, J., Reyes, A., Kinney, P.L., 2005. A Summary of Recent Findings on Birth Outcomes and Developmental Effects of Prenatal ETS, PAH, and Pesticide Exposures. NeuroToxicology, Infant and Child Neurotoxicity

StudiesTwenty-first International Neurotoxicology Conference 26, 573–587. doi:10.1016/j.neuro.2004.07.007

Perera, F.P., Whyatt, R.M., Jedrychowski, W., Rauh, V., Manchester, D., Santella, R.M., Ottman, R., 1998. Recent Developments in Molecular Epidemiology: A Study of the Effects of Environmental Polycyclic Aromatic Hydrocarbons on Birth Outcomes in Poland. Am. J. Epidemiol. 147, 309–314.

Perkins, A., Phillips, J., Kerkvliet, N., Tanguay, R., Perdew, G., Kolluri, S., Bisson, W., 2014. A Structural Switch between Agonist and Antagonist Bound Conformations for a Ligand-Optimized Model of the Human Aryl Hydrocarbon Receptor Ligand Binding Domain. Biology 3, 645–669. doi:10.3390/biology3040645

Petersen, K., Fetter, E., Kah, O., Brion, F., Scholz, S., Tollefsen, K.E., 2013. Transgenic (cyp19a1b-GFP) zebrafish embryos as a tool for assessing combined effects of oestrogenic chemicals. Aquat. Toxicol. 138–139, 88–97. doi:10.1016/j.aquatox.2013.05.001

Peterson, F.J., Mason, R.P., Hovsepian, J., Holtzman, J.L., 1979. Oxygen-sensitive and -insensitive nitroreduction by Escherichia coli and rat hepatic microsomes. J. Biol. Chem. 254, 4009–4014.

Philibert, D.A., Philibert, C.P., Lewis, C., Tierney, K.B., 2016. Comparison of Diluted Bitumen (Dilbit) and Conventional Crude Oil Toxicity to Developing Zebrafish. Environ. Sci. Technol. doi:10.1021/acs.est.6b00949

Pisharath, H., Parsons, M., 2009. Nitroreductase-Mediated Cell Ablation in Transgenic Zebrafish Embryos, in: Lieschke, G.J., Oates, A.C., Kawakami, K. (Eds.), Zebrafish, Methods in Molecular Biology. Humana Press, pp. 133–143. doi:10.1007/978-1-60327-977-2_9

Polidori, A., Kwon, J., Turpin, B.J., Weisel, C., 2010. Source proximity and residential outdoor concentrations of PM2.5, OC, EC, and PAHs. J. Expo. Sci. Environ. Epidemiol. 20, 457–468. doi:10.1038/jes.2009.39

Prasch, A.L., Teraoka, H., Carney, S.A., Dong, W., Hiraga, T., Stegeman, J.J., Heideman, W., Peterson, R.E., 2003. Aryl Hydrocarbon Receptor 2 Mediates 2,3,7,8-Tetrachlorodibenzo-p-dioxin Developmental Toxicity in Zebrafish. Toxicol. Sci. 76, 138–150. doi:10.1093/toxsci/kfg202

Puga, A., Ma, C., Marlowe, J.L., 2009. The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways. Biochem. Pharmacol., Biological Functions of the Ah Receptor: Beyond Induction of Cytochrome P450s 77, 713–722. doi:10.1016/j.bcp.2008.08.031

Purohit, V., Basu, A.K., 2000. Mutagenicity of Nitroaromatic Compounds. Chem. Res. Toxicol. 13, 673–692. doi:10.1021/tx000002x

Ramesh, A., Walker, S.A., Hood, D.B., Guillén, M.D., Schneider, K., Weyand, E.H., 2004. Bioavailability and Risk Assessment of Orally Ingested Polycyclic Aromatic Hydrocarbons. Int. J. Toxicol. 23, 301–333. doi:10.1080/10915810490517063

Ramírez, N., Cuadras, A., Rovira, E., Marcé, R.M., Borrull, F., 2011. Risk assessment related to atmospheric polycyclic aromatic hydrocarbons in gas and particle phases near industrial sites. Environ. Health Perspect. 119, 1110.

Rauh, V.A., Whyatt, R.M., Garfinkel, R., Andrews, H., Hoepner, L., Reyes, A., Diaz, D., Camann, D., Perera, F.P., 2004. Developmental effects of exposure to environmental tobacco smoke and material hardship among inner-city children. Neurotoxicol. Teratol. 26, 373–385. doi:10.1016/j.ntt.2004.01.002

Reisen, F., Arey, J., 2005. Atmospheric Reactions Influence Seasonal PAH and Nitro-PAH Concentrations in the Los Angeles Basin. Environ. Sci. Technol. 39, 64–73. doi:10.1021/es035454l

Ren, A., Qiu, X., Jin, L., Ma, J., Li, Z., Zhang, L., Zhu, H., Finnell, R.H., Zhu, T., 2011. Association of selected persistent organic pollutants in the placenta with the risk of neural tube defects. Proc. Natl. Acad. Sci. 108, 12770–12775. doi:10.1073/pnas.1105209108

Rennie, M.Y., Detmar, J., Whiteley, K.J., Yang, J., Jurisicova, A., Adamson, S.L., Sled, J.G., 2011. Vessel tortuousity and reduced vascularization in the fetoplacental arterial tree after maternal exposure to polycyclic aromatic hydrocarbons. Am. J. Physiol. - Heart Circ. Physiol. 300, H675–H684. doi:10.1152/ajpheart.00510.2010

RI DEM, 2008. Rhode Island Air Toxics Guideline.

Riedl, J., Altenburger, R., 2007. Physicochemical substance properties as indicators for unreliable exposure in microplate-based bioassays. Chemosphere 67, 2210–2220.

Rogge, W.F., Hildemann, L.M., Mazurek, M.A., Cass, G.R., Simoneit, B.R.T., 1993. Sources of fine organic aerosol. 2. Noncatalyst and catalyst-equipped automobiles and heavy-duty diesel trucks. Environ. Sci. Technol. 27, 636–651. doi:10.1021/es00041a007

Rosenkranz, H.S., Mermelstein, R., 1983. Mutagenicity and genotoxicity of nitroarenes. Mutat. Res. Genet. Toxicol. 114, 217–267. doi:10.1016/0165-1110(83)90034-9

Sabaté, J., Bayona, J.M., Solanas, A.M., 2001. Photolysis of PAHs in aqueous phase by UV irradiation. Chemosphere 44, 119–124. doi:10.1016/S0045-6535(00)00208-3

Satcher, D., Zeise, L., Dunn, A.J., 2000. Health Effects of Exposure to Environmental Tobacco Smoke: The Report of the California Environmental Protection Agency. DIANE Publishing.

Sauer, J.-M., Eversole, R.R., Lehmann, C.L., Johnson, D.E., Beuving, L.J., 1997. An ultrastructural evaluation of acute 1-nitronaphthalene induced hepatic and pulmonary toxicity in the rat. Toxicol. Lett. 90, 19–27.

Schauer, C., Niessner, R., Pöschl, U., 2003. Polycyclic Aromatic Hydrocarbons in Urban Air Particulate Matter: Decadal and Seasonal Trends, Chemical Degradation, and Sampling Artifacts. Environ. Sci. Technol. 37, 2861–2868. doi:10.1021/es034059s

Schlemitz, S., Pfannhauser, W., 1996. Analysis of nitro-PAHs in food matrices by on-line reduction and high performance liquid chromatography. Food Addit. Contam. 13, 969–977.

Scholz, S., Fischer, S., Gündel, U., Küster, E., Luckenbach, T., Voelker, D., 2008. The zebrafish embryo model in environmental risk assessment—applications beyond acute toxicity testing. Environ. Sci. Pollut. Res. 15, 394–404. doi:10.1007/s11356-008-0018-z

Schuetzle, D., Riley, T.J., Prater, T.J., Harvey, T.M., Hunt, D.F., 1982. Analysis of nitrated polycyclic aromatic hydrocarbons in diesel particulates. Anal. Chem. 54, 265–271. doi:10.1021/ac00239a028

Scornaienchi, M.L., Thornton, C., Willett, K.L., Wilson, J.Y., 2010. Functional differences in the cytochrome P450 1 family enzymes from Zebrafish (Danio rerio) using heterologously expressed proteins. Arch. Biochem. Biophys. 502, 17–22. doi:10.1016/j.abb.2010.06.018

Scott, J.A., Incardona, J.P., Pelkki, K., Shepardson, S., Hodson, P.V., 2011. AhR2-mediated, CYP1A-independent cardiovascular toxicity in zebrafish (Danio rerio) embryos exposed to retene. Aquat. Toxicol. 101, 165–174. doi:10.1016/j.aquatox.2010.09.016

Sehnert, A.J., Stainier, D.Y.R., 2002. A window to the heart: can zebrafish mutants help us understand heart disease in humans? Trends Genet. 18, 491–494. doi:10.1016/S0168-9525(02)02766-X

Shen, G., Xue, M., Wei, S., Chen, Y., Wang, B., Wang, R., Lv, Y., Shen, H., Li, W., Zhang, Y., Huang, Y., Chen, H., Wei, W., Zhao, Q., Li, B., Wu, H., Tao, S., 2013. Emissions of parent, nitrated, and oxygenated polycyclic aromatic hydrocarbons from indoor corn straw burning in normal and controlled combustion conditions. J. Environ. Sci. 25, 2072–2080. doi:10.1016/S1001-0742(12)60249-6

Shimada, T., Fujii-Kuriyama, Y., 2004. Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and1B1. Cancer Sci. 95, 1–6. doi:10.1111/j.1349-7006.2004.tb03162.x

Shimizu, Y., Nakatsuru, Y., Ichinose, M., Takahashi, Y., Kume, H., Mimura, J., Fujii-Kuriyama, Y., Ishikawa, T., 2000. Benzo [a] pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor. Proc. Natl. Acad. Sci. 97, 779–782.

Smith, K.E., Oostingh, G.J., Mayer, P., 2009. Passive dosing for producing defined and constant exposure of hydrophobic organic compounds during in vitro toxicity tests. Chem. Res. Toxicol. 23, 55–65.

Sogbanmu, T.O., Nagy, E., Phillips, D.H., Arlt, V.M., Otitoloju, A.A., Bury, N.R., 2016. Lagos lagoon sediment organic extracts and polycyclic aromatic hydrocarbons induce embryotoxic, teratogenic and genotoxic effects in Danio rerio (zebrafish) embryos. Environ. Sci. Pollut. Res. 1–13. doi:10.1007/s11356-016-6490-y

Swanson, H.I., 2002. DNA binding and protein interactions of the AHR/ARNT heterodimer that facilitate gene activation. Chem. Biol. Interact. 141, 63–76. doi:10.1016/S0009-2797(02)00066-2

Taga, R., Tang, N., Hattori, T., Tamura, K., Sakai, S., Toriba, A., Kizu, R., Hayakawa, K., 2005. Direct-acting mutagenicity of extracts of coal burning-derived particulates and contribution of nitropolycyclic aromatic hydrocarbons. Mutat. Res. Toxicol. Environ. Mutagen. 581, 91–95. doi:10.1016/j.mrgentox.2004.11.013

Tang, D., Li, T., Liu, J.J., Chen, Y., Qu, L., Perera, F., 2006. PAH-DNA Adducts in Cord Blood and Fetal and Child Development in a Chinese Cohort. Environ. Health Perspect. 114, 1297–1300.

Teraoka, H., Dong, W., Tsujimoto, Y., Iwasa, H., Endoh, D., Ueno, N., Stegeman, J.J., Peterson, R.E., Hiraga, T., 2003. Induction of cytochrome P450 1A is required for circulation failure and edema by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin in zebrafish. Biochem. Biophys. Res. Commun. 304, 223–228.

Titaley, I.A., Chlebowski, A., Truong, L., Tanguay, R.L., Massey Simonich, S.L., 2016. Identification and Toxicological Evaluation of Unsubstituted PAHs and Novel PAH

Derivatives in Pavement Sealcoat Products. Environ. Sci. Technol. Lett. doi:10.1021/acs.estlett.6b00116

Tobiszewski, M., Namieśnik, J., 2012. PAH diagnostic ratios for the identification of pollution emission sources. Environ. Pollut. 162, 110–119. doi:10.1016/j.envpol.2011.10.025

Truong, L., Bugel, S.M., Chlebowski, A., Usenko, C.Y., Simonich, M.T., Simonich, S.L.M., Tanguay, R.L., 2016. Optimizing multi-dimensional high throughput screening using zebrafish. Reprod. Toxicol. 65, 139–147. doi:10.1016/j.reprotox.2016.05.015

Uno, S., Tanaka, H., Miki, S., Kokushi, E., Ito, K., Yamamoto, M., Koyama, J., 2011. Bioaccumulation of nitroarenes in bivalves at Osaka Bay, Japan. Mar. Pollut. Bull., 6th International Conference on Marine Pollution and Ecotoxicology 63, 477–481. doi:10.1016/j.marpolbul.2011.02.044

US EPA, 2014. Priority Pollutant List [WWW Document]. URL https://www.epa.gov/sites/production/files/2015-09/documents/priority-pollutant-list-epa.pdf (accessed 11.2.16).

US EPA National Center for Environmental Assessment, I.O., Jones, S., n.d. Development of a Relative Potency Factor (RPF) Approach for Polycyclic Aromatic Hydrocarbon (PAH) Mixtures (External Review Draft) [WWW Document]. URL https://cfpub.epa.gov/ncea/iris_drafts/recordisplay.cfm?deid=194584 (accessed 2.3.17).

Usenko, S., Landers, D.H., Appleby, P.G., Simonich, S.L., 2007. Current and Historical Deposition of PBDEs, Pesticides, PCBs, and PAHs to Rocky Mountain National Park. Environ. Sci. Technol. 41, 7235–7241. doi:10.1021/es0710003

Van de Wiele, T.R., Peru, K.M., Verstraete, W., Siciliano, S.D., Headley, J.V., 2004. Liquid chromatography–mass spectrometry analysis of hydroxylated polycyclic aromatic hydrocarbons, formed in a simulator of the human gastrointestinal tract. J. Chromatogr. B 806, 245–253. doi:10.1016/j.jchromb.2004.04.001

Van Metre, P.C., Mahler, B.J., 2010. Contribution of PAHs from coal–tar pavement sealcoat and other sources to 40 U.S. lakes. Sci. Total Environ. 409, 334–344. doi:10.1016/j.scitotenv.2010.08.014

Van Tiem, L.A., Di Giulio, R.T., 2011. AHR2 knockdown prevents PAH-mediated cardiac toxicity and XRE- and ARE-associated gene induction in zebrafish (Danio rerio). Toxicol. Appl. Pharmacol. 254, 280–287. doi:10.1016/j.taap.2011.05.002

Vergauwen, L., Schmidt, S.N., Stinckens, E., Maho, W., Blust, R., Mayer, P., Covaci, A., Knapen, D., 2015. A high throughput passive dosing format for the Fish Embryo Acute Toxicity test. Chemosphere 139, 9–17.

Veyrand, B., Sirot, V., Durand, S., Pollono, C., Marchand, P., Dervilly-Pinel, G., Tard, A., Leblanc, J.-C., Le Bizec, B., 2013. Human dietary exposure to polycyclic aromatic hydrocarbons: Results of the second French Total Diet Study. Environ. Int. 54, 11–17. doi:10.1016/j.envint.2012.12.011

Wakeham, S.G., 1979. Azaarenes in recent lake sediments. Environ. Sci. Technol. 13, 1118–1123.

Walsh, C.D., Schrlau, J., Simonich, S.M., 2015. Development and Use of a Method for the Determination of Polycyclic Aromatic Hydrocarbon and Organochlorine Pesticide Concentrations in Freshly Fallen Snow. Polycycl. Aromat. Compd. 35, 57–73. doi:10.1080/10406638.2014.910239

Watanabe, M., Ishidate Jr., M., Nohmi, T., 1989. A sensitive method for the detection of mutagenic nitroarenes: construction of nitroreductase-overproducing derivatives of Salmonella typhimurium strains TA98 and TA100. Mutat. Res. Mutagen. Relat. Subj. 216, 211–220. doi:10.1016/0165-1161(89)90007-1

Wei, C., Bandowe, B.A.M., Han, Y., Cao, J., Zhan, C., Wilcke, W., 2015. Polycyclic aromatic hydrocarbons (PAHs) and their derivatives (alkyl-PAHs, oxygenated-PAHs, nitrated-PAHs and azaarenes) in urban road dusts from Xi'an, Central China. Chemosphere 134, 512–520. doi:10.1016/j.chemosphere.2014.11.052

Whitlock Jr, J.P., 1999. Induction of cytochrome P4501A1. Annu. Rev. Pharmacol. Toxicol. 39, 103–125.

WHO, 2015. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans [WWW Document]. URL http://monographs.iarc.fr/index.php (accessed 11.2.16).

Wickström, K., Pyysalo, H., Plaami-Heikkillä, S., Tuominen, J., 1986. Polycyclic aromatic compounds (PAC) in leaf lettuce. Z. Für Lebensm.-Unters. Forsch. 183, 182–185. doi:10.1007/BF01027443

Wilhelm, M., Ghosh, J.K., Su, J., Cockburn, M., Jerrett, M., Ritz, B., 2012. Traffic-related air toxics and term low birth weight in Los Angeles County, California. Environ. Health Perspect. 120, 132.

Wills, L.P., Matson, C.W., Landon, C.D., Di Giulio, R.T., 2010. Characterization of the recalcitrant CYP1 phenotype found in Atlantic killifish (Fundulus heteroclitus) inhabiting a Superfund site on the Elizabeth River, VA. Aquat. Toxicol. 99, 33–41. doi:10.1016/j.aquatox.2010.03.015

Wincent, E., Jönsson, M.E., Bottai, M., Lundstedt, S., Dreij, K., 2015. Aryl Hydrocarbon Receptor Activation and Developmental Toxicity in Zebrafish in Response to Soil Extracts Containing Unsubstituted and Oxygenated PAHs. Environ. Sci. Technol. 49, 3869–3877. doi:10.1021/es505588s

Wislocki, P.G., Bagan, E.S., Lu, A.Y.H., Dooley, K.L., Fu, P., Han-Hsu, H., Beland, F.A., Kadlubar, F.F., 1986. Tumorigenicity of nitrated derivatives of pyrene, benz[a]anthracene, chrysene and benzo[a]pyrene in the newborn mouse assay. Carcinogenesis 7, 1317–1322. doi:10.1093/carcin/7.8.1317

Wretling, S., Eriksson, A., Eskhult, G.A., Larsson, B., 2010. Polycyclic aromatic hydrocarbons (PAHs) in Swedish smoked meat and fish. J. Food Compos. Anal. 23, 264–272.

Wu, M.-T., Lee, T.-C., Wu, I.-C., Su, H.-J., Huang, J.-L., Peng, C.-Y., Wang, W., Chou, T.-Y., Lin, M.-Y., Lin, W.-Y., Huang, C.-T., Pan, C.-H., Ho, C.-K., 2011. Whole Genome Expression in Peripheral-Blood Samples of Workers Professionally Exposed to Polycyclic Aromatic Hydrocarbons. Chem. Res. Toxicol. 24, 1636–1643. doi:10.1021/tx200181q

Xiong, K.M., Peterson, R.E., Heideman, W., 2008. Aryl Hydrocarbon Receptor-Mediated Down-Regulation of Sox9b Causes Jaw Malformation in Zebrafish Embryos. Mol. Pharmacol. 74, 1544–1553. doi:10.1124/mol.108.050435

Xu, X., Hu, H., Kearney, G.D., Kan, H., Sheps, D.S., 2013. Studying the effects of polycyclic aromatic hydrocarbons on peripheral arterial disease in the United States. Sci. Total Environ. 461–462, 341–347. doi:10.1016/j.scitotenv.2013.04.089

Yang, L., Ho, N.Y., Alshut, R., Legradi, J., Weiss, C., Reischl, M., Mikut, R., Liebel, U., Müller, F., Strähle, U., 2009. Zebrafish embryos as models for embryotoxic and teratological effects of chemicals. Reprod. Toxicol., 37th Annual Conference of the European Teratology Society 28, 245–253. doi:10.1016/j.reprotox.2009.04.013

Yang, X.-Y., Igarashi, K., Tang, N., Lin, J.-M., Wang, W., Kameda, T., Toriba, A., Hayakawa, K., 2010. Indirect- and direct-acting mutagenicity of diesel, coal and wood burning-derived particulates and contribution of polycyclic aromatic hydrocarbons and nitropolycyclic aromatic hydrocarbons. Mutat. Res. Toxicol. Environ. Mutagen. 695, 29–34. doi:10.1016/j.mrgentox.2009.10.010

Yeager, R.L., Reisman, S.A., Aleksunes, L.M., Klaassen, C.D., 2009. Introducing the "TCDD-Inducible AhR-Nrf2 Gene Battery." Toxicol. Sci. 111, 238–246. doi:10.1093/toxsci/kfp115

Yoshikawa, T., Giamalva, D.H., Flory, W., Ruhr, L.P., Church, D.F., Pryor, W.A., 1988. Toxicity of aromatic hydrocarbons. VII. Hepatotoxicity of 9-nitrophenanthrene, and protection against it by beta-naphthoflavone. Biochem. Int. 16, 935–940.

Yunker, M.B., Macdonald, R.W., Vingarzan, R., Mitchell, R.H., Goyette, D., Sylvestre, S., 2002. PAHs in the Fraser River basin: a critical appraisal of PAH ratios as indicators of PAH source and composition. Org. Geochem. 33, 489–515. doi:10.1016/S0146-6380(02)00002-5

Zhang, Y., Tao, S., 2009. Global atmospheric emission inventory of polycyclic aromatic hydrocarbons (PAHs) for 2004. Atmos. Environ. 43, 812–819. doi:10.1016/j.atmosenv.2008.10.050

Zimmermann, K., Jariyasopit, N., Massey Simonich, S.L., Tao, S., Atkinson, R., Arey, J., 2013. Formation of nitro-PAHs from the heterogeneous reaction of ambient particle-bound PAHs with N2O5/NO3/NO2. Environ. Sci. Technol. 47, 8434–8442.

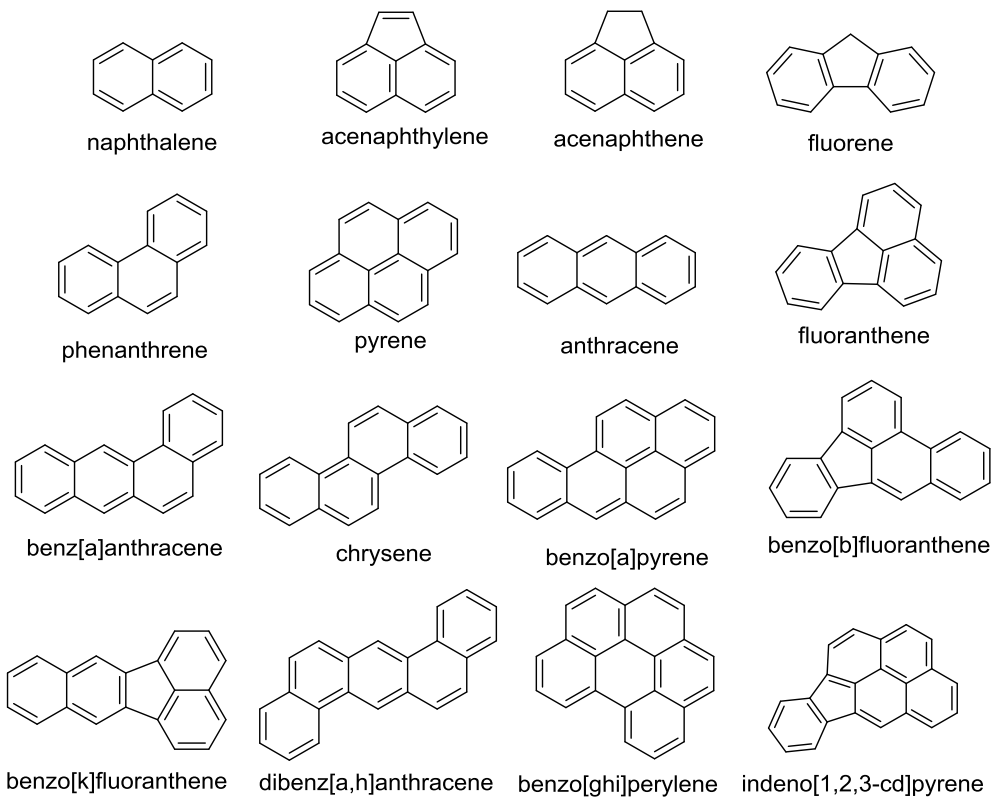

Figure 1.1. US EPA Priority Pollutant PAHs. Structures of the 16 PAHs classified as Priority Pollutants by the US EPA.

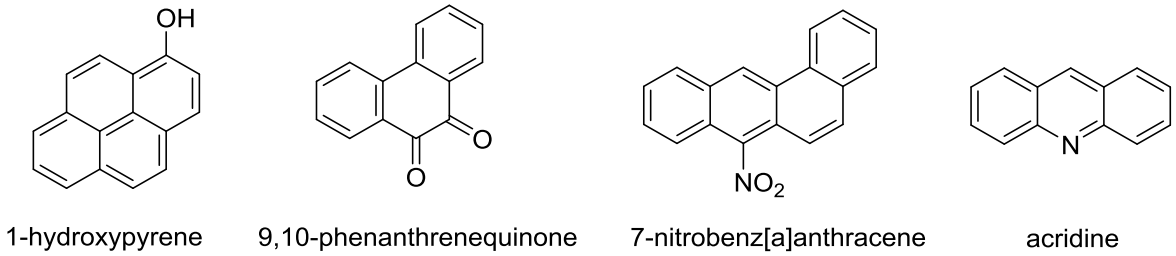

Figure 1.2.PAH derivative structures. Example structures of hydroxylated (1-hydroxypyrene), oxygenated (9,10-phenathrene quinone), nitrated (7-nitrobenz[a]anthracene), and heterocyclic (acridine) PAHs.

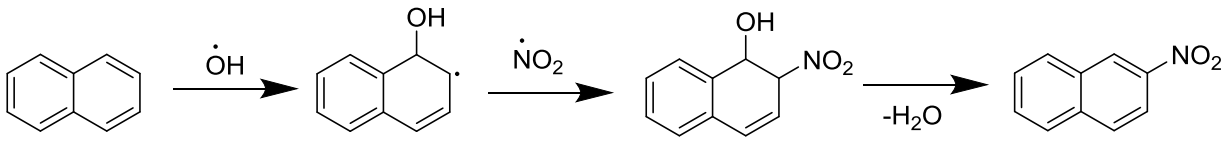

Figure 1.3. NPAH formation. General reaction schematic for the gas-phase reaction of PAHs with atmospheric radicals to generate nitrated PAHs.

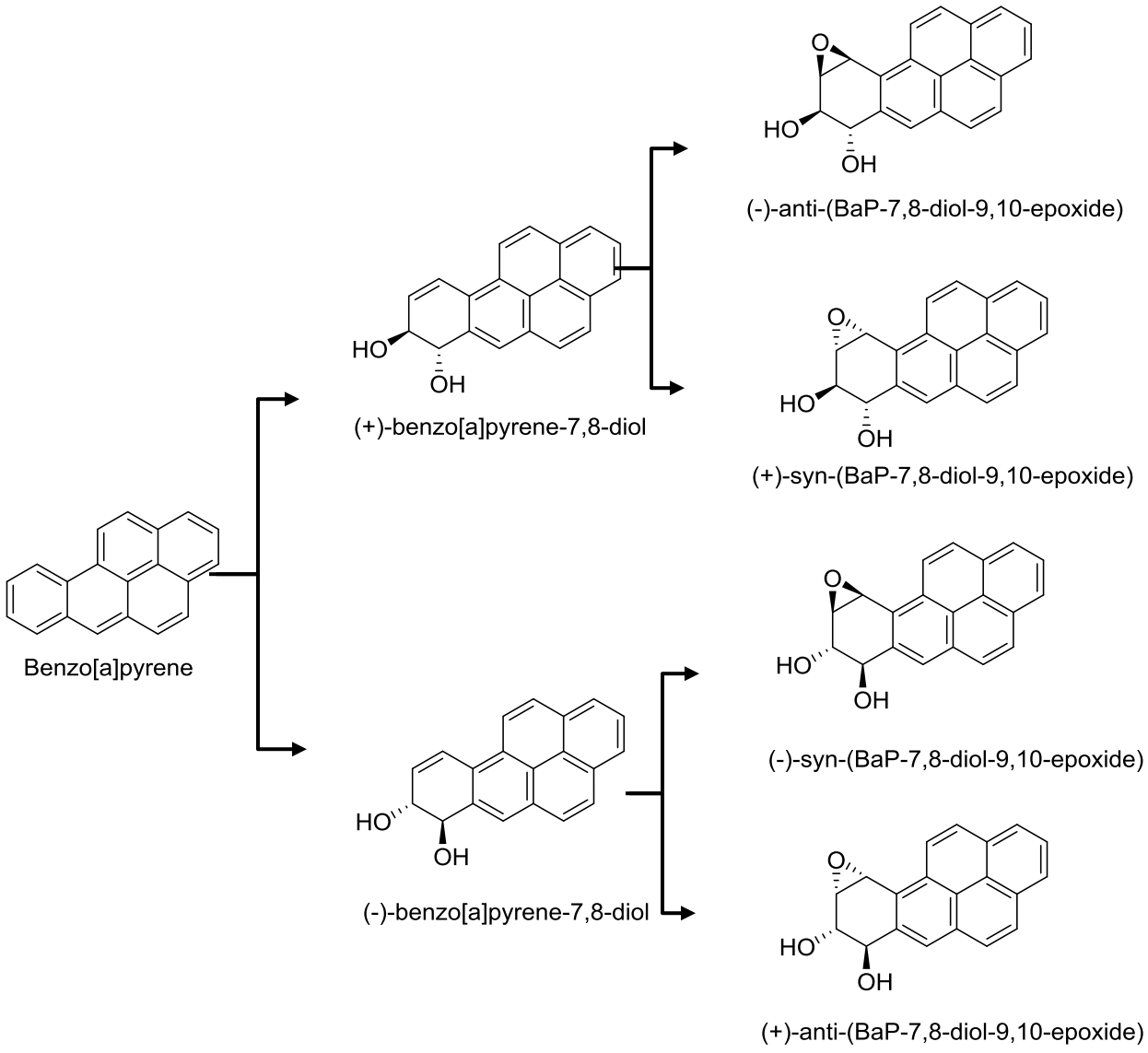

Figure 1.4. Cytochrome P450 metabolic pathway of benzo[a]pyrene.

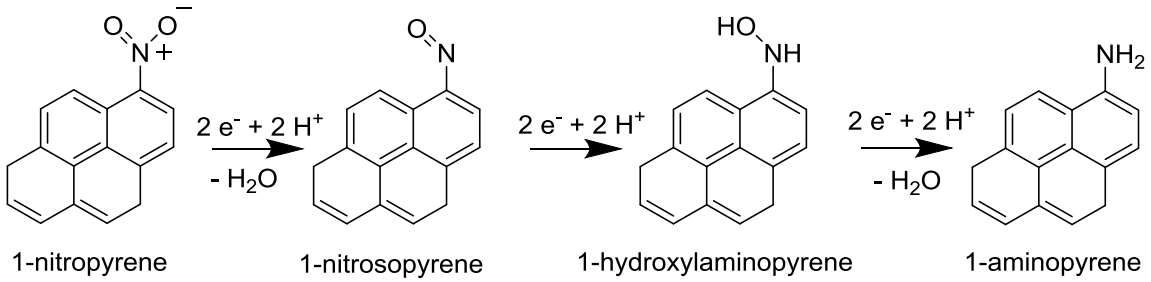

Figure 1.5. Nitroreduction. Reaction schematic for the reduction of 1-nitropyrene to 1-aminopyrene

Table 1.1. IARC PAH classification. Unsubstituted and nitrated PAHs listed in the International Agency for Research on Cancer (IARC) Monographs on the Evaluation of Carcinogenic Risk to Humans. Compounds are classified as Group 1 (carcinogenic to humans), Group 2A (probably carcinogenic to humans), Group 2B (possibly carcinogenic to humans), Group 3 (not classifiable as to its carcinogenicity to humans), or Group 4 (Probably not carcinogenic to humans).

Compound Name	IARC Classification	Compound Name	IARC Classification
Acenaphthene	3	3,7-Dinitrofluoranthene	2B
Anthanthrene	3	3,9-Dinitrofluoranthene	2B
Anthracene	3	1,3-Dinitropyrene	2B
Benz[a]anthracene	2B	1,6-Dinitropyrene	2B
Benzo[b]chrysene	3	1,8-Dinitropyrene	2B
Benzo[g]chrysene	3	5-Nitroacenaphthene	2B
Benzo[a]fluoranthene	3	9-Nitroanthracene	3
Benzo[b]fluoranthene	2B	3-Nitrobenzanthrone	2B
Benzo[ghi]fluoranthene	3	7-Nitrobenz[a]anthracene	3
Benzo[j]fluoranthene	2B	6-Nitrobenzo[a]pyrene	3
Benzo[k]fluoranthene	2B	4-Nitrobiphenyl	3
Benzo[a]fluorene	3	6-Nitrochrysene	2A
Benzo[b]fluorene	3	3-Nitrofluoranthene	3
Benzo[c]fluorene	3	2-Nitrofluorene	2B
Benzo[ghi]perylene	3	1-Nitronaphthalene	3
Benzo[c]phenanthrene	2B	2-Nitronaphthalene	3
Benzo[a]pyrene	1	3-Nitroperylene	3
Benzo[e]pyrene	3	1-Nitropyrene	2A
Chrysene	2B	2-Nitropyrene	3
4H-Cyclopenta[def]chrysene	3	4-Nitropyrene	2B
Cyclopenta[cd]pyrene	2A		
Dibenzo[a,e]fluoranthene	3		
13H-Dibenzo[a,g]fluorene	3		
Dibenzo[h,rst]pentaphene	3		
Dibenzo[a,e]pyrene	3		
Dibenzo[a,h]pyrene	2B		
Dibenzo[a,i]pyrene	2B		
Dibenzo[a,l]pyrene	2A		
Dibenzo[e,l]pyrene	3		
Dibenzothiophene	3		
Fluoranthene	3		

Fluorene	3
Indeno[1,2,3-cd]pyrene	2B
Naphthalene	2B
Naphtho[1,2-b]fluoranthene	3
Naphtho[2,1-a]fluoranthene	3
Naphtho[2,3-e]pyrene	3
Perylene	3
Phenanthrene	3
Picene	3
Pyrene	3

Table 1.2.BaP equivalency factors. Benzo[a]pyrene equivalency factors for unsubstituted and nitrated PAHs, compiled from the Rhode Island Department of Environmental Quality (RI DEQ) and the US Environmental Protection Agency (US EPA).

Compound Name	RI DEQ	US EPA	Compound Name	RI DEQ
Anthanthrene		0.4	1,6-Dinitropyrene	10
Anthracene	0.3	0	1,8-Dinitropyrene	1
Benz[a]anthracene	0.1	0.2	5-Nitroacenaphthene	0.03
11H-benz[b,c]aceanthrylene		0.05	6-Nitrochrysene	10
Benzo[b]fluoranthene	0.1	0.8	2-Nitrofluorene	0.01
Benzo[c]fluoranthene		20	1-Nitropyrene	0.1
Benz[e]aceanthrylene		0.8	4-Nitropyrene	0.1
Benzo[ghi]perylene		0.009		
Benz[j]aceanthrylene		60		
Benzo[j]fluoranthene	0.1	0.3		
Benzo[k]fluoranthene	0.1	0.03		
Benz[l]aceanthrylene		5		
Benzo[a]pyrene	1	1		
Chrysene	0.01	0.1		
Cyclopenta[c,d]pyrene	0.1	0.4		
4H-cyclopenta[d,e,f]chrysene		0.3		
Dibenz[a,h]acridine	0.1			
Dibenz[a,c]anthracene	0.1	4		
Dibenz[a,h]anthracene	1.1			
Dibenz[a,j]acridine	0.1			
Dibenzo[a,e]fluoranthene	1	0.9		
Dibenzo[a,e]pyrene	1	0.4		
Dibenz[a,h]anthracene		10		
Dibenzo[a,h]pyrene	10	0.9		
Dibenzo[a,i]pyrene	10	0.6		
Dibenzo[a,l]pyrene	10	30		
7H-dibenzo[c,g]carbazole	1			
7,12-Dimethylbenz[a]anthracene	64			
Fluoranthene	0.1	0.08		
Indeno[1,2,3-c,d]pyrene	0.1	0.07		
Naphtho[2,3-e]pyrene		0.3		
3-Methylcholanthrene	5.7			
5-Methylchrysene	1			
Phenanthrene		0		
Pyrene		0		

Chapter 2 – Quantitation and prediction of sorptive losses during toxicity testing of polycyclic aromatic hydrocarbon (PAH) and nitrated PAH (NPAH) using polystyrene 96-well plates

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Abstract

Developing zebrafish are increasingly being used for rapid assessments of chemical toxicity, and these assays are frequently conducted in multi-well plastic plates. This study investigated the sorptive behavior of polycyclic aromatic hydrocarbons (PAHs) and nitrated PAHs (NPAHs) to uncoated 96-well polystyrene plates typically used for zebrafish (*Danio rerio*) testing. We measured the percent sorption in the presence and absence of zebrafish embryos, at two exposure concentrations, as well as using two different procedures (addition of embryos to polystyrene plates either before analyte addition, or allowing 24 hours of equilibrium between analyte addition and embryo addition to the polystyrene plates). Following exposure, the plates were extracted with hexane and analyzed using gas chromatography coupled with mass spectrometry (GC/MS). Allowing 24 hours of pre-incubation between the addition of analytes and embryos did not significantly impact the percent sorption. The percent sorption was higher for both PAHs and NPAHs at the lower exposure concentration, and sorption was lower in the presence of zebrafish embryos. A mass balance model was developed to predict the sorption to polystyrene plates, based on the PAH and NPAH mass distribution ratios between polystyrene and water. While PAH sorption was significantly correlated with subcooled liquid solubility, NPAH sorption did not correlate with any of the physical-chemical properties investigated. This indicates the need to better understand the sorptive behavior of hydrophobic analytes to plastics, and to better account for sorptive losses during toxicity testing in polystyrene plates.

Introduction

Advances in high-throughput screening capabilities have led to an increase in the popularity of plastic plates as exposure vessels for numerous toxicity-testing platforms. These plates are inexpensive and disposable, available in a wide variety of sizes and volumes to fit a range of test systems and assays, and show low toxicity to cell cultures and model organisms (Hirmann et al., 2007; Truong et al., 2014). Whole-animal systems, such as zebrafish (*Danio rerio*), are increasing in popularity and are amenable to rapid and high-throughput phenotypic screening, as well as a range of other assays (Knecht et al., 2013; Reif et al., 2015; Truong et al., 2014). However, the hydrocarbon structure and composition of the plastic plates, commonly used for cell-based and whole-animal model system testing (e.g. zebrafish embryos), can be problematic for test analytes with low water solubility and/or high hydrophobicity (Gellert and Stommel, 1999; Hirmann et al., 2007; Incardona et al., 2006; Jarema et al., 2015; Sonnack et al., 2015). The ability of plastics to sequester hydrophobic analytes from aqueous environments is advantageous in some instances, such as passive sampling technologies (Fries and Zarfl, 2012), (García-Falcón et al., 2004; Kolahgar et al., 2002), and has been documented to occur with hydrophobic pollutants in the environment (Chandramouli et al., 2015; Rochman et al., 2013). However, use of plastic plates for the exposure of model systems (e.g., zebrafish embryos) to hydrophobic analytes in an aqueous media may result in the loss of these analytes from the exposure solution (Gellert and Stommel, 1999; Hirmann et al., 2007; Schreiber et al., 2008). Attempts at modeling the sorption of hydrophobic analytes to either glass or plastic containers have often used log K_{ow}, the octanol-water partition coefficient, or vapor pressure as predictive molecular characteristics, although with only moderate success (Riedl and Altenburger, 2007; Wolska et al., 2005). The conclusion from these studies is that properties such as lipophilicity or

volatility are related to sorption, but cannot completely account for the sorptive losses observed (Riedl and Altenburger, 2007).

Subcooled liquid solubility, the water solubility for a hypothetical state of a subcooled liquid (Liu et al., 2013), has been used to model multi-component non-aqueous phase liquids (NAPLs), containing polycyclic aromatic hydrocarbons (PAHs), in ground water and for predicting sorption to laboratory glassware (Liu et al., 2013; Qian et al., 2011). Subcooled liquid solubility can be derived from model system or environmental properties (such as organic carbon or mineral content of the soil), but can also be calculated using thermodynamic properties for the analytes of interest (Liu et al., 2013; Peters et al., 1999, 1997). Previous modeling of chemicals in the environment, including sorption to soil and organic matter, places the focus of the model on properties of the environmental system, rather than the analytes of interest (Karickhoff et al., 1979; Su et al., 2006). However, no models currently exist for the prediction of sorptive losses during chemical exposures that utilize plastic plates.

In the event that a significant amount of analyte sorbs to the plastic plate, the available concentration to which the zebrafish (or other model system) is exposed would be reduced. This unaccounted-for error would then be propagated through any data analysis, and would result in inaccurate assessment of toxicity metrics such as the concentration at which half of organisms show effects (EC_{50}). More accurate determination of analyte concentration to which the zebrafish are actually exposed would improve the translation of data from these high-throughput screening techniques to other systems, as well as lead to more accurate determination of potential health impacts as a result of exposure to environmental contaminants or mixtures.

The objective of this study was to determine the sorptive losses of PAHs and nitrated PAHs (NPAHs) to polystyrene 96-well plates (a common experimental format for zebrafish toxicity screening). This is the first study to compare sorptive losses in both the presence and absence of zebrafish embryos and at multiple exposure concentrations. We also compared two exposure protocols that differed in time between the addition of analytes and zebrafish embryos to the 96-well plates. The data derived herein was used to develop a predictive model that could be applied to structurally-related analytes to account for their sorptive losses to polystyrene plates.

Materials and Methods

Chemicals

Fluoranthene (FLA), pyrene (PYR), chrysene (CHR), benzo[a]pyrene (BaP), 3-nitrofluoranthene (3NF), 1,6-dinitropyrene (1,6DNP), and 6-nitrochrysene (6NC) were purchased from AccuStandard (New Haven, CT). 1-nitropyrene (1NP) and 6-nitrobenzo[a]pyrene (6NBaP) were purchased from Sigma-Aldrich (St. Louis, MO). All analytes were purchased as neat standards. Deuterated analytes, used as surrogates, (acenaphthene-d_{10}, fluoranthene-d_{10}, pyrene-d_{10}, benzo[a]pyrene-d_{12}, 2-nitrofluorene-d_9, 9-nitroanthracene-d_9, 3-nitrofluoranthene-d_9, 1-nitropyrene-d_9, 6-nitrochrysene-d_9) were purchased from CDN Isotopes (Point-Claire, Quebec, Canada) and Cambridge Isotope Laboratories (Andover, MA). Anhydrous dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich. Ethyl acetate and hexane were purchased from Fisher Scientific (Santa Clara, CA). Flat-bottom 300 µL total volume pre-sterilized Falcon® labware polystyrene tissue culture plates were purchased from VWR (Radnor, PA) and were not cleaned prior to use.

Zebrafish

All experiments were conducted with fertilized embryos according to Oregon State University Institutional Animal Care and Use Protocols. Embryos were collected following group spawning of adult wildtype 5D strain zebrafish as previously described (Reimers et al., 2006a; Truong et al., 2010). Briefly, zebrafish embryos were dechorionated using an automated dechorionation system at approximately 4 hours post fertilization (hpf) and added to the 96-well plates via automation (Mandrell et al., 2012a). For the pre-incubation protocol, the embryos were added to the plates manually. Embryos were evaluated for mortality at 24 hpf and 120 hpf to ensure adequate survival. Deceased embryos will disintegrate in the exposure media and would exhibit different partitioning behaviors than the live embryos.

Plate Exposure

The mass of individual PAH and NPAH standards were measured using an analytical balance (Mettler Toledo, Columbus, OH) and dissolved in anhydrous DMSO to create a stock solution of approximately 6 mM per analyte as a mixture. The DMSO solution was then diluted in E2 embryo media (the embryo media contained salts but no biologic materials, such as proteins) (Nusslein-Volhard and Dahm, 2002) to 10x the final exposure concentration as a mixture (in 6.4% DMSO by volume), and was utilized for the testing of both exposure procedures. The PAH and NPAH stock solution mixture and zebrafish embryos were added to the plates using our "Standard lab protocol" (Huang et al., 2010; Noyes et al., 2015; Truong et al., 2014) or a modified "Pre-incubation protocol" to investigate the degree of sorption which occurs under the test protocols:

"Standard lab protocol": The 10x analyte mixture was added to plates that already contained embryo media (and, where appropriate, 6 hpf dechorionated zebrafish embryos) to obtain a final DMSO concentration of 0.64%.

"Pre-incubation protocol": The 10x analyte mixture was added to the plates to obtain a DMSO concentration of 0.64%, the plates were wrapped in foil, and placed in the incubator for 24 hours, followed by addition of 6 hpf dechorionated zebrafish embryos (where appropriate).

The Pre-incubation protocol was investigated alongside the Standard lab protocol to investigate the reversibility of the sorption of the analytes to the polystyrene plates.

Final test concentrations of the individual PAHs and NPAHs were approximately 0.32 and 0.032 μM, termed "high" and "low" respectively, with 0.64% DMSO and a final liquid volume of 100 μL in each well. The nominal concentrations of each analyte in each exposure solution are given in Appendix A.1. These concentrations were above the experimental water solubilities of chrysene and benzo[a]pyrene (by a factor of two and six, respectively) and were within the range of estimated water solubilities for the remaining PAHs and NPAHs (measured or estimated at 25°C) (US EPA, 2015). However, no visible precipitate formed during the exposures, likely because the water solubility was increased because DMSO was used at 0.64% with embryo media in the wells and the temperature was set at 28°C in the incubator. These test concentrations are also consistent with those used for zebrafish toxicity screening of PAHs and oxy-PAHs (Hawliczek et al., 2012; Knecht et al., 2013; Noyes et al., 2015). Plates were covered in Parafilm®, wrapped in aluminum foil, and placed in a dark incubator at 28 °C for five days. For both protocols and concentrations, four plates were exposed to the analytes in the presence of zebrafish embryos, and three plates were exposed to analytes in the absence of zebrafish

embryos. Glass plates were also investigated initially; however, the resulting high sorptive losses led us to focus on the use of polystyrene plates (Appendix A.2).

Analyte Extraction from Exposure Plates

Each polystyrene 96-well plate contained one combination of exposure protocol, concentration, and presence or absence of zebrafish embryo making for a total of thirty-six 96-well polystyrene plates. Following the five-day exposure duration for both protocols, plates were removed from the incubator. For each plate, the exposure solutions were removed from each of the 96-wells using glass pipettes and combined in a clean amber glass vial and archived.

Due to the solubility of polystyrene in other organic solvents, including acetone, ethyl acetate, and dichloromethane, we were restricted to the use of hexane for the plate rinses. A hexane rinse of 100 µL was added to each of the individual 96-wells and allowed to sit in the dark for 20 min before the hexane rinse was removed from each of the 96-wells using glass pipettes and combined for each plate, for a total volume of 9.6 mL for each hexane rinse. Each plate was rinsed a total of three times and each rinse was stored and analyzed separately. One mL of each 9.6 mL hexane rinse was removed and surrogate standards were added, to account for analyte loss during sample preparation and to quantify the analyte. The extract was solvent exchanged into ethyl acetate and the volume reduced to 100 µL. Internal standards were added to track surrogate recovery, and the extracts analyzed using gas chromatography coupled with mass spectrometry (GC/MS). Surrogate standards and internal standards were added so that the final concentration was 500 pg/µL.

Analyte Extraction from Zebrafish

After five days of exposure, live embryos were pooled and collected into microcentrifuge tubes ($n \approx 30$, three replicates) from each of four plates and frozen. Prior to extraction, excess exposure media was removed from the embryos, surrogate standards were added, and the embryos were extracted using liquid-liquid extraction into ethyl acetate as previously described (Goodale et al., 2013). The final extracts (250 µL) were transferred into clean, amber glass vials, internal surrogate standards were added, and the extracts were analyzed by GC/MS. All of the analytes were detected below the limit of quantification in the zebrafish embryo extracts, therefore the amount of analyte sorbed and/or metabolized by the embryos was estimated as described in section 2.7.

GC/MS Analysis

Analysis of the hexane rinses and zebrafish extracts with GC/MS followed procedures previously described (Jariyasopit et al., 2014). Briefly, Agilent 6890 gas chromatographs coupled with Agilent 5973N mass spectrometers were operated in selected ion monitoring (SIM) and scan modes with ChemStation software (V. E.02.02.1431, Agilent Technologies). A 5%-phenyl-substituted methylpolysiloxane GC column (DB-5MS, 30 m x 0.25 mm I.D., 0.25 µm film thickness, J&W Scientific, USA) was used for chromatographic separations. PAHs were analyzed in electron impact (EI) mode, whereas NPAHs were analyzed in negative chemical ionization (NCI) mode, with methane as the reagent gas and a programmed temperature vaporization (PTV) inlet (Gerstel, Germany). For further detailed information regarding the

analytical analysis, QA/QC, ions monitored and estimated limits of detection (US EPA, n.d.), see Appendix A.3.

Modeling and Statistics

We assumed that mass balance was achieved for individual PAHs and NPAHs within the individual wells of the 96-well plates (Figure 2.1) (Wolska et al., 2005). The physical-chemical properties used for the modeling in this study are listed in Table 2.1 and were collected from the NIST web book (P.J. Lindstrom and W.G. Mallard, 2015) or estimated using the EPI Suite program (US EPA, 2015). Experimental values were used where available. However, in some cases, a lack of experimental data necessitated the use of estimated values. Subcooled liquid solubility was calculated as previously described (Mukherji et al., 1997; Peters et al., 1999) (see Appendix A. 4).

Values for the Henry's Law Constants were generated using the HenryWin program in EPI Suite (US EPA, 2015), and were converted from the provided units (atm m^3/mol) to the dimensionless form (concentration in air / aqueous concentration), with values shown in Table 2.1. The dimensionless coefficients were used to estimate the percent of each analyte that was lost to the headspace of the wells (the volume of the wells occupied by air rather than aqueous exposure media) based on the ratio of aqueous volume to headspace volume.

$$mass_{\text{air}} = H_{\text{c}} * V_{\text{air}} * C_{water,original} \qquad \textbf{(Equation 2.1)}$$

The mass of analyte in the headspace is represented by $mass_{\text{air}}$, the Henry's Law constant by H_{c}, the volume of the headspace by V_{air}, and the original concentration of analyte in the water by $C_{\text{water,original}}$. The amount of analyte measured in the zebrafish embryos was below the limit of

quantification. Therefore, we estimated the amount of each analyte sorbed and/or metabolized by the zebrafish embryos as the difference in the sorption in the presence and absence of zebrafish embryos:

$$mass_{\text{embryo}} = mass_{sorption-\text{embryo}} - mass_{\text{sorption+embryo}} \quad \textbf{(Equation 2.2)}$$

The mass of analyte sorbed and/or metabolized by the embryos is represented by $mass_{\text{embryo}}$, the mass sorbed to the walls of the plate in the absence of embryos by $mass_{\text{sorption-embryo}}$, and the mass sorbed to the walls of the plate by $mass_{\text{sorption+embryo}}$. Because the analyte concentration in the aqueous exposure solutions were not measured, the analyte mass that was not accounted for by either volatilization (Equation 2.1), sorption to the polystyrene walls of the plate (measured), and sorption and/or metabolism by the zebrafish embryos (Equation 2.2) was assumed to have remained in the aqueous exposure solution:

$$mass_{\text{total}} = mass_{\text{polystyrene}} + mass_{\text{water}} + mass_{\text{embryo}} + mass_{\text{headspace}} \quad \textbf{(Equation 2.3)}$$

The total mass added to the system is represented by $mass_{\text{total}}$, the mass sorbed to the polystyrene plate by $mass_{\text{polystyrene}}$, the mass of analyte remaining in the aqueous solution as $mass_{\text{water}}$, the mass of analyte sorbed and/or metabolized by the embryos as $mass_{\text{embryo}}$, and the mass of analyte in the headspace as $mass_{\text{headspace}}$. We defined a mass distribution ratio, D_{pw} (Nič et al., 2009), as the ratio of the mass of analyte sorbed to the walls of the polystyrene plate ($mass_{\text{polystyrene}}$) to the mass of analyte that was elsewhere in the system ($mass_{\text{system}}$):

$$D_{\text{pw}} = \frac{mass_{polystyrene}}{mass_{\text{system}}} \quad \textbf{(Equation 2.4)}$$

Student's t-tests were used to compare analyte concentrations and exposure scenarios, with a significance cut-off of $p<0.05$. Additionally, linear regressions between D_{pw} values and various

chemical properties (log K_{ow}, molecular weight, and subcooled liquid solubility) were performed using SigmaPlot 12.3.

Percent sorption was calculated as the mass of analyte sorbed to the polystyrene ($mass_{polystyrene}$) divided by the total mass dosed to the well ($mass_{total}$) multiplied by 100:

$$\%\ sorption = \frac{mass_{polystyrene}}{mass_{total}} * 100 \qquad \textbf{(Equation 2.5)}$$

Similarly, the percent distribution in the other compartments of the system were calculated as the mass of analyte in a given compartment divided by the total mass dosed to the plate, multiplied by 100.

Results

Comparison of Exposure Protocols

We successfully quantified sorptive losses of all PAHs and NPAHs to the polystyrene 96-well plates using both the Standard lab protocol and the Pre-incubation protocol. For most PAHs and NPAHs, no statistically significant difference in percent sorption (p<0.05) was observed between the two protocols at either of the exposure concentrations (Figure 2.2). The only compounds which did have a statistically significant difference were FLA and PYR at the higher concentration in the absence of zebrafish embryos, and 3NF and 1NP at the lower exposure concentration in the presence of zebrafish embryos. In the presence of zebrafish, the mean percent sorption for individual PAHs using the Standard lab protocol ranged from 1.6 to 39% and from 1.5 to 41% using the Pre-incubation protocols, while NPAHs ranged from 6.1 to 32% and 11 to 44% using the Standard lab and Pre-incubation protocols, respectively (Figure 2.2). In the absence of zebrafish, the mean percent sorption for individual PAHs using the Standard lab

protocol ranged from 5.2 to 63% and from 4.8 to 70% using the Pre-incubation protocols, while NPAHs ranged from 28 to 69% and 28 to 83% using the Standard lab and Pre-incubation protocols, respectively (Figure 2.2). Because there was no statistically significant difference between the two protocols with or without zebrafish embryos, the data for the two protocols for a given concentration and presence/absence of fish were combined.

Comparison of Exposure Concentrations

Figure 2.3 illustrates that a statistically significant higher percent sorption was observed for most PAHs and NPAHs under the lower exposure concentration (0.032 µM) compared to the higher exposure concentration (0.32 µM). The sorption at the lower exposure concentration, compared to the higher exposure concentration, was 7.9% higher for PAHs and 40% higher for NPAHs in the absence of zebrafish embryos, and 2.1% higher for PAHs and 9.6% higher for NPAHs in the presence of zebrafish embryos. However, the mass of each analyte sorbed at the lower exposure concentration was less than the mass sorbed at the higher exposure concentration.

Influence of Zebrafish Embryos

Figure 2.3 shows that the presence of zebrafish embryos significantly reduced the sorption of PAHs and NPAHs to the polystyrene plates at both exposure concentrations. The mean percent sorption of the PAHs and NPAHs was reduced by 9.6% and 25%, respectively, at the high exposure concentration and by 15% and 58%, respectively, at the lower exposure concentration. While the zebrafish embryos were collected and extracted, the amount of analyte present was below the limit of quantification, likely due to metabolism (Djomo et al., 1996).

Mass Distributions and Predictive Modeling

The PAHs and NPAHs were assumed to have reached equilibrium within each of the 96-wells, allowing us to calculate the mass balance distribution of our analytes within each of the various compartments shown in Figure 2.1. Table 2.2 shows this estimated distribution. As mentioned above, the measured percent sorption to the polystyrene wells ranged from 4.8 to 39% at the high exposure concentration, and 1.5 to 43% at the low exposure concentration. Based on the Henry's Law Constants and Equation 2.1, less than 0.1% of each analyte was present in the headspace above the aqueous media. Based on the difference in the sorption in the presence and absence of zebrafish embryos, the percent of each analyte sorbed and/or metabolized by the zebrafish embryos (Equation 2.2) ranged from 0.14% (FLA at the high exposure concentration) to 77% (1,6DNP at the low exposure concentration). The remainder of analyte not accounted for by either sorption to the walls of the plate, volatilization to the headspace, and sorption and/or metabolism by the zebrafish embryos was assumed to have remained in the aqueous exposure media (Equation 2.3). From the amount (grams) of analyte sorbed to the plates and the amount remaining in the aqueous exposure solution, we calculated values of D_{pw} (Equation 2.4). For PAHs, D_{pw} ranged from 0.05 to 0.8 and 0.016 to 1.2 under the high and low exposure concentrations, respectively. For the NPAHs, D_{pw} values ranged from 0.22 to 0.39 and 0.69 to 2.4 under the high and low exposure concentrations, respectively. While the mass of analyte sorbed to the polystyrene was measured directly, the concentration remaining in the aqueous media was estimated. This, along with the potential error associated with our estimated losses due to volatilization, as well as our assumption of sorption to and metabolism by the zebrafish embryos, must be acknowledged.

Figure 2.4 shows linear regressions between D_{pw} and log K_{ow}, the subcooled liquid solubility, and molecular weight. Only subcooled liquid solubility was a good predictor of D_{pw} for PAHs at both high and low concentrations and none of these properties were good predictors of D_{pw} for NPAHs (Table 2.3).

Discussion

We determined that sorption to polystyrene 96-well plates during toxicity testing can be significant (1.5-91%), which is consistent with previous studies in 96-well polystyrene plates (Hirmann et al., 2007) or in glass jars. (Hirmann et al., 2007; Wolska et al., 2005). While some analytes exhibited low sorption (e.g., FLA and PYR, sorption less than 5%), other analytes consistently showed sorption exceeding 40% (e.g., all NPAHs). Previous work by Wolska et al. with glass exposure vessels demonstrated that lower molecular weight PAHs exhibit lower sorption than higher molecular weight PAHs (Wolska et al., 2005). For example, the 2- and 3-ring PAHs and pyrene showed sorptive losses of 10% or less, whereas PAHs with 4 or more rings had sorptive losses of 40 to 70% (Wolska et al., 2005). We observed a similar pattern with the polystyrene plates, where FLA and PYR exhibited sorptive losses below 10%, while CHR, BaP, and all the NPAHs had much greater sorptive losses (up to 91%).

Diffusion into the bulk of the polystyrene, in addition to sorption on the polystyrene surface, is also possible (Schreiber et al., 2008). However, due to the structure of polystyrene as a rigid, glassy polymer with minimal void space between the polymer chains (George and Thomas, 2001), the relatively large size of the PAHs and NPAHs used in this study, and the relatively short time course of the exposure (Hopfenberg, 1978), we focused on sorptive losses to the surface of the polystyrene, rather than on diffusion into the bulk of the plastic.

We expected sorption to treated borosilicate glassware to be lower than sorption to polystyrene. This is based on previous studies, where sorption to glassware accounted for less than 10% of the total analyte. Using phenanthrene as a model analyte, Schreiber et al. noted that sorptive losses were relatively small when glass exposure vessels were used (1.8±1.6%), but were much greater when polystyrene plates were used (94±1.2%). Our initial experiments with 96-well glass exposure plates were abandoned because of prohibitively high sorptive losses (0.096-94%) (Appendix A.2). This was likely because the roughened surface inside the wells of the glass plates contributed to the enhanced sorptive losses due to increased surface area. Unfortunately, we were unable to source a suitable glass 96-well plate option with a smoothed surface, which would have allowed for more direct comparisons of PAH and NPAH sorptive losses to polystyrene 96-well plates. In addition, the cost of the 96-well glass plates exceeded the cost of the 96-well polystyrene plates by approximately 60 times, and the glass plates would require thorough cleaning between uses to ensure against contamination (in contrast, the polystyrene plates are disposable after a single use).

Comparison of Exposure Protocols

The majority of analytes did not have a statistically significant difference in percent sorption between the two exposure protocols (Figure 2.2). This indicates that sorption to the walls of the wells occurs on a fairly rapid timeframe (i.e., less than five days). This supports our assumption that equilibrium was reached within the sealed wells of the 96-well plate. The results obtained indicate that PAH and NPAH sorption to polystyrene 96-well plates is a reversible process, most

likely due to adsorption on the surface of the polystyrene, rather than partitioning into the bulk polystyrene (Schreiber et al., 2008).

Comparison of Exposure Concentrations

The higher measured percent sorption at the lower exposure concentration indicates that there is potential for saturation of the polystyrene at higher analyte concentrations. It is also likely that the percent sorption would be higher for even lower exposure concentrations (<0.032 μM). In developmental zebrafish toxicity studies, it is typical for analytes exhibiting high toxicity to be further tested at lower exposure concentrations (Knecht et al., 2013; Noyes et al., 2015). Thus, where toxic effects are observed in other studies at these very low concentrations, accounting for sorptive losses in excess of 50% is particularly important. We selected two relatively low exposure concentrations (0.32 and 0.032 μM) because they were within the range of concentrations usually evaluated in the developmental embryonic zebrafish model (Knecht et al., 2013; Noyes et al., 2015). In order to attempt to extract the embryos at 120 hpf, it was necessary for the majority of the embryos to survive the full duration of the exposure, because deceased embryos will disintegrate in the exposure media and would exhibit different partitioning behaviors than the live embryos.

Influence of Zebrafish Embryos

The percent sorption was significantly lower for both PAHs and NPAHs under both exposure concentrations when zebrafish embryos were present in the polystyrene plates. Embryonic zebrafish have a high lipid content and would provide an environment favorable for the

partitioning of hydrophobic analytes, such as PAHs and NPAHs, out of the aqueous exposure solution and onto the surface or into the body of the embryo, rather than onto the polystyrene plate.

While adequate survival was achieved during these experiments (>90%), the concentration of analytes in the embryos was below our limit of quantification, so we were unable to directly measure the body burden. Development of the liver begins at around 48 hpf; thus, metabolism is also possible during the latter half of the five-day exposure. It is likely that the embryos metabolized the analytes following development of the liver at 48 hpf (Elke A Ober et al., 2003), leaving little of the original analytes in the zebrafish embryo. Metabolism of PAHs and NPAHs also likely contributed to the observed decrease in sorptive losses to the polystyrene plates, in the presence of zebrafish embryos. Metabolism would decrease the aqueous analyte concentration, which would impact the partitioning of the analyte between the polystyrene and aqueous media.

Mass Distributions and Predictive Modeling

We also estimated the losses of these analytes to the headspace in the sealed 96-well plates using estimated Henry's Law Constants, as volatilization is another potential source of analyte loss, and approximately two-thirds of the volume of each well of the polystyrene plate was occupied with air rather than aqueous media. Although losses due to volatilization were relatively small, less than 0.1% (Table 2.2), they are likely to be more significant for analytes with lower molecular weights or higher vapor pressure (Riedl and Altenburger, 2007).

Linear regressions between D_{pw} and log K_{ow}, molecular weight, and the subcooled liquid solubility indicated that the subcooled liquid water solubility was a good predictor of D_{pw} for

PAHs, but that none of those properties were useful predictors of D_{pw} for NPAHs (Figure 2.4). Parameters of statistically significant (p<0.05) models are shown in Table 2.3. We propose several possible explanations for the lack of statistically significant correlations for the NPAHs: 1.) The lack of experimental data for NPAH physical/chemical properties inhibited the modeling capabilities, because experimental log K_{ow} values were not available for most NPAHs. Additionally, estimated values for water solubility were used in the calculations of the subcooled liquid solubility (Appendix A.4); 2.) While evidence from the literature (Wolska et al., 2005) suggests that PAHs reach equilibrium within five days, NPAHs are less well-studied and equilibrium between the polystyrene 96-well plate, zebrafish embryos, and aqueous exposure solution may require more than five days to fully establish; and 3.) We speculate NPAHs may have a different mechanism of sorption to polystyrene than PAHs due to the nature of the nitro functional group.

Conclusions

If multi-well plates made of polystyrene or other plastics are to continue being used as exposure vessels during toxicity screening (because of their low cost and ease-of-use), losses of hydrophobic analytes to the plastic need to be accounted for to more accurately characterize the concentration of analyte remaining in the exposure solution, for not only zebrafish toxicity testing, but for any test system, such as cell culture, which also utilizes polystyrene plates for the testing of hydrophobic environmental pollutants. One potential option currently under investigation involves the use of passive dosing to maintain the exposure concentration for the duration of the experiment, although analyte losses using phenanthrene as a model analyte are

still reported to occur (Mayer et al., 1999b; K. E. C. Smith et al., 2009; Vergauwen et al., 2015b). Group exposures in glass petri dishes or tanks can also be conducted; however, this method does not allow for tracking the development of a single animal over time (Jin et al., 2015; Massarsky et al., 2015; Oliveri et al., 2015). Other potential methods for reduction of sorptive losses include pre-treatment of the plastic plates (Sonnack et al., 2015), or the use of renewal (Padilla et al., 2012; Stanley et al., 2009), rather than non-renewal assay design. However, the use of renewal systems would be costly, both from a human labor and chemical use perspective. Additionally, previous toxicity screening results for hydrophobic analytes and environmental samples may need to be re-examined with the knowledge that upwards of 50% of the analyte originally introduced to the exposure solution may have sorbed to the plastic and were no longer available to the test system. Previous experiments comparing the toxicity of hydrophobic analytes, such as PAHs, in glass and plastic exposure vessels have demonstrated that plastic exposure vessels led to an underestimation of toxicity in bacterial test systems (Gellert and Stommel, 1999; Hirmann et al., 2007). Loss of analyte due to sorption and the resulting decrease in exposure concentration would impact data analysis, as the true exposure concentrations could, in some cases, be less than half of what was added to the assay. This would have implications for downstream data analysis and determination of toxicity indicator values, such as EC_{50}, which could be used in risk assessment applications. In cases where significant sorption occurs, values such as the EC_{50} calculated based on the theoretical exposure concentration would be higher than the true value, based on the sorption-corrected exposure concentrations, and would therefore under-estimate toxicity. This work highlights the need for further research into this ongoing research challenge.

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References

Chandramouli, B., Benskin, J.P., Hamilton, M.C., Cosgrove, J.R., 2015. Sorption of per- and polyfluoroalkyl substances (PFASs) on filter media: Implications for phase partitioning studies. Environ. Toxicol. Chem. 34, 30–36. doi:10.1002/etc.2751

Djomo, J.E., Garrigues, P., Narbonne, J.F., 1996. Uptake and depuration of polycyclic aromatic hydrocarbons from sediment by the zebrafish (Brachydanio Rerio). Environ. Toxicol. Chem. 15, 1177–1181. doi:10.1002/etc.5620150724

Fries, E., Zarfl, C., 2012. Sorption of polycyclic aromatic hydrocarbons (PAHs) to low and high density polyethylene (PE). Environ. Sci. Pollut. Res. 19, 1296–1304. doi:10.1007/s11356-011-0655-5

García-Falcón, M.., Cancho-Grande, B., Simal-Gándara, J., 2004. Stirring bar sorptive extraction in the determination of PAHs in drinking waters. Water Res. 38, 1679–1684. doi:10.1016/j.watres.2003.12.034

Gellert, G., Stommel, A., 1999. Influence of microplate material on the sensitivity of growth inhibition tests with bacteria assessing toxic organic substances in water and waste water. Environ. Toxicol. 14, 424–428. doi:10.1002/(SICI)1522-7278(1999)14:4<424::AID-TOX8>3.0.CO;2-4

George, S.C., Thomas, S., 2001. Transport phenomena through polymeric systems. Prog. Polym. Sci. 26, 985–1017. doi:10.1016/S0079-6700(00)00036-8

Goodale, B.C., Tilton, S.C., Corvi, M.M., Wilson, G.R., Janszen, D.B., Anderson, K.A., Waters, K.M., Tanguay, R.L., 2013. Structurally distinct polycyclic aromatic hydrocarbons induce differential transcriptional responses in developing zebrafish. Toxicol. Appl. Pharmacol. 272, 656–670. doi:10.1016/j.taap.2013.04.024

Hawliczek, A., Nota, B., Cenijn, P., Kamstra, J., Pieterse, B., Winter, R., Winkens, K., Hollert, H., Segner, H., Legler, J., 2012. Developmental toxicity and endocrine disrupting potency of 4-azapyrene, benzo[b]fluorene and retene in the zebrafish Danio rerio. Reprod. Toxicol., Zebrafish Teratogenesis 33, 213–223. doi:10.1016/j.reprotox.2011.11.001

Hirmann, D., Loibner, A.P., Braun, R., Szolar, O.H.J., 2007. Applicability of the bioluminescence inhibition test in the 96-well microplate format for PAH-solutions and elutriates of PAH-contaminated soils. Chemosphere 67, 1236–1242. doi:10.1016/j.chemosphere.2006.10.047

Hopfenberg, H.B., 1978. The effect of film thickness and sample history on the parameters describing transport in glassy polymers. J. Membr. Sci. 3, 215–230. doi:10.1016/S0376-7388(00)83023-9

Huang, H., Huang, C., Wang, L., Ye, X., Bai, C., Simonich, M.T., Tanguay, R.L., Dong, Q., 2010. Toxicity, uptake kinetics and behavior assessment in zebrafish embryos following exposure to perfluorooctanesulphonicacid (PFOS). Aquat. Toxicol. 98, 139–147. doi:10.1016/j.aquatox.2010.02.003

Incardona, J.P., Day, H.L., Collier, T.K., Scholz, N.L., 2006. Developmental toxicity of 4-ring polycyclic aromatic hydrocarbons in zebrafish is differentially dependent on AH receptor isoforms and hepatic cytochrome P4501A metabolism. Toxicol. Appl. Pharmacol. 217, 308–321. doi:10.1016/j.taap.2006.09.018

Jarema, K.A., Hunter, D.L., Shaffer, R.M., Behl, M., Padilla, S., 2015. Acute and developmental behavioral effects of flame retardants and related chemicals in zebrafish. Neurotoxicol.

Teratol., Investigating the neurotoxicity of past, present, and future flame retardants 52, Part B, 194–209. doi:10.1016/j.ntt.2015.08.010

Jariyasopit, N., Zimmermann, K., Schrlau, J., Arey, J., Atkinson, R., Yu, T.-W., Dashwood, R.H., Tao, S., Simonich, S.L.M., 2014. Heterogeneous Reactions of Particulate Matter-Bound PAHs and NPAHs with NO3/N2O5, OH Radicals, and O3 under Simulated Long-Range Atmospheric Transport Conditions: Reactivity and Mutagenicity. Environ. Sci. Technol. doi:10.1021/es5015407

Jin, Y., Liu, Z., Liu, F., Ye, Y., Peng, T., Fu, Z., 2015. Embryonic exposure to cadmium (II) and chromium (VI) induce behavioral alterations, oxidative stress and immunotoxicity in zebrafish (Danio rerio). Neurotoxicol. Teratol. 48, 9–17. doi:10.1016/j.ntt.2015.01.002

Karickhoff, S.W., Brown, D.S., Scott, T.A., 1979. Sorption of hydrophobic pollutants on natural sediments. Water Res. 13, 241–248. doi:10.1016/0043-1354(79)90201-X

Knecht, A.L., Goodale, B.C., Truong, L., Simonich, M.T., Swanson, A.J., Matzke, M.M., Anderson, K.A., Waters, K.M., Tanguay, R.L., 2013. Comparative developmental toxicity of environmentally relevant oxygenated PAHs. Toxicol. Appl. Pharmacol. 271, 266–275. doi:10.1016/j.taap.2013.05.006

Kolahgar, B., Hoffmann, A., Heiden, A.C., 2002. Application of stir bar sorptive extraction to the determination of polycyclic aromatic hydrocarbons in aqueous samples. J. Chromatogr. A 963, 225–230. doi:10.1016/S0021-9673(02)00361-8

Liu, L., Wu, F., Haderlein, S., Grathwohl, P., 2013. Determination of the subcooled liquid solubilities of PAHs in partitioning batch experiments. Geosci. Front. 4, 123–126. doi:10.1016/j.gsf.2012.03.010

Mandrell, D., Truong, L., Jephson, C., Sarker, M.R., Moore, A., Lang, C., Simonich, M.T., Tanguay, R.L., 2012. Automated Zebrafish Chorion Removal and Single Embryo Placement Optimizing Throughput of Zebrafish Developmental Toxicity Screens. J. Lab. Autom. 17, 66–74. doi:10.1177/2211068211432197

Massarsky, A., Jayasundara, N., Bailey, J.M., Oliveri, A.N., Levin, E.D., Prasad, G.L., Di Giulio, R.T., 2015. Teratogenic, bioenergetic, and behavioral effects of exposure to total particulate matter on early development of zebrafish (Danio rerio) are not mimicked by nicotine. Neurotoxicol. Teratol. 51, 77–88. doi:10.1016/j.ntt.2015.09.006

Mayer, P., Wernsing, J., Tolls, J., de Maagd, P.G.-J., Sijm, D.T.H.M., 1999. Establishing and Controlling Dissolved Concentrations of Hydrophobic Organics by Partitioning from a Solid Phase. Environ. Sci. Technol. 33, 2284–2290. doi:10.1021/es9808898

Mukherji, S., Peters, C.A., Weber, W.J., 1997. Mass Transfer of Polynuclear Aromatic Hydrocarbons from Complex DNAPL Mixtures. Environ. Sci. Technol. 31, 416–423. doi:10.1021/es960227n

Nič, M., Jirát, J., Košata, B., Jenkins, A., McNaught, A. (Eds.), 2009. IUPAC Compendium of Chemical Terminology: Gold Book, 2.1.0. ed. IUPAC, Research Triagle Park, NC.

Noyes, P.D., Haggard, D.E., Gonnerman, G.D., Tanguay, R.L., 2015. Advanced Morphological — Behavioral Test Platform Reveals Neurodevelopmental Defects in Embryonic Zebrafish Exposed to Comprehensive Suite of Halogenated and Organophosphate Flame Retardants. Toxicol. Sci. 145, 177–195. doi:10.1093/toxsci/kfv044

Nusslein-Volhard, C., Dahm, R., 2002. Zebrafish. OUP Oxford.

Ober, E.A., Field, H.A., Stainier, D.Y.R., 2003. From endoderm formation to liver and pancreas development in zebrafish. Mech. Dev. 120, 5–18. doi:10.1016/S0925-4773(02)00327-1

Oliveri, A.N., Bailey, J.M., Levin, E.D., 2015. Developmental exposure to organophosphate flame retardants causes behavioral effects in larval and adult zebrafish. Neurotoxicol. Teratol., Investigating the neurotoxicity of past, present, and future flame retardants 52, Part B, 220–227. doi:10.1016/j.ntt.2015.08.008

Padilla, S., Corum, D., Padnos, B., Hunter, D.L., Beam, A., Houck, K.A., Sipes, N., Kleinstreuer, N., Knudsen, T., Dix, D.J., Reif, D.M., 2012. Zebrafish developmental screening of the ToxCastTM Phase I chemical library. Reprod. Toxicol., Zebrafish Teratogenesis 33, 174–187. doi:10.1016/j.reprotox.2011.10.018

Peters, C.A., Knightes, C.D., Brown, D.G., 1999. Long-Term Composition Dynamics of PAH-Containing NAPLs and Implications for Risk Assessment. Environ. Sci. Technol. 33, 4499–4507. doi:10.1021/es981203e

Peters, C.A., Mukherji, S., Knightes, C.D., Weber, W.J., 1997. Phase Stability of Multicomponent NAPLs Containing PAHs. Environ. Sci. Technol. 31, 2540–2546. doi:10.1021/es960948m

P.J. Lindstrom and W.G. Mallard (Ed.), 2015. NIST Chemistry WebBook, NIST Standard Reference Database Number 69. National Institute of Standards and Technology, Gaithersburg, MD.

Qian, Y., Posch, T., Schmidt, T.C., 2011. Sorption of polycyclic aromatic hydrocarbons (PAHs) on glass surfaces. Chemosphere 82, 859–865. doi:10.1016/j.chemosphere.2010.11.002

Reif, D.M., Truong, L., Mandrell, D., Marvel, S., Zhang, G., Tanguay, R.L., 2015. High-throughput characterization of chemical-associated embryonic behavioral changes predicts teratogenic outcomes. Arch. Toxicol. 1–12. doi:10.1007/s00204-015-1554-1

Reimers, M.J., La Du, J.K., Periera, C.B., Giovanini, J., Tanguay, R.L., 2006. Ethanol-dependent toxicity in zebrafish is partially attenuated by antioxidants. Neurotoxicol. Teratol. 28, 497–508. doi:10.1016/j.ntt.2006.05.007

Riedl, J., Altenburger, R., 2007. Physicochemical substance properties as indicators for unreliable exposure in microplate-based bioassays. Chemosphere 67, 2210–2220. doi:10.1016/j.chemosphere.2006.12.022

Rochman, C.M., Manzano, C., Hentschel, B.T., Simonich, S.L.M., Hoh, E., 2013. Polystyrene Plastic: A Source and Sink for Polycyclic Aromatic Hydrocarbons in the Marine Environment. Environ. Sci. Technol. 47, 13976–13984. doi:10.1021/es403605f

Schreiber, R., Altenburger, R., Paschke, A., Küster, E., 2008. How to deal with lipophilic and volatile organic substances in microtiter plate assays. Environ. Toxicol. Chem. 27, 1676–1682. doi:10.1897/07-504.1

Smith, K.E.C., Oostingh, G.J., Mayer, P., 2009. Passive Dosing for Producing Defined and Constant Exposure of Hydrophobic Organic Compounds during in Vitro Toxicity Tests. Chem. Res. Toxicol. 23, 55–65. doi:10.1021/tx900274j

Sonnack, L., Kampe, S., Muth-Köhne, E., Erdinger, L., Henny, N., Hollert, H., Schäfers, C., Fenske, M., 2015. Effects of metal exposure on motor neuron development, neuromasts and the escape response of zebrafish embryos. Neurotoxicol. Teratol. 50, 33–42. doi:10.1016/j.ntt.2015.05.006

Stanley, K.A., Curtis, L.R., Massey Simonich, S.L., Tanguay, R.L., 2009. Endosulfan I and endosulfan sulfate disrupts zebrafish embryonic development. Aquat. Toxicol., Zebrafish Issue 95, 355–361. doi:10.1016/j.aquatox.2009.10.008

Su, Y.-H., Zhu, Y.-G., Sheng, G., Chiou, C.T., 2006. Linear Adsorption of Nonionic Organic Compounds from Water onto Hydrophilic Minerals: Silica and Alumina. Environ. Sci. Technol. 40, 6949–6954. doi:10.1021/es0609809

Truong, L., Moody, I.S., Stankus, D.P., Nason, J.A., Lonergan, M.C., Tanguay, R.L., 2010. Differential stability of lead sulfide nanoparticles influences biological responses in embryonic zebrafish. Arch. Toxicol. 85, 787–798. doi:10.1007/s00204-010-0627-4

Truong, L., Reif, D.M., Mary, L.S., Geier, M.C., Truong, H.D., Tanguay, R.L., 2014. Multidimensional In Vivo Hazard Assessment Using Zebrafish. Toxicol. Sci. 137, 212–233. doi:10.1093/toxsci/kft235

US EPA, 2015. Estimation Programs Interface Suite [TM] for Microsoft Windows. United States Environmental Protection Agency, Washington, DC, USA.

US EPA, n.d. Method 8280A [WWW Document]. Anal. Polychlorinated Dibenzo-P-Dioxins Polychlorinated Dibenzofurans High-Resolut. Gas Chromatogr. Resolut. Mass Spectrom. HRGCLRMS. URL http://www.accustandard.com/assets/8280a.pdf (accessed 3.8.16).

Vergauwen, L., Schmidt, S.N., Stinckens, E., Maho, W., Blust, R., Mayer, P., Covaci, A., Knapen, D., 2015. A high throughput passive dosing format for the Fish Embryo Acute Toxicity test. Chemosphere 139, 9–17. doi:10.1016/j.chemosphere.2015.05.041

Wolska, L., Rawa-Adkonis, M., Namieśnik, J., 2005. Determining PAHs and PCBs in aqueous samples: finding and evaluating sources of error. Anal. Bioanal. Chem. 382, 1389–1397. doi:10.1007/s00216-005-3280-7

Figure 2.1. Mass balance model**.** Schematic of a single well within a sealed 96-well polystyrene plate, showing potential partitioning of a model compound (1NP) within the system. Following addition of the embryos and analyte, the wells are sealed using Parafilm® to prevent evaporative loss. Volumes of the aqueous exposure media and headspace are drawn to scale.

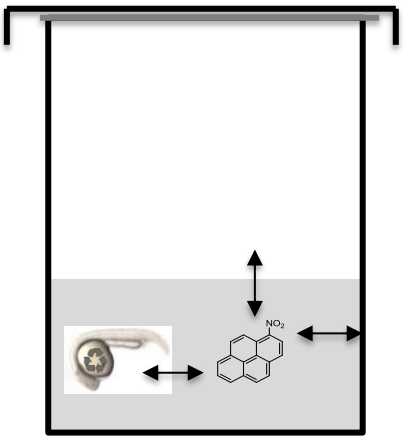

Figure 2.2. Measured sorptive losses for two exposure protocols. Comparison of the Standard lab protocol and Pre-incubation protocol, at both exposure concentrations (high concentration ≈0.32 μM, low concentration ≈0.032 μM) in the presence and absence of zebrafish embryos for PAHs (A-D) and NPAHs (E-H) Shown as mean ± standard error. * indicates statistical significance, $p<0.05$; $n=4$ with fish present, $n=3$ without fish present.

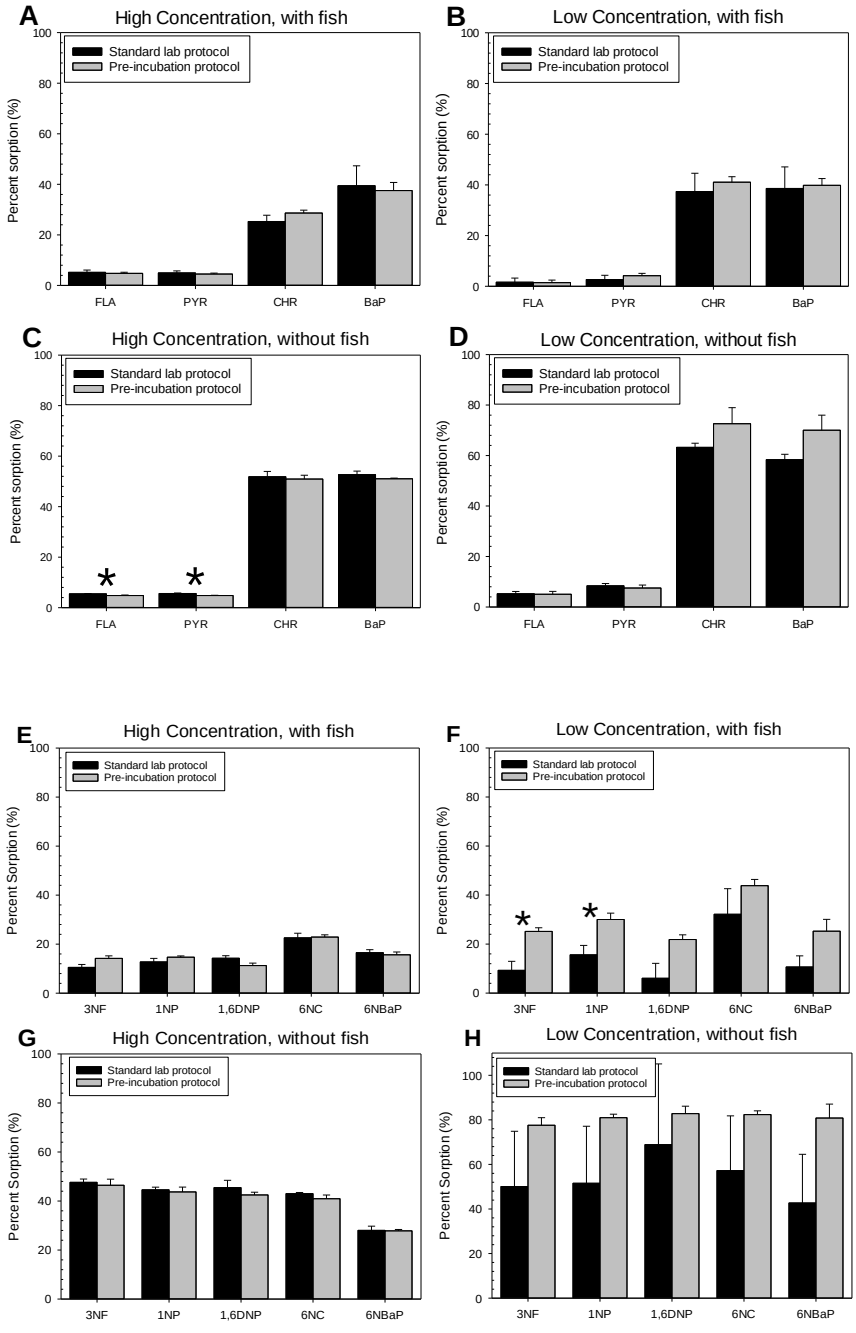

Figure 2.3. Measured percent sorption for PAHs and NPAHs. Mean ± SE percent sorption measured for PAHs (A) and NPAHs (B) at both the high (0.32 µM) and low (0.032 µM) exposure concentrations. Letters indicate statistically significant difference (p<0.05) against high concentration with fish (a), high concentration without fish (b), and low concentration with fish (c). n=8 with fish, n=6 without fish.

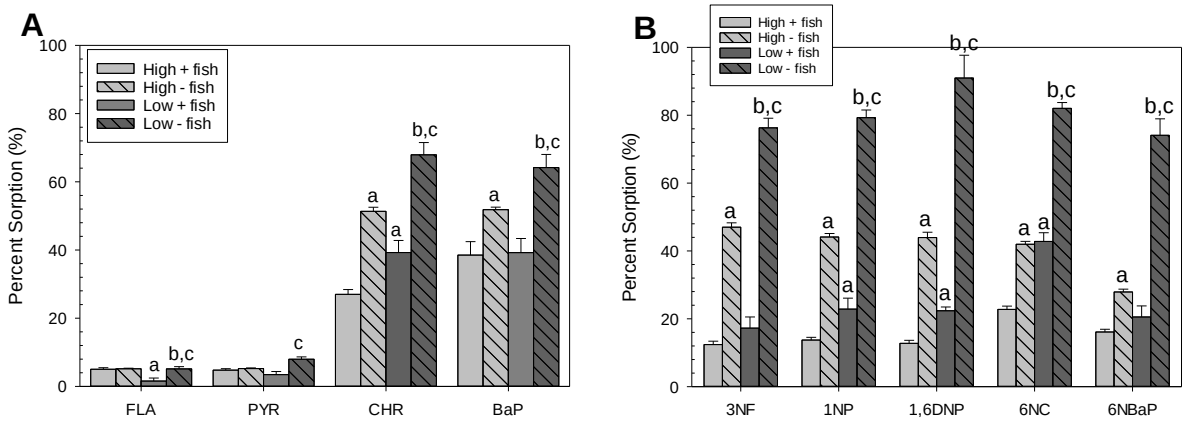

Figure 2.4. Modeling of sorptive losses. Linear regressions between log K_{ow} (A, D), subcooled liquid water solubility (B, E), and molecular weight (C, F) against calculated D_{pw} values, for both PAHs (A-C) and NPAHs (D-F). Parameters of statistically significant (p<0.05) models (indicated by *) are shown in Table 2.3.

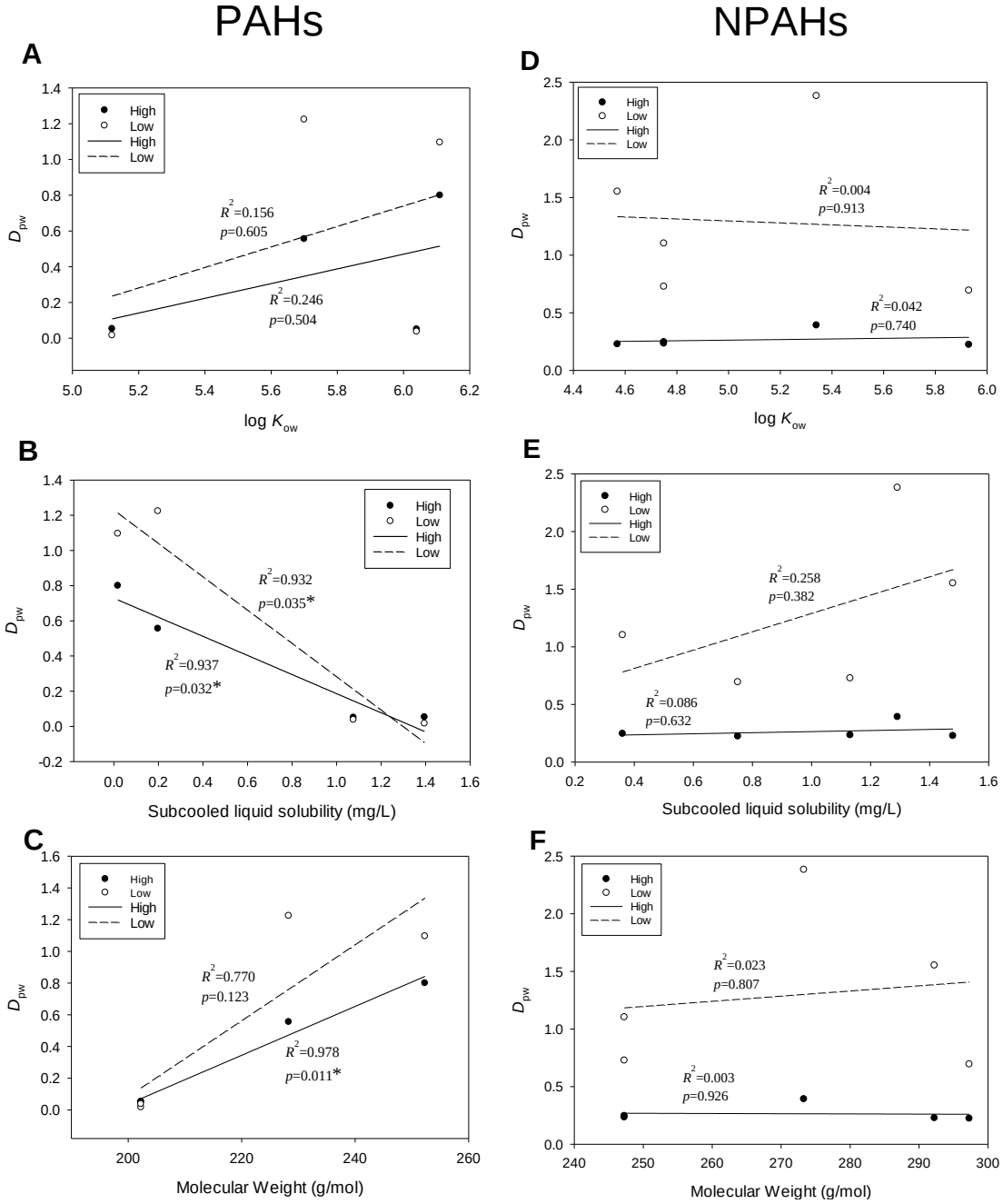

Table 2.1. Information on PAHs and NPAHs used in sorption study**.** Abbreviations and physical-chemical properties of PAHs and NPAHs used in this study. Log K_{ow} values are experimental (from the EPI Suite database), except where indicated by *(estimated values were generated using EPI Suite software). Subcooled liquid solubility was calculated using a method previously described (Appendix A. 4). Henry's Law Constants were estimated using the EPI Suite software.

Analyte Name	Abbreviation	Molecular weight (g/mol)	log K_{ow}	Subcooled liquid solubility (mg/L)	Henry's Law Constant (H_c) (dimensionless)
Fluoranthene	FLA	202.3	5.16	1.4	$3.4x10^{-4}$
3-nitrofluoranthene	3NF	247.1	4.8*	1.1	$1.3x10^{-5}$
Pyrene	PYR	202.1	4.88	1.1	$3.4x10^{-4}$
1-nitropyrene	1NP	247.1	4.8*	0.36	$1.3x10^{-6}$
1,6-dinitropyrene	1,6DNP	292.3	4.6*	1.5	$5.3x10^{-9}$
Chrysene	CHR	228.3	5.81	0.20	$2.1x10^{-4}$
6-nitrochrysene	6NC	273.3	5.3*	1.3	$8.1x10^{-7}$
Benzo[a]pyrene	BaP	252.1	6.13	0.018	$3.3x10^{-5}$
6-nitrobenzo[a]pyrene	6NBaP	297.3	5.9*	0.75	$1.3x10^{-7}$

Table 2.2. Estimated mass balance. Estimated percent, mass (μg), and D_{pw} for each analyte in a well (Figure 2.1).

	Analyte	% walls (μg)	% embryos (μg)	% headspace (μg)	% water (μg)	D_{pw}
High exposure concentration	FLA	5.0 (57)	0.14 (1.6)	$7.1x10^{-4}$ (0.008)	95 (1090)	0.053
	3NF	12 (76)	35 (210)	$2.7x10^{-3}$ (0.016)	53 (330)	0.23
	PYR	4.8 (67)	0.45 (6.3)	$7.1x10^{-4}$ (0.01)	95 (1340)	0.050
	1NP	14 (110)	30 (250)	$2.7x10^{-4}$ (0.002)	56 (460)	0.25
	1,6DNP	13 (79)	31 (190)	$1.1x10^{-6}$ ($6.5x10^{-6}$)	56 (340)	0.23
	CHR	27 (160)	24 (150)	$4.3x10^{-4}$ (0.003)	49 (290)	0.56
	6NC	23 (140)	19 (120)	$1.6x10^{-4}$ (0.001)	58 (360)	0.39
	BaP	39 (220)	13 (75)	$6.9x10^{-5}$ (0.0003)	48 (270)	0.80
	6NBaP	16 (61)	12 (45)	$2.6x10^{-5}$ ($1x10^{-4}$)	72 (280)	0.22
Low exposure concentration	FLA	1.5 (1.8)	3.6 (4.1)	$7.1x10^{-4}$ (0.001)	95 (110)	0.016
	3NF	17 (11)	59 (36)	$2.9x10^{-3}$ (0.002)	24 (15)	0.73
	PYR	3.4 (4.9)	4.5 (6.4)	$7.1x10^{-4}$ (0.001)	92 (130)	0.037
	1NP	23 (19)	57 (46)	$2.7x10^{-4}$ (0.0002)	21 (17)	1.1
	1,6DNP	14 (8.6)	77 (47)	$1.1x10^{-6}$ ($6.5x10^{-7}$)	9.0 (5.5)	1.6
	CHR	39 (24)	29 (17)	$4.3x10^{-4}$ (0.0002)	32 (19)	1.2
	6NC	43 (26)	39 (26)	$1.6x10^{-4}$ ($1x10^{-4}$)	18 (11)	2.4
	BaP	39 (22)	25 (22)	$6.9x10^{-5}$ ($4x10^{-5}$)	36 (20)	1.1
	6NBaP	18 (6.9)	56 (6.9)	$2.6x10^{-5}$ ($1x10^{-5}$)	26 (9.9)	0.69

Table 2.3. Parameters for statistically significant models. Parameters for statistically significant (p<0.05) models investigated for PAH (Figure 2.4). The y-intercept is indicated by y_0, the independent variable by x, and the slope of the linear fit by a, $D_{pw} = y_0 + ax$. No models for NPAHs were statistically significant.

PAH Exposure concentration	y_0	a	x	p
High (0.32 µM)	0.73	-0.54	Subcooled liquid solubility	0.032
	-3.0	0.015	Molecular weight	0.011
Low (0.032 µM)	1.2	-0.95	Subcooled liquid solubility	0.035

Chapter 3 – Mechanistic investigations into the developmental toxicity of nitrated and heterocyclic PAHs

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Abstract.

Nitrated polycyclic aromatic hydrocarbons (NPAHs) and heterocyclic PAHs (HPAHs) are recognized environmental pollutants. However, the health risks of NPAHs and HPAHs to humans and environmental systems are not well-studied. The developmental zebrafish (*Danio rerio*) model was used to evaluate the toxicity of a structurally diverse set of 27 NPAHs and 10 HPAHs. The individual activity of each compound towards the aryl hydrocarbon receptor (AHR), including the role of the AHR in observed toxicity, and genetic markers of oxidative stress and cardiac toxicity were evaluated. Zebrafish embryos were exposed from 6 to 120 hours post fertilization (hpf), to a broad concentration range of individual compounds, and evaluated for 22 developmental endpoints. The potential role of AHR was determined using the transgenic *Tg(cyp1a:nls-egfp)* reporter zebrafish line. All compounds were screened computationally through molecular docking using a previously developed AHR models of zebrafish isoforms 1A, 1B, and 2. Some compounds did not induce observable developmental toxic responses, while others produced statistically significant concentration-dependent toxicity. The tested compounds also exhibited a range of predicted AHR binding and *cyp1a/*GFP induction patterns, including *cyp1a* expression in the liver, vasculature, skin, and yolk, which we determined to be due to distinct isoforms of the AHR, using morpholino oligonucleotide knockdown. Furthermore, we investigated mRNA expression of oxidative and cardiac stress genes at 48 and 120 hpf, which indicated several potential mechanisms-of-action for NPAHs. Overall, we observed a range of developmental toxicities, *cyp1a*/GFP expression patterns, and gene expression profiles, suggestive of several potential mechanisms of action.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are well-known and established environmental contaminants with a range of sources to the environment (Khalili et al., 1995; Yunker et al., 2002). Exposure to PAHs has been linked with a range of health effects, including cancer and developmental challenges (Bernstein et al., 2004; Hardin et al., 1992; Irigaray et al., 2007; Perera et al., 2005, 1998; Sydbom et al., 2001; WHO, 2015).

Despite the toxicity of PAHs, the overall toxicity or mutagenicity of complex environmental mixtures often cannot be explained by PAHs alone (Chibwe et al., 2015; Toftgård et al., 1985). This has led to an increasing interest in PAH derivatives, including oxygenated PAHs (OPAHs), nitrated PAHs (NPAHs) and heterocyclic PAHs (HPAHs), which contain one or more heteroatomic substitutions within or on the aromatic ring system. These PAH derivatives are less studied than the corresponding unsubstituted, or parent, PAHs, both with regards to toxicity and environmental occurrence.

PAH derivatives can occur in the environment from a range of primary and secondary sources, including biotic and abiotic transformation processes (Cochran et al., 2016; W. Li et al., 2015; Shailaja et al., 2006; Vicente et al., 2015; Wei et al., 2015). NPAHs are known to be formed through reactions with particulate-bound PAHs and NO_x radicals during atmospheric transport (Arey et al., 1986; Jariyasopit et al., 2014; Zimmermann et al., 2013), as well as being common pollutants in diesel vehicle exhaust (Holly A. Bamford et al., 2003; Schuetzle et al., 1982). Both NPAHs and HPAHs are also known constituents of petroleum-based products, such as sealcoat pavement sealant (Titaley et al., 2016; Wei et al., 2015).

Limited toxicological data exists for NPAHs and HPAHs, most of which has been focused on mutagenicity. Many NPAHs and HPAHs are highly mutagenic in the Ames Salmonella assay

(Ball et al., 1984; Jariyasopit et al., 2013; Rosenkranz and Mermelstein, 1983), and several NPAHs are known or suspected human carcinogens (RI DEM, 2008; WHO, 2015).

Limited data exists for NPAHs and HPAHs with other acute or developmental toxicity endpoints. However, these studies have been limited in the number of compounds investigated, or the non-mortality endpoints observed, leading to a lack of comparable information across a large set of compounds (Dumont et al., 1979; Iwanari et al., 2002; Onduka et al., 2012; Peddinghaus et al., 2012).

Aromatic compounds, such as PAHs, are well-known and established to activate the aryl hydrocarbon receptor (AHR) pathway and, in many cases, the observed toxicity is *AHR*-dependent (Goodale et al., 2013; Knecht et al., 2013; Prasch et al., 2003). Binding of a ligand, such as a PAH, to the AHR results in up-regulation of numerous downstream genes, including the cytochrome P450s (*cyp* genes), which metabolize certain AHR ligands, including PAHs (Denison and Nagy, 2003; Miranda et al., 2006; Schmidt and Bradfield, 1996).

Other mechanisms of action for PAHs and PAH-containing mixtures have also been commonly observed, including oxidative stress and cardiac stress. Oxidative stress pathways are known to be linked with activation of the AHR pathway (Dalton et al., 2002), and AHR knockout mice have a reduced ability to generate an antioxidant response following exposure to TCDD, a known AHR agonist (Senft et al., 2002). Cardiac toxicity following exposure to 5-ring PAHs can be mediated by the AHR pathway, but these effects were AHR-independent for other structurally similar compounds (Brown et al., 2014; Incardona et al., 2011; Scott et al., 2011). Cardiac toxicity is also commonly observed following exposure to PAH-containing mixtures (Hicken et al., 2011; Jung et al., 2013).

Zebrafish (*Danio rerio*) are an increasingly popular model system for high-throughput screening of compounds for developmental toxicity and mechanistic investigations (Garcia et al., 2016). Zebrafish embryos develop rapidly and externally, with their major organs and systems formed within 5 days post-fertilization, and are amenable to cell culture techniques, including growth in 96-well tissue culture plates (Bugel et al., 2014; Jones et al., 2010; MacRae and Peterson, 2015; Noyes et al., 2015; Truong et al., 2014). Developing zebrafish embryos have a high degree of homology to humans, and this model has previously been used for the evaluation of PAH and OPAH toxicity (Brown et al., 2014; Huang et al., 2012; Incardona et al., 2006b 2004, Knecht et al., 2016, 2013).

The goal of this study was to determine the developmental toxicity of a suite of NPAHs and HPAHs using the embryonic zebrafish model. In addition to the developmental toxicity screen, potential mechanisms of action for NPAH and HPAH toxicity were investigated, including activation of the AHR pathway, oxidative stress, and cardiac stress.

Materials and Methods

Fish care and husbandry.

Adult zebrafish were maintained with a water temperature of 28° ±1°C on a recirculating system with a 14h light : 10h dark photoperiod at the Sinnhuber Aquatic Research Laboratory (SARL). All experiments were conducted with the wild type Tropical 5D strain or *Tg(cyp1a:nls-egfp)* (background strain TL) zebrafish (Kim et al., 2013). Adult care and reproductive techniques were conducted according to the Institutional Animal Care and Use Committee protocols at Oregon State University (OSU). All 5D embryos used in exposures were collected following group spawning of adult zebrafish as described previously (Reimers et al., 2006). Embryos from

the *Tg(cyp1a:nls-egfp)* reporter line were collected following incross or outcross small group spawns.

Chemicals and developmental exposures.

Analytical-grade standards were obtained from several vendors, including Sigma (St. Louis, MO), AccuStandard (New Haven, CT) and Chiron AS (Trondheim, Norway). Dimethyl sulfoxide (DMSO) was obtained from Sigma. Two compounds, 7-nitrobenzo[k]fluoranthene and 3,7-dinitrobenzo[k]fluoranthene, were custom synthesized in-house at OSU (Jariyasopit et al., 2013). For a complete list of chemicals tested, see Appendix B.1. In total, 27 NPAHs, two amino PAHs (potential metabolites of NPAHs), and 10 heterocyclic PAHs were tested. Individual compounds were dissolved in DMSO to make stock concentrations of either 10 or 1 mM, dependent on solubility (concentrations are listed in Appendix B.1). For the static exposures to zebrafish, compounds were dispensed into polystyrene 96-well plates pre-loaded with 100 µL of embryo media and individual zebrafish embryos, which had been enzymatically dechorionated and loaded into the plates using automation (Mandrell et al., 2012). Chemicals were dispensed into the plates using an HP D300 Digital Dispenser and shaken using pre-optimized protocols (Titaley et al., 2016; Truong et al., 2016). Two plates were treated with each chemical at a range of initial exposure concentrations (50, 35.6, 11.2, 5, 1.12 µM for chemicals with a 10 mM DMSO stock, or 5, 3.56, 1.12, 0.5, 0.1 µM for chemicals with a 1 mM DMSO stock), for a total number of 32 animals exposed to each concentration. 1% DMSO vehicle controls were also exposed on each plate. Following chemical addition, plates were moved to a temperature-controlled room (28 °C) and were placed on a custom-modified rotating shaker table overnight

(Truong et al., 2016). Embryos were evaluated at 24 hpf for developmental progress, spontaneous movement, notochord malformations and mortality. At 120 hpf, embryos were screened for total mortality and a suite of morphological endpoints, including pericardial and yolk sac edemas, malformations of the eye, snout, jaw, pectoral and caudal fins, axial malformations, and touch response (Knecht et al., 2013; Truong et al., 2011). Compounds were bi-hierarchically clustered based on similar malformation profiles using the "aheatmap" function in the NMF R package, in order to better evaluate structure-activity relationships.

Cyp1a/GFP reporter fish.

For CYP1A evaluation using the *Tg(cyp1a:nls-egfp)* transgenic reporter fish (Kim et al., 2013), 8-12 embryos per compound for all 39 compounds were exposed as described previously in section 2.2, to a single concentration selected to maximize malformations while minimizing mortality. Concentrations evaluated are listed in Appendix B.1. Following chemical addition using the HP D300 Digital Dispenser, plates were shaken overnight as described. Embryos were evaluated following continuous chemical exposure at 48 (prior to liver development and metabolic capacity) and 120 hpf for *cyp1a/*GFP expression using a Keyence BZ-X700 fluorescence microscope (Keyence North America, Itasca, IL). The *cyp1a*/GFP reporter line harbors a fosmid construct with GFP under control of a recombineered zebrafish *cyp1a* promoter, and recapitulates *cyp1a* induction by TCDD with high sensitivity (Kim et al., 2013). Immunohistochemistry (IHC) was also performed for all compounds following exposure in the wild type 5D zebrafish from 6-120 hpf to validate results from the reporter line. Further information can be found in Appendix B.2.

Antisense morpholino injections.

Morpholinos designed against zebrafish AHR1A, AHR1B, and AHR2, as well as a standard injection control morpholino were purchased from Gene Tools (Philomath, OR), and sequences are provided in Table 3.1 (Gerlach et al., 2014; Goodale et al., 2012). Morpholinos were dissolved in ultrapure water, and heterozygous *Tg(cyp1a:nls-egfp)* transgenic reporter embryos were injected at the 1-2 cell stage with 2 nL of morpholino (concentrations give in Table 3.1) and 0.5% phenol red. Embryos developing normally with morpholino incorporation were exposed to single concentrations of selected compounds, representing each of the previously observed *cyp1a*/GFP expression patterns: 5-nitroacenaphthalene, 1,6-dinitropyrene, 1,3-dinitropyrene, 7-nitrobenzo[k]fluoranthene, 9-nitrophenanthrene, 7-nitrobenz[a]anthracene, 3-nitrofluoranthene, 3,7-dinitrodibenzo[k]fluoranthene, and DMSO. Embryos were chemically exposed from 6 to 48 or 6 to 120 hpf and imaged at 48 or 120 hpf, respectively, using a Keyence BZ-X700 fluorescence microscope, and evaluated for developmental toxicity and *cyp1a* expression.

AHR docking predictions.

Docking of individual compounds to *in silico* models of the Per-AHR/Arnt-Sim (PAS)-B domains of each of the zebrafish AHR isoforms, as well as human AHR, was performed as previously described (Bisson et al., 2009; Goodale et al., 2015; Perkins et al., 2014). Docking scores were assigned for each compound and AHR isoform, overall priority for docking to any AHR isoforms were assigned, and predictions were compared to *in vivo cyp1a*/GFP expression.

RNA extraction.

For RNA samples, dechorionated 5D embryos were exposed from 6 to 48 hpf or 6 to 120 hpf to a subset of NPAHs at the same concentration used for *Tg(cyp1a:nls-egfp)* transgenic reporter fish evaluations (Appendix B.1). Compounds were selected for qPCR analysis in order to sample a range of developmental toxicities and AHR activation patterns: 5-nitroacenaphthalene, 1,6-dinitropyrene, 1,3-dinitropyrene, 7-nitrobenzo[k]fluoranthene, and vehicle control. Briefly, 48 hpf or 120 hpf embryos were rinsed in embryo media and four samples of 20 embryos each were collected on ice in snap-safe Eppendorf tubes with 0.5 mm zirconium oxide beads. 500 µL RNAzol was added and samples were homogenized with a Bullet Blender (Next Advance) for 3 minutes at speed 8, and then stored at -20°C until RNA isolation.

For total RNA isolation, RNA was extracted via RNAzol/isopropanol precipitation as previously described (Knecht et al., 2013). RNA was quantified using a Synergy/Mxmicroplate reader (Biotek) with the Gen5 Take3 module to calculate 260/280 O.D. ratios and RNA concentration. RNA was stored at -80°C until use.

Quantitative RT-qPCR.

Gene expression of a suite of 21 AHR-related, cardiac stress-related and oxidative stress gene transcripts was assessed in whole-embryo homogenates for 6-48 hpf or 6-120 hpf exposures of single concentrations of selected compounds. Gene-specific primers (MWG Operon) are listed in Appendix B.3. All qRT-PCR experiments were performed in 10 µL reactions consisting of 5 µL Power SYBR Green PCR one-step master mix (Applied Biosystems), 0.4 µL of each 10 mM primer in 0.1% Triton X, 4.2 µL H_2O and 2 µg of cDNA in 0.1% Triton X, which were

dispensed using the PCR program on the HP D300 Digital Dispenser. The temperature program consisted of 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 58 or 60 °C (dependent on primer set), with a final melt curve consisting of a 15 second hold at 95 °C, 2 minutes at 70 °C, and a 0.2 °C per minute ramp, with a final hold at 95 °C for 15 seconds.

Statistics and data analysis.

Data analysis. Statistical analysis and data visualization for developmental toxicity screening was done using code developed in R, using the custom Zebrafish Analysis and Acquisition (ZAAP) program (Truong et al., 2014). Concentrations which induced 50 percent effect (EC_{50}) were calculated by fitting dose-response data with a binomial logit regression using custom R scripts and the *dose.p* function (Haggard et al., 2016; R Core Team, 2013). Venn diagram representations of *cyp1a*/GFP expression patterns were generated using the Draw Venn Diagrams software from the Ghent University Bioinformatics Evolutionary Genomics group.

PCR statistics. qRT-PCR analysis was performed with StepOne software (Applied Biosystems), using the $\Delta\ \Delta C_T$ method with genes normalized to β-actin (Pfaffl, 2001). Four biological replicates of 20 embryos each were analyzed by comparing NPAH treated to DMSO control with a one-way ANOVA following normalization with β-actin, using SigmaPlot V11.0 software.

Results

Developmental toxicity screen.

Concentration-dependent morphological responses were observed at 24 hpf and 120 hpf in the zebrafish embryos exposed to a dilution series of NPAHs and HPAHs, compared to the DMSO-exposed control, for which no significant developmental toxicity was observed. This data is presented in Figure 3.1 as a heatmap, showing the relative potency derived from the lowest effect level (LEL) value for each endpoint evaluated, as well as any effect including and excluding mortality, for each compound tested.

Based on the clustering of compounds with similar developmental toxicity endpoints, several groups of compounds, with similar toxicity profiles and commonly observed endpoints, emerged. The most toxic group of compounds, as indicated by lowest effect levels at the lowest concentrations, were 3-nitrobenzanthrone, 1,6-dinitropyrene and 1,3-dinitropyrene (labeled as group 1 in Figure 3.1), which caused the common endpoints of pericardial and yolk sac edemas and craniofacial malformations at 120 hpf with high potency. Similar endpoints, in addition to mortality at 24 hpf, were observed for 1-aminopyrene, acridine, and 5,6-benzoquinoline (group 2). However, for this group of compounds, these effects were observed at higher concentrations. The next group of compounds included 5-nitroacenaphthalene, 9-nitrophenanthrene, and 1,8-dinitropyrene (group 3), displayed a higher degree of lethality and a lower incidence of non-mortality endpoints, at relatively higher concentrations. The next group of chemicals (9-aminophenanthrene, 7-nitrobenz[a]anthracene, carbazole, and 2-nitroanthracene; group 4) resulted in stronger effects at 24 hpf, including developmental delay and mortality, as well as pericardial edema at 120 hpf. Defects in circulation, marked by pooling of blood in the body of

the zebrafish embryos, was the only endpoint observed following exposure to 1-nitropyrene and 3-nitrofluoranthene (group 5). Two compounds (2-nitropyrene and dibenzofuran; group 6) had a significant effect for the "any effect" or "any effect including mortality" endpoints, but not for any of the individual endpoints. The remaining 22 compounds (group 7), greater than half of those tested, did not result in significant toxicity to the zebrafish embryos following exposure at the tested concentrations.

The EC_{50} values were calculated for compounds with observed toxicity and are shown in Table 3.2. Values ranged from 0.096 µM (1,3-dinitropyrene) to 44.8 µM (1-nitropyrene). Three compounds (2-nitroanthracene, 7-nitrobenz[a]anthracene, and dibenzofuran) had EC_{50} values estimated to be beyond the tested range of concentrations, and beyond the range of accuracy of the EC_{50} model.

CYP expression as a biomarker for AHR pathway activation.

To investigate the potential role of AHR, we utilized the transgenic *Tg(cyp1a:nls-egfp)* zebrafish line to evaluate expression of *cyp1a*/GFP, a known biomarker for AHR activation. Several distinct *cyp1a*/GFP expression patterns were observed following exposure to each of the 39 individual compounds from samples collected at 48 and 120 hpf (Figure 3.2). Compared to the DMSO-exposed control in which no distinct *cyp1a*/GFP expression was observed (Figure 3.2a), *cyp1a*/GFP expression was observed in the liver Figure 3.2b), vasculature (Figure 3.2c), yolk sac (Figure 3.2d), skin, and neuromasts (Figure 3.2e) following exposure to certain NPAHs. There were also a number of compounds for which no *cyp1a*/GFP expression was observed. Tissues displaying fluorescence, indicating *cyp1a*/GFP expression and activation of the AHR pathway,

for all compounds tested are listed in Table 3.3. Several compounds also showed *cyp1a*/GFP expression in multiple tissues simultaneously. For example, expression in the neuromasts was always observed alongside expression in the skin. Unique and overlapping *cyp1a*/GFP expression patterns are shown in Venn diagrams, for expression at 48 hpf (Figure 3.2f) and 120 hpf (Figure 3.2g).

Knock-down of AHR to elucidate its role in developmental toxicity.

Embryos injected with any of the morpholinos did not display an increase in background developmental toxicity, compared to non-injected controls (data not shown). *cyp1a*/GFP expression in the livers of fish exposed to 5-nitroacenaphthalene, 9-nitrophenanthrene, and 7-nitrobenzo[k]fluoranthene was eliminated in AHR1A morphants (Figure 3.3 b-c), but did not result in a substantial decrease in observed developmental toxicity following exposure to 5-nitroacenaphthalene.

Decreased GFP expression in AHR2 morphants *cyp1a*/GFP reporter embryos exposed to 7-nitrobenzo[k]fluoranthene in the skin and neuromasts, and 7-nitrobenz[a]anthracene in the vasculature, indicating AHR2 dependence for *cyp1a*/GFP expression (Figure 3.3 d-e), at both 48 and 120 hpf. Expression in the skin and vasculature following exposure to 1,6-dinitropyrene and 3,7-dinitrobenzo[k]fluoranthene were also reduced in AHR2 morphants (not shown). Injection of the AHR1B morpholino alone did not visibly reduce *cyp1a*/GFP expression following exposure to any of the compounds investigated. However, co-injection of AHR2 and AHR1B morpholinos appeared to reduce GFP expression slightly more than the AHR2 morpholino alone (Figure 3.3f).

In silico docking to AHR active site models.

Of the set of 39 compounds investigated, 22 were predicted to dock to one or more isoforms of the zebrafish AHR. Table 3.4 shows the docking scores, (given in kilocalories/mol), for all three zebrafish AHR isoforms, as well as human AHR, along with the overall docking priority to the zebrafish AHR isoforms. The more negative the docking score, the higher the predicted binding and activation. Of the 22 compounds predicted to dock to one or more isoforms of the zebrafish AHR, 16 displayed *in vivo cyp1a*/GFP expression, indicative of AHR activation, for a success rate of 73%. Of the 23 compounds which showed *in vivo cyp1a*/GFP expression, 16 were predicted to dock to the AHR, for a success rate of 70%.

Differential gene expression to elucidate other potential mechanisms of action.

To further investigate other potential mechanisms of action for NPAHs, qPCR analysis was performed for a suite of 21 genes selected to investigate cardiac toxicity, oxidative stress, and additional potential mechanisms of action. Significantly increased expression of *cyp1a*, *cyp1b1*, *cyp1c1*, and *cyp1c2* was observed following exposure to 5 µM 1,6-dinitropyrene at 48 and 120 hpf, and at 120 hpf following exposure to 50 µM 7-nitrobenzo[k]fluoranthene for *cyp1a*, *cyp1b1*, and *cyp1c1* (Figure 3.4 a-b). Expression of *cyp1a1* at 48 hpf was increased by 1,963-fold following exposure to 1,6-dinitropyrene, compared to the DMSO exposed controls. In the genes related to cardiac stress, expression of *nppb* (natriuretic peptide b, which encodes a small peptide responsible for the regulation of homeostatic contractility and response to cardiac stress) was increased by 12-fold in animals exposed to 1.12 µM 1,8-dinitropyrene at 48 hpf, and 23-fold in animals exposed to 3.56 µM 1,3-dinitropyrene at 120 hpf. Additionally, expression of *myl6*

(cardiac myosin light chain 6, which encodes the essential cardiac myosin light chain) was significantly decreased by 0.9-fold in the 1,8-dinitropyrene exposed animals at 48 hpf, whereas expression of *myl7* (cardiac myosin light chain 7, which encodes the regulatory cardiac myosin light chain) was significantly increased by 2-fold in animals exposed to 1,3-dinitropyrene at 48 hpf (Figure 3.4 c-d). Other genes important in detoxification and cellular protection from oxidative stress were also differentially expressed following developmental exposure to NPAHs (Figure 3.4 e). These included members of the superoxide dismutase (*sod*) family (an antioxidant enzyme responsible for the dismutation of superoxide radical into molecular oxygen or hydrogen peroxide), glutathione transferases (*gst*, phase II enzymes responsible for the conjugation of reduced glutathione to xenobiotic substrates), and glutathione peroxidases (*gpx*, which protect against oxidative stress by reducing peroxides). 1,3-dinitropyrene at 48 hpf resulted in differential expression of the greatest number of genes (4), while *arg2* (arginase) was the gene most commonly mis-expressed at both 48 hpf and 120 hpf.

Discussion

While unsubstituted PAHs are known to elicit a range of human and environmental health effects, NPAHs and HPAHs are not as thoroughly studied. Investigation of the developmental toxicity of these compounds, as well as potential contributing mechanisms of action, is important in order to more accurately determine potential human and environmental health hazards as a result of exposure. A range of developmental toxicity endpoints and *cyp1a*/GFP expression patterns were observed following NPAH or HPAH exposure. Selected compounds also resulted

in a variety of differentially expressed genes, indicating multiple potential contributing mechanisms of action.

Differential developmental toxicity.

Of the 39 compounds screened, 17 (44%) resulted in significant developmental toxicity of at least one observed endpoint. The profile of developmental toxicity malformations observed is similar to what has been previously observed with PAHs and oxygenated PAHs, as well as PAH-containing oil preparations (Goodale et al., 2013; Incardona et al., 2013; Jung et al., 2013; Knecht et al., 2013).

Comparing the calculated EC_{50} values with the log K_{ow}, molecular weight, and water solubility of compounds tested did not result in any significant correlation (Appendix B.1). However, experimentally derived values for the log K_{ow} and water solubility do not exist for many of the compounds in this data set, resulting in a reliance on estimated values from the EPI Suite program (US EPA, n.d.). Previous studies had shown relationships between the observed toxicity of N-heterocyclic (Schultz et al., 1980) and substituted anilines (Zok et al., 1991) with molecular weight, partition coefficients, and/or bioconcentration factors; however, experimentally determined values were available for the compounds under investigation in those studies. The reliability of our modeling would likely be improved if experimentally determined values had been available.

There is also significant uncertainly in the aqueous concentrations and uptake of these compounds during the assay. Sorption of hydrophobic analytes to the polystyrene plates commonly used for zebrafish testing is known to occur, and can result in significant analyte loss,

with upwards of fifty percent of the analyte sorbing to the plastic rather than remaining in the aqueous exposure media (Chlebowski et al., 2016; Peddinghaus et al., 2012). While the protocols have been optimized to minimize the sorptive losses which occur (Truong et al., 2016), and maximize the analyte concentration in the exposure solution, the actual analyte concentration remaining in the exposure media is uncertain. Furthermore, the availability and uptake of PAHs and PAH derivatives can be variable (Goodale et al., 2013), and is unknown for the NPAHs and HPAHs studied here.

Activation of the AHR pathway and cyp1a expression.

Fluorescence indicating *cyp1a* expression and activation of one or more AHR isoforms was observed at 48 and/or 120 hpf for 23 of the 39 compounds tested, suggesting the involvement of AHR in the toxicity of at least some NPAHs and HPAHs. Other mechanisms for the induction of *cyp1a*, such as cellular stress, could potentially be contributing as well (Behrendt et al., 2010), but the selective elimination of c*yp1a*/GFP expression in AHR2 morphants suggests the AHR isoform dependence of *cyp1a* expression.

The near-complete inhibition of *cyp1a*/GFP expression in the vasculature and/or skin (and neuromasts, for 7-nitrobenzo[k]fluoranthene) of embryos exposed to 7-nitrobenzo[k]fluoranthene, 1,6-dinitropyrene, 7-nitrobenz[a]anthracene and 3,7-dinitrobenzo[k]fluoranthene, following knockdown of AHR2, indicates that AHR2 is the primary isoform responsible for expression in these tissues. Decreased toxicity was observed in 1,6-dinitropyrene exposed AHR2 morphants, indicating a role for AHR in the toxicity of this compound. No substantial change in toxicity was observed for the other three compounds

investigated, which were all relatively non-toxic in the developmental toxicity screen. This is consistent with previous research on PAHs and oxygenated PAHs, where knockdown of AHR2 results in the inhibition of the skin or vasculature *cyp1a*/GFP expression patterns (Goodale et al., 2013; Incardona et al., 2011; Knecht et al., 2013). Expression of *cyp1a* has been observed previously in the neuromasts of transgenic Japanese Medaka (*Oryzias latipes*) following exposure to TCDD (Ng and Gong, 2013), although no further mechanistic information has been elucidated. Based on our results, the inhibition of *cyp1a*/GFP expression in the neuromasts, in AHR2 morphants, indicates AHR2 dependence of this expression pattern as well.

The decrease in *cyp1a*/GFP expression in AHR1A morphants is also consistent with previous research (Knecht et al., 2013), which implicated AHR1A as the dominant isoform present in the liver. Fluorescence in the liver was not observed in 48 hpf embryos, due to the majority of liver development occurring after 48 hpf (Elke A. Ober et al., 2003). We did not observe a substantial decrease in toxicity in AHR1A morphants, indicating that the toxicity observed is not completely AHR-dependent.

cyp1a/GFP expression in the yolk of embryos exposed to 1,3-dinitropyrene or 3-nitrofluoranthene did not appear to decrease as a result of injection with any of the morpholinos. *cyp1a*/GFP expression in the syncytial layer of the yolk had been previously observed following exposure to 1,9-benz-10-anthrone (Goodale et al., 2015), although this was not determined to be due to a specific isoform of the AHR. Another isoform of *cyp*, *cyp11a1*, is expressed in the yolk syncytial layer (Goldstone et al., 2010; Hsu et al., 2002), although this isoform has not been linked with xenobiotic metabolism.

Overall, the *in silico* model for the AHR1A isoform had the highest predictive success rate, where eight of the twelve compounds with *in vivo cyp1a*/GFP expression were predicted to dock to the AHR, in most cases to the AHR1A with the highest affinity. In contrast, the predicted docking scores to the AHR2 isoform did not align well with the observed *cyp1a*/GFP expression patterns in the skin and vasculature. The AHR docking models were developed and refined using TCDD as the guide ligand for AHR2 and AHR1B, and leflunomide for AHR1A (Gerlach et al., 2014). Both TCDD and leflunomide are structurally distinct from PAHs. While the AHR docking models performed adequately for the NPAHs and HPAHs studied here, there is room for further optimization of these models for PAHs and PAH derivatives, in particular the larger and more potent compounds. Binding to the different isoforms of the AHR appears to be the major factor contributing to the differential *cyp1a*/GFP expression patterns. Bioavailability and uptake for hydrophobic compounds can vary greatly and is not known for NPAHs and HPAHs, the larger and more hydrophobic compounds, such as 7-nitrobenzo[k]fluoranthene and the dinitropyrenes were among the most potent both with regards to *cyp1a*/GFP expression and developmental toxicity, suggesting that bioavailability and uptake is not necessarily a limiting factor for bioactivity of these compounds.

NPAHs differentially alter gene expression.

PAH and oxygenated PAH exposures have been previously linked with alterations in the expression of genes related to phase I metabolism (*cyp* genes), Phase II metabolism and oxidative stress, and cardiac toxicity (Goodale et al., 2015; Huang et al., 2012; Incardona et al., 2006, 2004; Knecht et al., 2013). Compared to oxygenated PAHs, fewer of the NPAHs studied

resulted in significantly altered expression of the four *cyp* genes investigated. However, the induction was a similar order of magnitude. This could indicate that phase I metabolism by the *cyp* genes as a result of AHR activation is not a major detoxification pathway for many NPAHs. While our data do suggest that the *cyp* metabolism pathways and phase II metabolism and oxidative stress could contribute to the toxicity of NPAHs, other mechanisms of action are likely also contributing. Metabolism and oxidative stress may be regulated, at least in part, by the *AHR* pathway, but further studies would be required to confirm this.

Cardiac toxicity has been previously observed following exposure to PAHs and PAH-containing products (Incardona et al., 2014, 2004; Jung et al., 2013; McIntyre et al., 2016). We observed altered expression of each of the three genes investigated (*nppb*, *myl6*, and *myl7*) following exposure to 1,8-dinitropyrene and 1,3-dinitropyrene at 48 and 120 hpf, indicating that cardiac toxicity may play a role in the developmental toxicity of at least some NPAHs. Zebrafish exposed to these compounds also displayed pericardial edema and circulatory malfunction (indicated by pooling of blood in the body). However, these malformations were also observed following exposure to other compounds which did not alter expression of these cardiac genes. Of these two compounds, only 1,3-dinitropyrene also resulted in altered *cyp1a* expression, indicated by visual expression in the yolk sac, which indicates that the observed cardiac toxicity is, at least in part, not AHR-dependent. Previous work has shown a diversity in cardiac toxicity mechanisms among PAHs acting as AHR agonists (Brown et al., 2014). Therefore, investigation of a wider number of genes and toxicity phenotypes is necessary to more fully elucidate the role of cardiac toxicity in the observed developmental toxicity of NPAHs and HPAHs, as well as the role of the AHR pathway.

The gene most commonly differentially expressed following NPAH exposure was *arg2*, the gene encoding the protein arginase 2. Arginases catalyze the hydrolysis of the amino acid arginine to urea and ornithine, and has a role in nitric oxide and polyamine metabolism (Durante et al., 2007). Arginase expression is positively correlated to expression of nitric oxide synthase (NOS), and, therefore, increased production of nitric oxide. Nitric oxide plays regulatory roles on various physiological and pathophysiological properties, including vascular and neurological functions (Sousa et al., 2010). We observed significantly decreased expression of *arg2* at 48 hpf following exposure to all seven NPAHs tested, and significantly increased expression at 120 hpf following exposure to 1,6-dinitropyrene, 5-nitroacenaphthalene, and 7-nitrobenzo[k]fluoranthene. Previous work (Goodale et al., 2015) showed that *arg2* expression was significantly elevated by 1,9-benz-10-anthrone, but not by benz[a]anthracene-7,12-dione, in 48 hpf embryos, and was dependent on AHR2. While we did not investigate AHR dependency of gene expression, two of the three compounds which had elevated *arg2* expression at 120 hpf (1,6-dinitropyrene and 7-nitrobenzo[k]fluoranthene) also displayed AHR2-dependent *cyp1a* expression. However, since many of the compounds under investigation were not observed to be AHR2-agonists, the altered expression of *arg2* at 48 hpf appears to be via an AHR2-independent mechanism.

Conclusion.

NPAHs and HPAHs, like the more thoroughly-studied PAHs and oxygenated PAHs, are toxicologically a non-homogenous group of compounds in the developing zebrafish model, as summarized in Appendix B.4. NPAHs and HPAHs can elicit a variety of developmental toxicity endpoints, at a range of concentrations, demonstrating potential for ecological and human health

impacts. NPAH and HPAH exposure also resulted in a range of *cyp1a*/GFP expression patterns, indicative of differential activation of AHR isoforms. Select NPAHs caused altered gene expression of a diverse set of genes, suggesting contributions of several potential mechanisms of action, including cardiac stress, *cyp* metabolism pathways, and oxidative stress. When considering health risks from PAH-containing complex environmental mixtures, PAH derivatives, including NPAHs and HPAHs, should be included. Further research into the toxicity and mechanisms of action of these compounds is warranted.

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References

Arey, J., Zielinska, B., Atkinson, R., Winer, A.M., Ramdahl, T., Pitts, J.N., 1986. The formation of nitro-PAH from the gas-phase reactions of fluoranthene and pyrene with the OH radical in the presence of NO x. Atmospheric Environ. 1967 20, 2339–2345.

Ball, L.M., Kohan, M.J., Claxton, L.D., Lewtas, J., 1984. Mutagenicity of derivatives and metabolites of 1-nitropyrene: activation by rat liver S9 and bacterial enzymes. Mutat. Res. Toxicol. 138, 113–125. doi:10.1016/0165-1218(84)90033-8

Bamford, H.A., Bezabeh, D.Z., Schantz, M.M., Wise, S.A., Baker, J.E., 2003. Determination and comparison of nitrated-polycyclic aromatic hydrocarbons measured in air and diesel particulate reference materials. Chemosphere 50, 575–587.

Behrendt, L., Jönsson, M.E., Goldstone, J.V., Stegeman, J.J., 2010. Induction of cytochrome P450 1 genes and stress response genes in developing zebrafish exposed to ultraviolet radiation. Aquat. Toxicol. 98, 74–82. doi:10.1016/j.aquatox.2010.01.008

Bernstein, J.A., Alexis, N., Barnes, C., Bernstein, I.L., Nel, A., Peden, D., Diaz-Sanchez, D., Tarlo, S.M., Williams, P.B., Bernstein, J.A., 2004. Health effects of air pollution. J. Allergy Clin. Immunol. 114, 1116–1123. doi:10.1016/j.jaci.2004.08.030

Bisson, W.H., Koch, D.C., O'Donnell, E.F., Khalil, S.M., Kerkvliet, N.I., Tanguay, R.L., Abagyan, R., Kolluri, S.K., 2009. Modeling of the aryl hydrocarbon receptor (AhR) ligand binding domain and its utility in virtual ligand screening to predict new AhR ligands. J. Med. Chem. 52, 5635–5641.

Brown, D.R., Clark, B.W., Garner, L.V.T., Giulio, R.T.D., 2014. Zebrafish cardiotoxicity: the effects of CYP1A inhibition and AHR2 knockdown following exposure to weak aryl hydrocarbon receptor agonists. Environ. Sci. Pollut. Res. 22, 8329–8338. doi:10.1007/s11356-014-3969-2

Bugel, S.M., Tanguay, R.L., Planchart, A., 2014. Zebrafish: A Marvel of High-Throughput Biology for 21st Century Toxicology. Curr. Environ. Health Rep. 1, 341–352. doi:10.1007/s40572-014-0029-5

Chibwe, L., Geier, M.C., Nakamura, J., Tanguay, R.L., Aitken, M.D., Simonich, S.L.M., 2015. Aerobic Bioremediation of PAH Contaminated Soil Results in Increased Genotoxicity and Developmental Toxicity. Environ. Sci. Technol. 49, 13889–13898. doi:10.1021/acs.est.5b00499

Chlebowski, A.C., Tanguay, R.L., Simonich, S.L.M., 2016. Quantitation and prediction of sorptive losses during toxicity testing of polycyclic aromatic hydrocarbon (PAH) and nitrated PAH (NPAH) using polystyrene 96-well plates. Neurotoxicol. Teratol. 57, 30–38. doi:10.1016/j.ntt.2016.05.001

Cochran, R.E., Jeong, H., Haddadi, S., Fisseha Derseh, R., Gowan, A., Beránek, J., Kubátová, A., 2016. Identification of products formed during the heterogeneous nitration and ozonation of polycyclic aromatic hydrocarbons. Atmos. Environ. 128, 92–103. doi:10.1016/j.atmosenv.2015.12.036

Dalton, T.P., Puga, A., Shertzer, H.G., 2002. Induction of cellular oxidative stress by aryl hydrocarbon receptor activation. Chem. Biol. Interact. 141, 77–95. doi:10.1016/S0009-2797(02)00067-4

Denison, M.S., Nagy, and S.R., 2003. Activation of the Aryl Hydrocarbon Receptor by Structurally Diverse Exogenous and Endogenous Chemicals. Annu. Rev. Pharmacol. Toxicol. 43, 309–334. doi:10.1146/annurev.pharmtox.43.100901.135828

Dumont, J.N., Schultz, T.W., Jones, R.D., 1979. Toxicity and teratogenicity of aromatic amines toXenopus laevis. Bull. Environ. Contam. Toxicol. 22, 159–166. doi:10.1007/BF02026923

Durante, W., Johnson, F.K., Johnson, R.A., 2007. Arginase: a critical regulator of nitric oxide synthesis and vascular function. Clin. Exp. Pharmacol. Physiol. 34, 906–911.

Garcia, G.R., Noyes, P.D., Tanguay, R.L., 2016. Advancements in zebrafish applications for 21st century toxicology. Pharmacol. Ther. 161, 11–21. doi:10.1016/j.pharmthera.2016.03.009

Gerlach, C.V., Das, S.R., Volz, D.C., Bisson, W.H., Kolluri, S.K., Tanguay, R.L., 2014. Mono-substituted isopropylated triaryl phosphate, a major component of Firemaster 550, is an AHR agonist that exhibits AHR-independent cardiotoxicity in zebrafish. Aquat. Toxicol. 154, 71–79. doi:10.1016/j.aquatox.2014.05.007

Goldstone, J.V., McArthur, A.G., Kubota, A., Zanette, J., Parente, T., Jönsson, M.E., Nelson, D.R., Stegeman, J.J., 2010. Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. BMC Genomics 11, 643. doi:10.1186/1471-2164-11-643

Goodale, B.C., Du, J.L., Tilton, S.C., Sullivan, C.M., Bisson, W.H., Waters, K.M., Tanguay, R.L., 2015. Ligand-Specific Transcriptional Mechanisms Underlie Aryl Hydrocarbon Receptor-Mediated Developmental Toxicity of Oxygenated PAHs. Toxicol. Sci. 147, 397–411. doi:10.1093/toxsci/kfv139

Goodale, B.C., La Du, J.K., Bisson, W.H., Janszen, D.B., Waters, K.M., Tanguay, R.L., 2012. AHR2 Mutant Reveals Functional Diversity of Aryl Hydrocarbon Receptors in Zebrafish. PLoS ONE 7, e29346. doi:10.1371/journal.pone.0029346

Goodale, B.C., Tilton, S.C., Corvi, M.M., Wilson, G.R., Janszen, D.B., Anderson, K.A., Waters, K.M., Tanguay, R.L., 2013. Structurally distinct polycyclic aromatic hydrocarbons induce differential transcriptional responses in developing zebrafish. Toxicol. Appl. Pharmacol. 272, 656–670

Haggard, D.E., Noyes, P.D., Waters, K.M., Tanguay, R.L., 2016. Phenotypically anchored transcriptome profiling of developmental exposure to the antimicrobial agent, triclosan, reveals hepatotoxicity in embryonic zebrafish. Toxicol. Appl. Pharmacol. 308, 32–45. doi:10.1016/j.taap.2016.08.013

Hardin, J.A., Hinoshita, F., Sherr, D.H., 1992. Mechanisms by which benzo[a]pyrene, an environmental carcinogen, suppresses B cell lymphopoiesis. Toxicol. Appl. Pharmacol. 117, 155–164. doi:10.1016/0041-008X(92)90232-H

Hicken, C.E., Linbo, T.L., Baldwin, D.H., Willis, M.L., Myers, M.S., Holland, L., Larsen, M., Stekoll, M.S., Rice, S.D., Collier, T.K., Scholz, N.L., Incardona, J.P., 2011. Sublethal exposure to crude oil during embryonic development alters cardiac morphology and reduces aerobic capacity in adult fish. Proc. Natl. Acad. Sci. 108, 7086–7090. doi:10.1073/pnas.1019031108

Hsu, H.-J., Hsiao, P., Kuo, M.-W., Chung, B., 2002. Expression of zebrafish cyp11a1 as a maternal transcript and in yolk syncytial layer. Gene Expr. Patterns 2, 219–222. doi:10.1016/S1567-133X(02)00059-5

Huang, L., Wang, C., Zhang, Y., Li, J., Zhong, Y., Zhou, Y., Chen, Y., Zuo, Z., 2012. Benzo[a]pyrene exposure influences the cardiac development and the expression of cardiovascular relative genes in zebrafish (Danio rerio) embryos. Chemosphere 87, 369–375. doi:10.1016/j.chemosphere.2011.12.026

Incardona, J.P., Collier, T.K., Scholz, N.L., 2004. Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. Toxicol. Appl. Pharmacol. 196, 191–205. doi:10.1016/j.taap.2003.11.026

Incardona, J.P., Day, H.L., Collier, T.K., Scholz, N.L., 2006. Developmental toxicity of 4-ring polycyclic aromatic hydrocarbons in zebrafish is differentially dependent on AH receptor isoforms and hepatic cytochrome P4501A metabolism. Toxicol. Appl. Pharmacol. 217, 308–321.

Incardona, J.P., Gardner, L.D., Linbo, T.L., Brown, T.L., Esbaugh, A.J., Mager, E.M., Stieglitz, J.D., French, B.L., Labenia, J.S., Laetz, C.A., Tagal, M., Sloan, C.A., Elizur, A., Benetti, D.D., Grosell, M., Block, B.A., Scholz, N.L., 2014. Deepwater Horizon crude oil impacts the developing hearts of large predatory pelagic fish. Proc. Natl. Acad. Sci. 111, E1510–E1518. doi:10.1073/pnas.1320950111

Incardona, J.P., Linbo, T.L., Scholz, N.L., 2011. Cardiac toxicity of 5-ring polycyclic aromatic hydrocarbons is differentially dependent on the aryl hydrocarbon receptor 2 isoform during zebrafish development. Toxicol. Appl. Pharmacol. 257, 242–249. doi:10.1016/j.taap.2011.09.010

Incardona, J.P., Swarts, T.L., Edmunds, R.C., Linbo, T.L., Aquilina-Beck, A., Sloan, C.A., Gardner, L.D., Block, B.A., Scholz, N.L., 2013. Exxon Valdez to Deepwater Horizon: Comparable toxicity of both crude oils to fish early life stages. Aquat. Toxicol. 142–143, 303–316. doi:10.1016/j.aquatox.2013.08.011

Irigaray, P., Newby, J.A., Lacomme, S., Belpomme, D., 2007. Overweight/obesity and cancer genesis: More than a biological link. Biomed. Pharmacother. 61, 665–678. doi:10.1016/j.biopha.2007.10.008

Iwanari, M., Nakajima, M., Kizu, R., Hayakawa, K., Yokoi, T., 2002. Induction of CYP1A1, CYP1A2, and CYP1B1 mRNAs by nitropolycyclic aromatic hydrocarbons in various human tissue-derived cells: chemical-, cytochrome P450 isoform-, and cell-specific differences. Arch. Toxicol. 76, 287–298. doi:10.1007/s00204-002-0340-z

Jariyasopit, N., McIntosh, M., Zimmermann, K., Arey, J., Atkinson, R., Cheong, P.H.-Y., Carter, R.G., Yu, T.-W., Dashwood, R.H., Massey Simonich, S.L., 2013. Novel nitro-PAH formation from heterogeneous reactions of PAHs with NO2, NO3/N2O5, and OH radicals: prediction, laboratory studies, and mutagenicity. Environ. Sci. Technol. 48, 412–419.

Jariyasopit, N., Zimmermann, K., Schrlau, J., Arey, J., Atkinson, R., Yu, T.-W., Dashwood, R.H., Tao, S., Simonich, S.L.M., 2014. Heterogeneous Reactions of Particulate Matter-Bound PAHs and NPAHs with NO3/N2O5, OH Radicals, and O3 under Simulated Long-Range Atmospheric Transport Conditions: Reactivity and Mutagenicity. Environ. Sci. Technol. 48, 10155–10164.

Jones, H.S., Panter, G.H., Hutchinson, T.H., Chipman, J.K., 2010. Oxidative and Conjugative Xenobiotic Metabolism in Zebrafish Larvae In Vivo. Zebrafish 7, 23–30. doi:10.1089/zeb.2009.0630

Jung, J.-H., Hicken, C.E., Boyd, D., Anulacion, B.F., Carls, M.G., Shim, W.J., Incardona, J.P., 2013. Geologically distinct crude oils cause a common cardiotoxicity syndrome in developing zebrafish. Chemosphere 91, 1146–1155. doi:10.1016/j.chemosphere.2013.01.019

Khalili, N.R., Scheff, P.A., Holsen, T.M., 1995. PAH source fingerprints for coke ovens, diesel and, gasoline engines, highway tunnels, and wood combustion emissions. Atmos. Environ. 29, 533–542. doi:10.1016/1352-2310(94)00275-P

Kim, K.-H., Park, H.-J., Kim, J.H., Kim, S., Williams, D.R., Kim, M.-K., Jung, Y.D., Teraoka, H., Park, H.-C., Choy, H.E., Shin, B.A., Choi, S.-Y., 2013. Cyp1a reporter zebrafish reveals target tissues for dioxin. Aquat. Toxicol. Amst. Neth. 134–135, 57–65. doi:10.1016/j.aquatox.2013.03.010

Knecht, A.L., Goodale, B.C., Truong, L., Simonich, M.T., Swanson, A.J., Matzke, M.M., Anderson, K.A., Waters, K.M., Tanguay, R.L., 2013. Comparative developmental toxicity of environmentally relevant oxygenated PAHs. Toxicol. Appl. Pharmacol. 271, 266–275.

Knecht, A.L., Truong, L., Simonich, M.T., Tanguay, R.L., 2016. Developmental benzo[a]pyrene (B[a]P) exposure impacts larval behavior and impairs adult learning in zebrafish. Neurotoxicol. Teratol. doi:10.1016/j.ntt.2016.10.006

Li, W., Wang, C., Shen, H., Su, S., Shen, G., Huang, Y., Zhang, Y., Chen, Y., Chen, H., Lin, N., Zhuo, S., Zhong, Q., Wang, X., Liu, J., Li, B., Liu, W., Tao, S., 2015. Concentrations and origins of nitro-polycyclic aromatic hydrocarbons and oxy-polycyclic aromatic hydrocarbons in ambient air in urban and rural areas in northern China. Environ. Pollut. 197, 156–164. doi:10.1016/j.envpol.2014.12.019

MacRae, C.A., Peterson, R.T., 2015. Zebrafish as tools for drug discovery. Nat. Rev. Drug Discov. 14, 721–731. doi:10.1038/nrd4627

Mandrell, D., Truong, L., Jephson, C., Sarker, M.R., Moore, A., Lang, C., Simonich, M.T., Tanguay, R.L., 2012. Automated Zebrafish Chorion Removal and Single Embryo Placement Optimizing Throughput of Zebrafish Developmental Toxicity Screens. J. Lab. Autom. 17, 66–74.

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. Environ. Sci. Technol. 50, 1561–1569. doi:10.1021/acs.est.5b04786

Miranda, C.L., Chung, W.G., Wang-Buhler, J.-L., Musafia-Jeknic, T., Baird, W.M., Buhler, D.R., 2006. Comparative in vitro metabolism of benzo[a]pyrene by recombinant zebrafish CYP1A and liver microsomes from β-naphthoflavone-treated rainbow trout. Aquat. Toxicol. 80, 101–108. doi:10.1016/j.aquatox.2006.07.018

Ng, G.H.B., Gong, Z., 2013. GFP Transgenic Medaka (Oryzias latipes) under the Inducible cyp1a Promoter Provide a Sensitive and Convenient Biological Indicator for the Presence of TCDD and Other Persistent Organic Chemicals. PLOS ONE 8, e64334. doi:10.1371/journal.pone.0064334

Noyes, P.D., Haggard, D.E., Gonnerman, G.D., Tanguay, R.L., 2015. Advanced morphological-behavioral test platform reveals neurodevelopmental defects in embryonic zebrafish

exposed to comprehensive suite of halogenated and organophosphate flame retardants. Toxicol. Sci. kfv044.

Ober, E.A., Field, H.A., Stainier, D.Y., 2003. From endoderm formation to liver and pancreas development in zebrafish. Mech. Dev. 120, 5–18.

Onduka, T., Ojima, D., Kakuno, A., Ito, K., Koyama, J., Fujii, K., 2012. Nitrated polycyclic aromatic hydrocarbons in the marine environment: acute toxicities for organisms at three trophic levels. Jpn. J. Environ. Toxicol. 15, 1–10. doi:10.11403/jset.15.1

Peddinghaus, S., Brinkmann, M., Bluhm, K., Sagner, A., Hinger, G., Braunbeck, T., Eisenträger, A., Tiehm, A., Hollert, H., Keiter, S.H., 2012. Quantitative assessment of the embryotoxic potential of NSO-heterocyclic compounds using zebrafish (Danio rerio). Reprod. Toxicol., Zebrafish Teratogenesis 33, 224–232. doi:10.1016/j.reprotox.2011.12.005

Perera, F.P., Rauh, V., Whyatt, R.M., Tang, D., Tsai, W.Y., Bernert, J.T., Tu, Y.H., Andrews, H., Barr, D.B., Camann, D.E., Diaz, D., Dietrich, J., Reyes, A., Kinney, P.L., 2005. A Summary of Recent Findings on Birth Outcomes and Developmental Effects of Prenatal ETS, PAH, and Pesticide Exposures. NeuroToxicology, Infant and Child Neurotoxicity StudiesTwenty-first International Neurotoxicology Conference 26, 573–587. doi:10.1016/j.neuro.2004.07.007

Perera, F.P., Whyatt, R.M., Jedrychowski, W., Rauh, V., Manchester, D., Santella, R.M., Ottman, R., 1998. Recent Developments in Molecular Epidemiology: A Study of the Effects of Environmental Polycyclic Aromatic Hydrocarbons on Birth Outcomes in Poland. Am. J. Epidemiol. 147, 309–314.

Perkins, A., Phillips, J.L., Kerkvliet, N.I., Tanguay, R.L., Perdew, G.H., Kolluri, S.K., Bisson, W.H., 2014. A Structural Switch between Agonist and Antagonist Bound Conformations for a Ligand-Optimized Model of the Human Aryl Hydrocarbon Receptor Ligand Binding Domain. Biology 3, 645–669. doi:10.3390/biology3040645

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 29, e45–e45. doi:10.1093/nar/29.9.e45

Prasch, A.L., Teraoka, H., Carney, S.A., Dong, W., Hiraga, T., Stegeman, J.J., Heideman, W., Peterson, R.E., 2003. Aryl Hydrocarbon Receptor 2 Mediates 2,3,7,8-Tetrachlorodibenzo-p-dioxin Developmental Toxicity in Zebrafish. Toxicol. Sci. 76, 138–150. doi:10.1093/toxsci/kfg202

R Core Team, 2013. R: A language and environment for statistical computing. R Found. Stat. Comput. Vienna Austria.

Reimers, M.J., La Du, J.K., Periera, C.B., Giovanini, J., Tanguay, R.L., 2006. Ethanol-dependent toxicity in zebrafish is partially attenuated by antioxidants. Neurotoxicol. Teratol. 28, 497–508.

RI DEM, 2008. Rhode Island Air Toxics Guideline.

Rosenkranz, H.S., Mermelstein, R., 1983. Mutagenicity and genotoxicity of nitroarenes. Mutat. Res. Genet. Toxicol. 114, 217–267. doi:10.1016/0165-1110(83)90034-9

Schmidt, J.V., Bradfield, C.A., 1996. Ah Receptor Signaling Pathways. Annu. Rev. Cell Dev. Biol. 12, 55–89. doi:10.1146/annurev.cellbio.12.1.55

Schuetzle, D., Riley, T.J., Prater, T.J., Harvey, T.M., Hunt, D.F., 1982. Analysis of nitrated polycyclic aromatic hydrocarbons in diesel particulates. Anal. Chem. 54, 265–271. doi:10.1021/ac00239a028

Schultz, T.W., Cajina-Quezada, M., Dumont, J.N., 1980. Structure-toxicity relationships of selected nitrogenous heterocyclic compounds. Arch. Environ. Contam. Toxicol. 9, 591–598. doi:10.1007/BF01056938

Scott, J.A., Incardona, J.P., Pelkki, K., Shepardson, S., Hodson, P.V., 2011. AhR2-mediated, CYP1A-independent cardiovascular toxicity in zebrafish (Danio rerio) embryos exposed to retene. Aquat. Toxicol. 101, 165–174. doi:10.1016/j.aquatox.2010.09.016

Senft, A.P., Dalton, T.P., Nebert, D.W., Genter, M.B., Puga, A., Hutchinson, R.J., Kerzee, J.K., Uno, S., Shertzer, H.G., 2002. Mitochondrial reactive oxygen production is dependent on the aromatic hydrocarbon receptor. Free Radic. Biol. Med. 33, 1268–1278. doi:10.1016/S0891-5849(02)01014-6

Shailaja, M.S., Rajamanickam, R., Wahidulla, S., 2006. Formation of Genotoxic Nitro-PAH Compounds in Fish Exposed to Ambient Nitrite and PAH. Toxicol. Sci. 91, 440–447. doi:10.1093/toxsci/kfj151

Sousa, M.S.A., Latini, F.R.M., Monteiro, H.P., Cerutti, J.M., 2010. Arginase 2 and nitric oxide synthase: Pathways associated with the pathogenesis of thyroid tumors. Free Radic. Biol. Med. 49, 997–1007. doi:10.1016/j.freeradbiomed.2010.06.006

Sydbom, A., Blomberg, A., Parnia, S., Stenfors, N., Sandström, T., Dahlén, S.-E., 2001. Health effects of diesel exhaust emissions. Eur. Respir. J. 17, 733–746.

Titaley, I.A., Chlebowski, A., Truong, L., Tanguay, R.L., Massey Simonich, S.L., 2016. Identification and Toxicological Evaluation of Unsubstituted PAHs and Novel PAH Derivatives in Pavement Sealcoat Products. Environ. Sci. Technol. Lett. doi:10.1021/acs.estlett.6b00116

Toftgård, R., Franzén, B., Gustafsson, J.-Å., Löfroth, G., 1985. Characterization of TCDD-receptor ligands present in extracts of urban particulate matter. Environ. Int., Genotoxic Air Pollutants 11, 369–374. doi:10.1016/0160-4120(85)90031-5

Truong, L., Bugel, S.M., Chlebowski, A., Usenko, C.Y., Simonich, M.T., Simonich, S.L.M., Tanguay, R.L., 2016. Optimizing multi-dimensional high throughput screening using zebrafish. Reprod. Toxicol. 65, 139–147. doi:10.1016/j.reprotox.2016.05.015

Truong, L., Harper, S.L., Tanguay, R.L., 2011. Evaluation of embryotoxicity using the zebrafish model. Methods Mol. Biol. Clifton NJ 691, 271–279. doi:10.1007/978-1-60761-849-2_16

Truong, L., Reif, D.M., St Mary, L., Geier, M.C., Truong, H.D., Tanguay, R.L., 2014. Multidimensional in vivo hazard assessment using zebrafish. Toxicol. Sci. 137, 212–233.

US EPA, n.d. Estimation Programs Interface Suite [TM] for Microsoft Windows. United States Environmental Protection Agency, Washington DC, USA.

Vicente, E.D., Vicente, A.M., Bandowe, B.A.M., Alves, C.A., 2015. Particulate phase emission of parent polycyclic aromatic hydrocarbons (PAHs) and their derivatives (alkyl-PAHs, oxygenated-PAHs, azaarenes and nitrated PAHs) from manually and automatically fired combustion appliances. Air Qual. Atmosphere Health 1–16. doi:10.1007/s11869-015-0364-1

Wei, C., Bandowe, B.A.M., Han, Y., Cao, J., Zhan, C., Wilcke, W., 2015. Polycyclic aromatic hydrocarbons (PAHs) and their derivatives (alkyl-PAHs, oxygenated-PAHs, nitrated-

PAHs and azaarenes) in urban road dusts from Xi'an, Central China. Chemosphere 134, 512–520. doi:10.1016/j.chemosphere.2014.11.052

WHO, 2015. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans [WWW Document]. URL http://monographs.iarc.fr/index.php (accessed 11.2.16).

Yunker, M.B., Macdonald, R.W., Vingarzan, R., Mitchell, R.H., Goyette, D., Sylvestre, S., 2002. PAHs in the Fraser River basin: a critical appraisal of PAH ratios as indicators of PAH source and composition. Org. Geochem. 33, 489–515. doi:10.1016/S0146-6380(02)00002-5

Zimmermann, K., Jariyasopit, N., Massey Simonich, S.L., Tao, S., Atkinson, R., Arey, J., 2013. Formation of nitro-PAHs from the heterogeneous reaction of ambient particle-bound PAHs with N2O5/NO3/NO2. Environ. Sci. Technol. 47, 8434–8442.

Zok, S., Görge, G., Kalsch, W., Nagel, R., 1991. Bioconcentration, metabolism and toxicity of substituted anilines in the zebrafish (Brachydanio rerio). Sci. Total Environ., QSAR in Environmental Toxicology 109, 411–421. doi:10.1016/0048-9697(91)90196-L

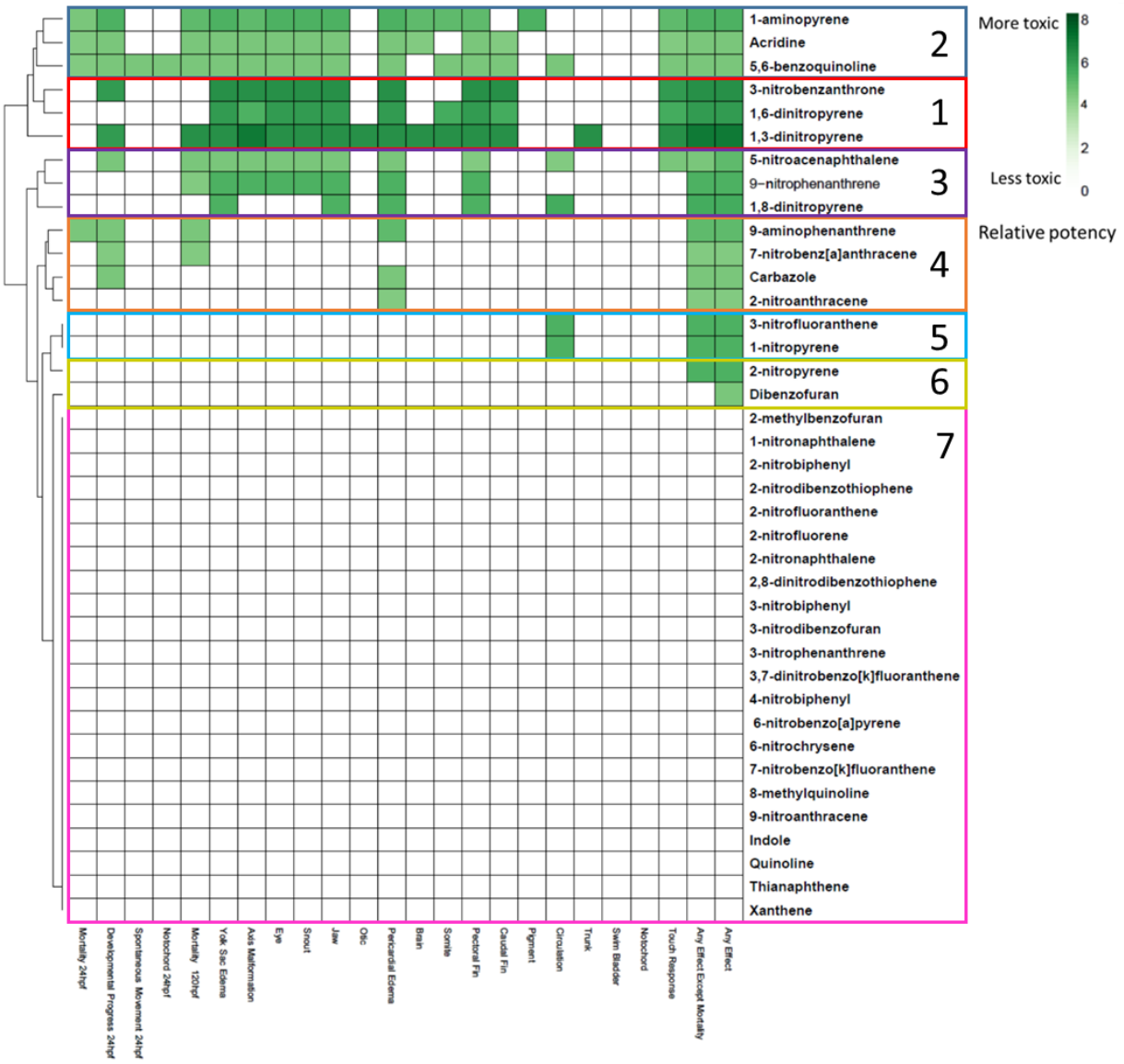
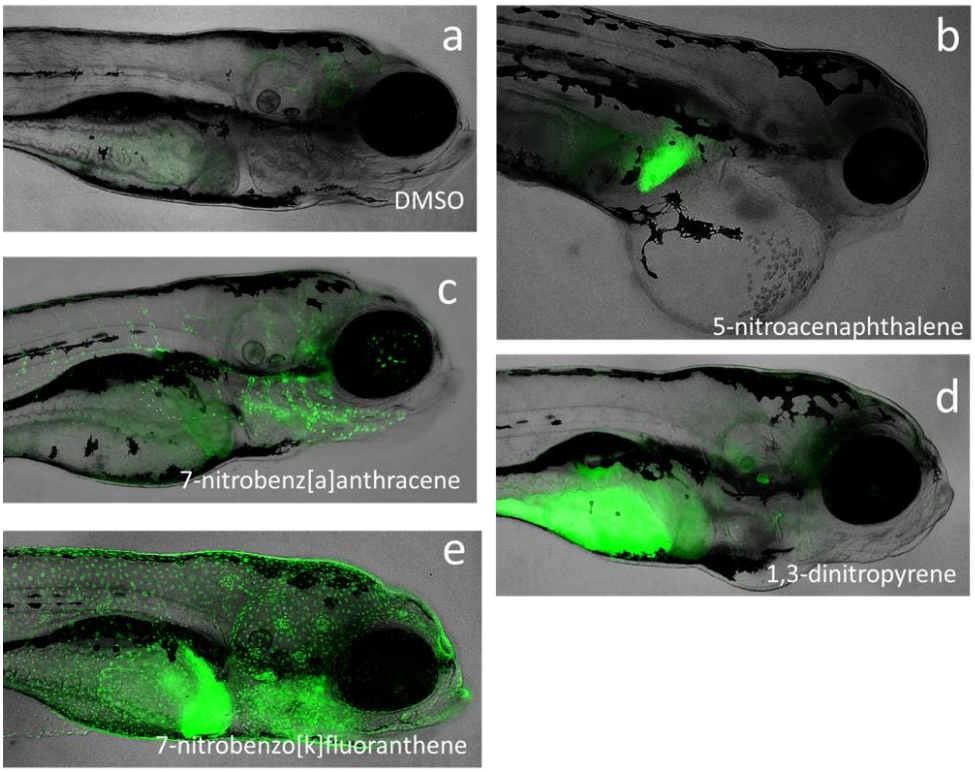


Figure 3.1. Developmental toxicity heatmap. Heatmap displaying developmental toxicity of all 39 compounds investigated. Color scaling indicates relative potency, based on the lowest effect level (LEL) observed for a given compound and endpoint. Darker shades of green indicate a higher relative potency, and therefore a more toxic response, while lighter shades indicate a lower relative potency and less toxic response. White indicates that there was no significant observable LEL for that endpoint, or that values were incalculable due to high mortality. Compounds are vertically clustered based on the observation of similar developmental endpoints. Groups of compounds with similar toxicity profiles are indicated by boxes and group numbers.



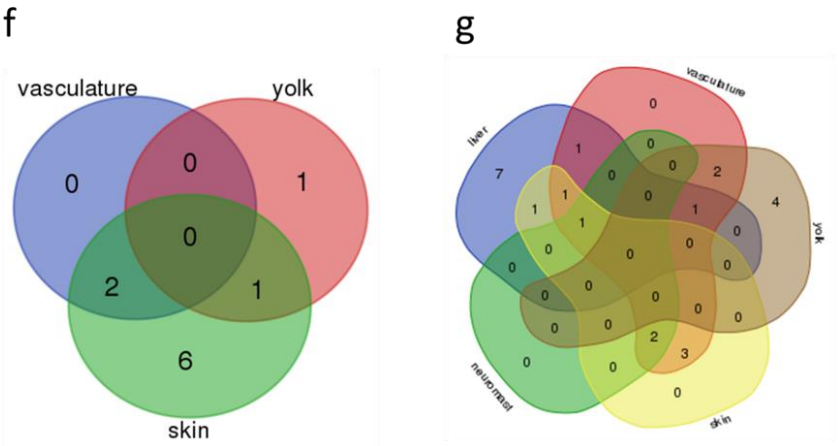


Figure 3.2. *cyp1a*/GFP expression. Selected representative images of *cyp1a*/GPF expression in *Tg(cyp1a:nls-egfp)* transgenic zebrafish following chemical exposures. Examples of *cyp1a*/GFP expression patterns in the DMSO-exposed animals (a), liver (b), vasculature (c), yolk sac (d) and skin, neuromasts and liver (e) following exposure to indicated NPAHs. Expression patterns observed at 48 hpf (f) and 120 hpf (g), showing unique and overlapping *cyp1a*/GFP expression patterns for all compounds where expression in one or more tissues was observed. All results are summarized in Table 3.3.

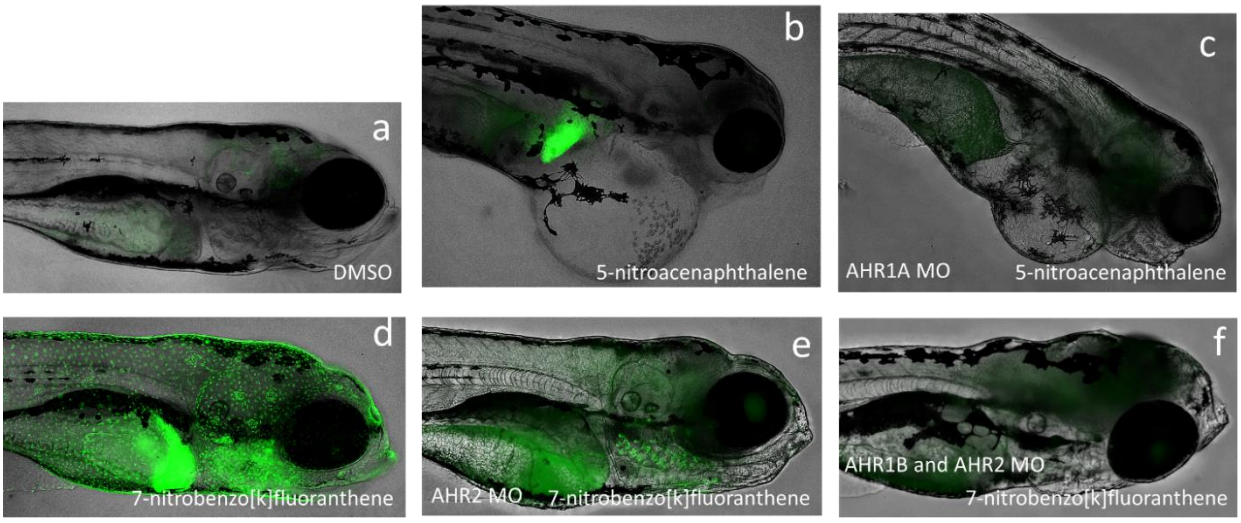


Figure 3.3. *cyp1a*/GFP expression in AHR morphants. Selected representative images of *cyp1a*/GPF expression in *Tg(cyp1a:nls-egfp)* transgenic zebrafish following chemical exposures and morpholino oligonucleotide injections. Examples of *cyp1a*/GFP expression patterns in the DMSO-exposed animals (a), liver (b), absence liver expression in AHR1A morphants (c), skin, neuromasts and liver (d), near-complete elimination of skin, neuromast, and vascular expression in AHR2 morphants (e), and complete skin, neuromast, and vasculature expression elimination (f) following co-injection of the AHR2 and AHR1B morpholinos, following exposure to indicated NPAHs.

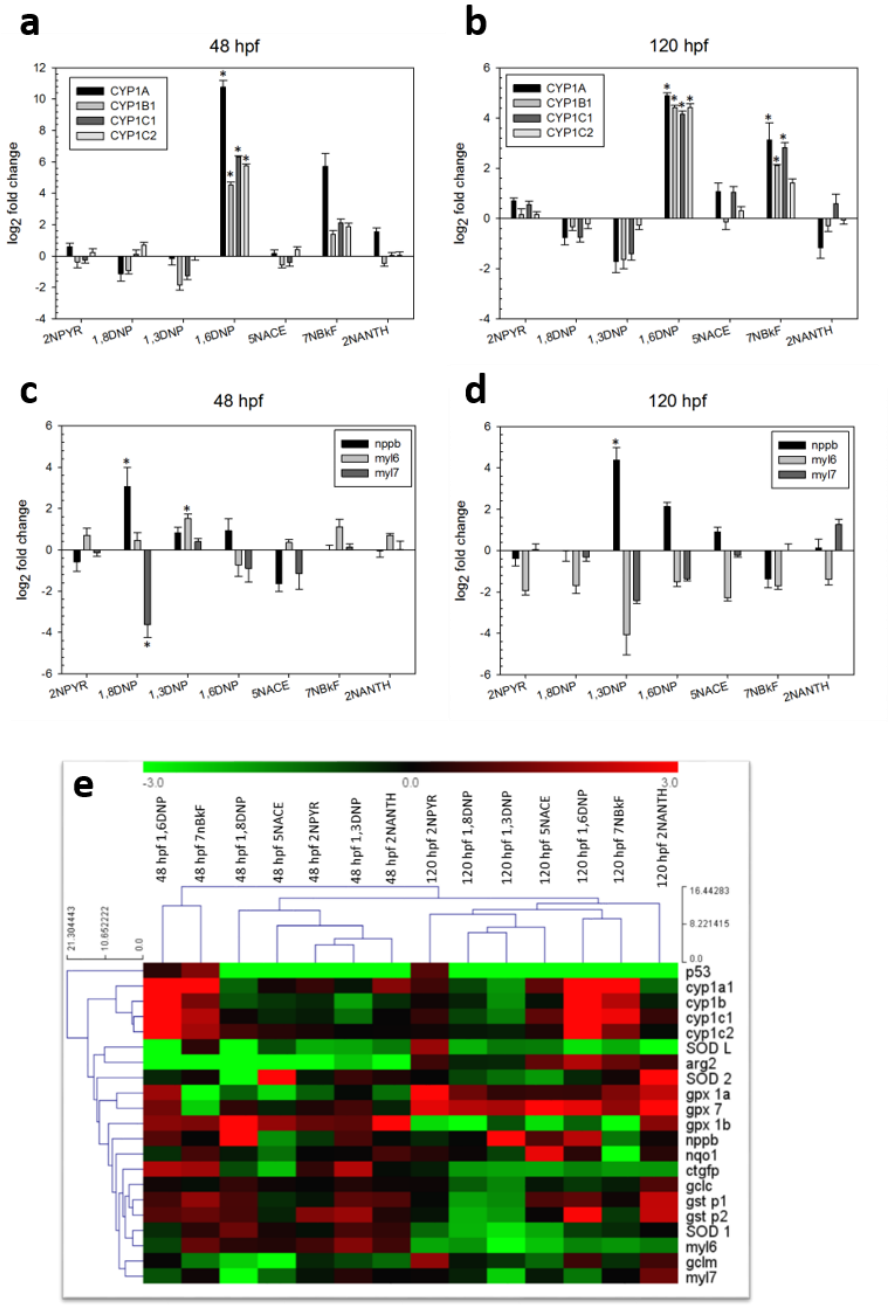


Figure 3.4. Changes in gene expression. Changes in gene expression induced by exposure to selected NPAHs. Graphs indicate changes in expression to selected *cyp* genes at 48 hpf (a) and 120 hpf (b), and in selected cardiac stress genes at 48 hpf (c) and 120 hpf (d). Asterisks indicate statistical significant at a $p<0.05$ level, based on one-way ANOVA. All genes investigated are shown in the heatmap (e) for 48 and 120 hpf, where red indicates increased expression, and green indicates decreased expression.

Table 3.1. Morpholino oligonucleotide sequences. Sequences of morpholino oligonucleotides used, with appropriate injection concentrations.

AHR Isoform	**Sequence**	**Concentration**
AHR1A	CTTTTGAAGTGACTTTTGGCCCGCA	1.5 mM
AHR1B	ACACAGTCGTCCATGATTACTTTGC	0.75 mM
AHR2	TGTACCGATACCCGCCGACATGGTT	0.75 mM
Control	CCTCTTACCTCAGTTACAATTTATA	1.5 mM
AHR1B/AHR2 mixture		0.75 mM each

Table 3.2. Calculated EC_{50} values. EC_{50} (μM) values for all compounds, with standard error. Blank cells indicate insufficient data to calculate EC_{50}. † indicates calculated value is beyond the range of concentrations tested, and beyond the range of accuracy of the EC_{50} model.

	EC_{50} (μM)	Std Error
NPAHs		
1-nitronaphthalene		
2-nitronaphthalene		
2-nitrobiphenyl		
3-nitrobiphenyl		
4-nitrobiphenyl		
3-nitrodibenzofuran		
5-nitroacenaphthalene	13.41	0.78
2-nitrofluorene		
9-nitroanthracene	222.13†	189
9-nitrophenanthrene	1.81	0.78
2-nitrodibenzothiophene		
3-nitrophenanthrene		
2-nitroanthracene		
2-nitrofluoranthene		
3-nitrofluoranthene	2.04	0.85
1-nitropyrene	44.82	8.34
2-nitropyrene		
7-nitrobenz[a]anthracene	237.6†	108
2,8-dinitrodibenzothiophene		
6-nitrochrysene		
3-nitrobenzanthrone	0.2	0.17
1,3-dinitropyrene	0.1	0.05
1,6-dinitropyrene	0.88	0.22
1,8-dinitropyrene	3.33	1.18
6-nitrobenzo[a]pyrene		
7-nitrobenzo[k]fluoranthene		
3,7-dinitrobenzo[k]fluoranthene		
Amino PAHs		
1-aminopyrene	1.85	0.74
9-aminophenanthrene	8.68	0.87
HPAHs		
Indole		
Quinoline		
2-Methylbenzofuran		
Thianaphthene		
8-Methylquinoline		
Carbazole	15.15	0.96
Dibenzofuran	228.3†	227.4
5,6-Benzoquinoline	10.35	1.04
Acridine	13.49	0.96
Xanthene		

Table 3.3. Summary of *cyp1a*/GFP expression patterns. Summary of *cyp1a*/GFP expression patterns observed using *Tg(cyp1a:nls-egfp)* transgenic zebrafish following individual exposure to all NPAHs and HPAHs studied, at 48 hpf and 120 hpf. Tissues where *cyp1a*/GFP expression are observed are designated with an "x".

	48 hpf						120 hpf					
NPAHs	Liver	Vasculature	Yolk	Skin	Neuromast	None	Liver	Vasculature	Yolk	Skin	Neuromast	None
1-nitronaphthalene						x						x
2-nitronaphthalene						x						x
2-nitrobiphenyl						x						x
3-nitrobiphenyl						x	x					
4-nitrobiphenyl						x						x
3-nitrodibenzofuran						x						x
5-nitroacenaphthalene						x	x					
2-nitrofluorene						x						x
9-nitroanthracene						x						x
9-nitrophenanthrene						x	x					
2-nitrodibenzothiophene						x	x					
3-nitrophenanthrene						x	x					
2-nitroanthracene			x						x			
2-nitrofluoranthene		x		x			x	x	x			
3-nitrofluoranthene						x		x	x			
1-nitropyrene						x			x			
2-nitropyrene						x						x
7-nitrobenz[a]anthracene				x				x		x		
2,8-dinitrodibenzothiophene						x						x
6-nitrochrysene				x				x		x		
3-nitrobenzanthrone			x	x					x			
1,3-dinitropyrene						x			x			
1,6-dinitropyrene				x				x		x		
1,8-dinitropyrene						x						x
6-nitrobenzo[a]pyrene						x	x	x		x		
7-nitrobenzo[k]fluoranthene				x			x	x		x	x	
3,7-dinitrobenzo[k]fluoranthene						x		x		x	x	
Amino PAHs												
1-aminopyrene						x		x	x			
9-aminophenanthrene						x	x					
HPAHs												
Indole				x				x		x	x	
Quinoline						x						x
2-Methylbenzofuran						x						x
Thianaphthene						x						x
8-Methylquinoline						x						x
Carbazole						x	x	x				
Dibenzofuran						x						x
5,6-Benzoquinoline				x			x			x		
Acridine		x		x			x					x
Xanthene						x						x

Table 3.4.*In silico* predicted AHR docking scores. Predicted AHR docking scores (kilocalories/mol) for all three zebrafish AHR isoforms, as well as human AHR. ND indicates the compound was not predicted to dock, or docked with non-favorable score/energy to the AHR active site model. Asterisk indicates that docking was predicted, but with an unfavorable pose in the active site, despite the promising binding energy. Compounds were classified as "high priority" for AHR activation (indicated by †) based on overall AHR binding favorability to the three zebrafish isoforms.

Docking Scores					
NPAHs	Human	Zebrafish AHR2	Zebrafish AHR 1B	Zebrafiah AHR1A	High Priority
1-nitronaphthalene	-14.32	-16.08	-13.01	-19.36	
2-nitronaphthalene	-13.43	-14.78	-18.03	-17.97	
2-nitrobiphenyl	-11.83	-15.5	-19.13	-15.43	
3-nitrobiphenyl	-14.94	-17.73	-18.01	-15.42	
4-nitrobiphenyl	-18.12	-12.71	-14.19	-17.2	
3-nitrodibenzofuran	-19.52	-19.09	-14.9	-18.07	†
5-nitroacenaphthalene	-5.91	-11.29	-15.46	-17.04	†
2-nitrofluorene	-12.97	-9.1	-9.72	-17.07	†
9-nitroanthracene	-9.89	-10.15	-12.06	-14.84	†
9-nitrophenanthrene	-3.08	-12.25	- 15.91*	-15.72	†
2-nitrodibenzothiophene	-15.85	-17.06	-10.85	-17.92	†
3-nitrophenanthrene	-12.91	-15.17	- 16.31*	-16.34	†
2-nitroanthracene	-16.55	-14.81	-8.2	-19.02	†
2-nitrofluoranthene	-8.33	-12.79	- 15.25*	-12.43	
3-nitrofluoranthene	-9.12	-7.91	- 16.55*	-17.14	†
1-nitropyrene	ND	-6.91	- 15.67*	-12.07	†
2-nitropyrene	-3.54	-4.4	- 14.36*	-17.92	
7-nitrobenz[a]anthracene	-9.97	-5.53	- 18.08*	ND	†
2,8-dinitrodibenzothiophene	-15.14	-18.19	-10.85	-17.31	†
6-nitrochrysene	-11.37	-5.47	- 16.91*	-11.6	†
3-nitrobenzanthrone	-5.64	-3.86	- 17.15*	-6.66	†
1,3-dinitropyrene	ND	ND	- 15.13*	ND	
1,6-dinitropyrene	ND	-5.71	- 15.85*	ND	
1,8-dinitropyrene	-3.69	-5.66	- 16.23*	-11.64	
6-nitrobenzo[a]pyrene	ND	-1.64	- 18.13*	ND	
7-nitrobenzo[k]fluoranthene	-5.27	ND	- 16.05*	ND	
3,7-dinitrobenzo[k]fluoranthene	-5.27	ND	- 16.05*	ND	
Amino PAHs					
1-aminopyrene	ND	-5.3	- 15.06*	-11.09	†
9-aminophenanthrene	-7.77	-10.99	-11.89	-21.81	†
HPAHs					
Indole	-11.14	-15.57	-13.94	-19.94	†
Quinoline	-12.29	-15.18	-17.52	-15.83	
2-Methylbenzofuran	-15.38	-17.2	-16.96	-14.17	
Thianaphthene	-13.89	-12.07	-14.46	-17.7	
8-Methylquinoline	-7.85	-13.23	-12.8	-17.29	
Carbazole	-11.86	-14.85	-15.81	-19.78	†
Dibenzofuran	-15.02	-15.82	-14.07	-16.12	†
5,6-Benzoquinoline	-14.18	-11.97	-14.57	-20.46	†
Acridine	-15.68	-15.48	-11.31	-15.09	†
Xanthene	-16.26	-14.69	-11.64	-14.18	†

CHAPTER 4 – INVESTIGATING THE APPLICATION OF A NITROREDUCTASE-EXPRESSING TRANSGENIC ZEBRAFISH LINE FOR HIGH-THROUGHPUT TOXICITY TESTING

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Abstract

Nitroreductase enzymes are responsible for the reduction of nitro functional groups to amino functional groups, and are found in a range of animal models, zebrafish (*Danio rerio*) excluded. Transgenic zebrafish models have been developed for tissue-specific cell ablation, which use nitroreductase to ablate specific tissues or cell types following exposure to the non-toxic pro-drug metronidazole (MTZ). When metabolized by nitroreductase, MTZ produces a potent cytotoxin, which specifically ablates the tissue in which metabolism occurs. Uses, beyond tissue-specific cell ablation, are possible for the hepatocyte-specific *Tg(l-fabp:CFP-NTR)*^{s891} zebrafish line, including investigations of the role of nitroreductase in the toxicity of nitrated compounds. The hepatic ablation characteristics of this transgenic line were explored, in order to expand its potential uses. Embryos were exposed at 48, 72, or 96 hours post fertilization (hpf) to a range of MTZ concentrations, and the ablation profiles were compared. Ablation occurred at a 10-fold lower concentration than previously reported. Embryos were exposed to a selection of other compounds, with and without MTZ, in order to investigate alternative uses for this transgenic line. Test compounds were selected based on: their ability to undergo nitroreduction, known importance of hepatic metabolism to toxicity, and known pharmaceutical hepatotoxins. Selected compounds included nitrated polycyclic aromatic hydrocarbons (nitro-PAHs), the PAHs retene and benzo[a]pyrene, and the pharmaceuticals acetaminophen and flutamide. The results suggest a range of potential roles of the liver in the toxicity of these compounds, and highlight the additional uses of this transgenic model in toxicity testing.

Keywords: zebrafish, transgenic, nitroreductase, nitrated polycyclic aromatic hydrocarbon, tissue ablation, pharmaceuticals

Introduction

The zebrafish (*Danio rerio*) is unique among vertebrate model organism systems in that it is amendable to high-throughput developmental toxicity testing (Bugel et al., 2014; Garcia et al., 2016; Truong et al., 2014). Zebrafish are easy to cultivate in a laboratory setting, have a high fecundity, develop externally, and the embryos are transparent during development. Zebrafish are also metabolically competent, in particular following development of the liver between 48 and 72 hours post fertilization (hpf) (Kimmel et al., 1995; Elke A. Ober et al., 2003). Zebrafish have high genetic homology to humans, with approximately 70% of human genes and about 82% of potential human disease-related genes having at least one zebrafish orthologue (Howe et al., 2013). This model is also highly amenable to genetic manipulation, with the addition or removal of genes of interest being relatively easy to achieve, including those involved in metabolism (Garcia et al., 2016). However, for some chemicals and chemical classes, there may be discordance in toxicity response between other model systems.

One difference in the metabolic capability of zebrafish compared to other model organisms is the enzyme nitroreductase. Nitroreductases are responsible for the reduction of nitro functional groups to amino functional groups. This has been implicated as an important component for the mechanism of toxicity for some compounds, such as nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) (Ball et al., 1984). Zebrafish are not believed to have nitroreductase activity. This is supported by the distinctly different toxicological profiles for the corresponding pairs of amino- and nitro- compounds in zebrafish (Chlebowski et al., 2017). Some evidence suggests zebrafish may have nitroreductase activity in the yolk (Z. Li et al., 2015), and it is possible that zebrafish, like humans and other model organisms, may have nitroreductase activity in the intestine due to the presence of microbiota (Fu, 1990; Salem et al., 1981).

The lack of nitroreductase activity in zebrafish has been used to develop transgenic lines for tissue-specific cell ablation. Tissue-specific cell ablation has previously been used in other model systems (Lewandoski, 2001; McGuire et al., 2004), with more recent developments in zebrafish as well. One technique showing success in the zebrafish model uses the expression of nitroreductase genes controlled by tissue-specific promoters, to create the desired tissue specificity (Curado et al., 2008, 2007; Mathias et al., 2014; White and Mumm, 2013). Animals are treated with a non-toxic pro-drug containing a nitro functional group, commonly metronidazole (MTZ), which becomes cytotoxic when reduced by nitroreductase (Bridgewater et al., 1995). Models have been developed where nitroreductase is expressed in a range of tissues, including hepatocytes, cardiomyocytes, pancreatic β-islet cells, oocytes and testis (Curado et al., 2008, 2007; Hsu et al., 2009; Pisharath et al., 2007; White et al., 2011). Specificity of the nitroreductase promoter is essential, as well as containment of the cytotoxin, to prevent ablation of other tissues and off-target effects, and the lack of endogenous nitroreductase expression in zebrafish makes them well-suited to this model for tissue ablation.

The use of transgenic zebrafish lines previously developed for nitroreductase-based tissue-specific cell ablation would be ideal for further investigating the role of nitroreductase in toxicity and metabolism. Of the previously developed nitroreductase-expressing transgenic zebrafish lines, those which express *Escherichia coli* nitroreductase using a hepatocyte-specific promoter most closely resembles nitroreductase expression in humans, with nitroreductase expressed in the liver, as humans do. This allows for a whole-animal system with a metabolic capability more similar to humans and mammalian model systems than the standard zebrafish lines.

Aside from the use in tissue-specific cell ablation, a zebrafish line with nitroreductase ability in the liver, and a more human-like metabolic capability, can have other uses as well. The nitroreductase capability makes this transgenic zebrafish a valuable resource in gaining insight on the metabolism of nitro-containing compounds, as well as identifying potential hepatotoxins. One potential use is the toxicity screening of nitrated environmental contaminants, such as nitrated polycyclic aromatic hydrocarbons (nitro-PAHs), for which nitroreduction has been implicated as a component of toxicity (Chlebowski et al., 2017; Fu et al., 1988; Möller, 1994). Previous data in the zebrafish model has indicated that amino-PAHs elicit a greater toxicity response than the corresponding nitro-PAH, with greater incidences of developmental malformations as well as mortality occurring following exposure to 1-aminopyrene and 9-aminophenanthrene, compared to embryos exposed to 1-nitropyrene and 9-nitrophenanthrene, respectively (Chlebowski et al., 2017). Zebrafish with this more human-like metabolic capability could also be useful in the investigation of pharmaceuticals and hepatotoxins, as well as investigating the role of the liver and hepatic metabolism in toxicity. Other PAHs, such as benzo[a]pyrene and retene, are known to undergo hepatic metabolism (Baird et al., 2005; Billiard et al., 1999; Shimada and Fujii-Kuriyama, 2004), and exposure results in developmental malformations, including edemas and craniofacial malformations, in the developing zebrafish (Hawliczek et al., 2012; Incardona et al., 2011; Scott et al., 2011). Certain pharmaceuticals, including acetaminophen and flutamide, are known to result in human hepatotoxicity (Gomez et al., 1992; Mitchell et al., 1973; Wysowski and Fourcroy, 1996), and can result in developmental toxicity in the zebrafish model (David and Pancharatna, 2009).

The purpose of this study was to explore the importance of nitroreductase and hepatic metabolism in the developmental toxicity observed in zebrafish. Prior to adapting this line for use in high-throughput assays, further characterization of the *Tg(l-fabp:CFP-NTR)*^{s891} zebrafish line was necessary. Following more thorough line characterization, the role of nitroreductase and hepatic metabolism was investigated for a subset of selected chemicals, for which nitroreduction or hepatic metabolism had been previously implicated as an important component of toxicity.

Materials and Methods

Fish care and husbandry

Adult zebrafish were maintained with a water temperature of 28° ±1°C on a recirculating system with a 14h light 10h dark photoperiod at the Sinnhuber Aquatic Research Laboratory (SARL). All experiments were conducted with wild-type 5D strain or *Tg(l-fabp:CFP-NTR)*^{s891} (background strain TL) (Curado et al., 2007). Adult care and reproductive techniques were conducted according to the Institutional Animal Care and Use Committee protocols at Oregon State University (OSU). All 5D embryos used in exposures were collected following group spawning of adult zebrafish as described previously. Embryos from the *Tg(l-fabp:CFP-NTR)*^{s891} transgenic strain were collected following incross or outcross small group spawns (Reimers et al., 2006).

Chemicals

Analytical-grade metronidazole (MTZ, CAS # 443-48-1), acetaminophen (CAS # 103-90-2), benzo[a]pyrene (CAS #50-32-8), 1-nitropyrene (CAS # 5522-43-0), 1-aminopyrene (CAS # 1606-67-3), 9-aminophenanthrene (CAS # 947-73-9), and dimethyl sulfoxide (DMSO, CAS #

67-68-5) were obtained from Sigma-Aldrich (St. Louis, MO). Analytical-grade 9-nitrophenanthrene (CAS # 954-46-1) was obtained from AccuStandard (New Haven, CT). Analytical-grade retene (CAS # 483-65-8) was purchased from Santa Cruz Biotechnology (Dallas, TX). Flutamide (CAS # 13311-84-7) was provided by the NIEHS National Toxicology Project (NTP). The ROS-ID® Hypoxia/Oxidative Stress Detection Kit was purchased from Enzo Life Sciences (Farmingdale, NY). For each experiment, a fresh solution of MTZ was made in DMSO immediately prior to exposure, and protected from light prior to and during the course of the exposure to prevent photodegradation.

Basic Tg(l-fabp:CFP-NTR)^{s891} embryo exposure

Unless otherwise noted, embryos were exposed in 20 mL amber glass vials in groups of 10-12 animals per treatment, in 10 mL total volume of exposure solution (7 mL for flutamide exposure, with 7-8 embryos). Embryos were added to the vial prior to addition of appropriate chemical treatments. For experiments where exposures started at 6 hpf, embryos were distributed into vials prior to exposure. For experiments where exposures started at 48 hpf or later, embryos were kept in clean petri dishes of embryo media until prior to exposure, at which time embryos displaying normal development were placed into amber glass vials for treatment. During exposure, the amber glass vials containing embryos were rocked at 28 °C. Following exposure, embryos were evaluated for liver presence/ablation and imaged using a Keyence BZ-X700 fluorescence microscope (Keyence North America, Itasca, IL) with a green fluorescent protein (GFP) filter.

Initial characterization

Tg(l-fabp:CFP-NTR)^{s891} embryos (incross and outcross) were distributed into amber glass vials and exposed to 10 mM MTZ at 48, 72, or 96 hpf. Embryos were observed daily following exposure, until 120 hpf.

MTZ dilutions

Embryos were continuously exposed from 96 to 120 hpf to 1 µM to 10 mM MTZ on a 10-fold dilution scale. Based on the results from this experiment, a second group of 96 hpf embryos were exposed to a refined dilution series from 100-1000 µM MTZ, and evaluated at 120 hpf.

Liver ablation time course

96 hpf embryos were exposed to 10 mM MTZ, and evaluated at 30 minutes, every hour from 1-8, 12, and 24 hours after MTZ exposure.

Ablation recovery time course

96 hpf embryos were exposed to 10 mM MTZ until 120 hpf, at which point the MTZ solution was removed, and the embryos rinsed three times and placed in clean embryo media (EM). Embryos were evaluated for regeneration of the liver 0 min, 30 min, and every hour from 1-12, 24, 36, 48, 60, and 72 hours after MTZ removal.

The ROS-ID® Hypoxia/Oxidative Stress Detection Kit

Exposures were conducted based on the manufacturer's suggested protocols for cells in suspension, with evaluation using fluorescent microscopy. Either wild-type or *Tg(l-fabp:CFP-NTR)*^{s891} embryos were distributed into individual wells of a 96-well plate containing 100 µL EM. At 48, 72, or 96 hpf, 56 µL of the EM was removed, and replaced with 56 µL of either EM, EM containing the hypoxia detection reagent at the suggested concentration (0.5 µM), or EM containing the detection reagent and the provided hypoxia inducer (deferoxamine, DFO) at the suggested concentration (200 µM). The plates were wrapped in foil, and embryos were exposed for 1, 3, 5, or 24 hours in the 28 °C incubator. Following exposure, embryos were rinsed three times to remove excess reagent, and imaged using fluorescence microscopy.

Exposure using 96-well plates

For windows of exposure experiments using the wild-type or *Tg(l-fabp:CFP-NTR)*^{s891} zebrafish line, exposures were performed as previously described (Chlebowski et al., 2017; Truong et al., 2016). Briefly, embryos (dechorionated if wild-type, chorions on for the *Tg(l-fabp:CFP-NTR)*^{s891} line) were added to individual wells of a polystyrene 96-well plate already containing 100 µL of embryo media. Wild-type embryos were dechorionated and loaded in the plates using automation (Mandrell et al., 2012c), while *Tg(l-fabp:CFP-NTR)*^{s891} embryos were not dechorionated due to low embryo production, and to minimize stress. Chemicals were dispensed into the plates using an HP D300 Digital Dispenser, utilizing previously optimized protocols, at 6, 24, 48, 72, or 96 hpf (Truong et al., 2016). Following chemical addition, plates were moved to a

temperature-controlled room (28 °C), and were placed on a custom-modified rotating shaker table from 6 to 24 hpf, and in a stationary incubator thereafter (Truong et al., 2016). Embryos were evaluated at 24 and 120 hpf for mortality and a suite of developmental malformations (Chlebowski et al., 2017; Knecht et al., 2013; Truong et al., 2011).

Chemical co-exposures

At 48 hpf, *Tg(l-fabp:CFP-NTR)*^{s891} embryos were distributed into amber glass vials containing EM, and were exposed to either 10 mM MTZ in DMSO, or an equal volume of DMSO. At 72 hpf, embryos were exposed to retene (10, 12, 14, 16, 18 µM), benzo[a]pyrene (50, 65, 100 µM), acetaminophen (0.125, 2.5, 5, 10, 25 mM), or flutamide (5, 7, 10, 12 µM), or an equivalent volume of DMSO. Wild-type 5D embryos were also exposed to 5, 7, 10, and 12 µM flutamide in the presence and absence of MTZ. The final volume of DMSO in each vial was 1% for retene and flutamide exposures, and 1.5% for acetaminophen and benzo[a]pyrene exposures. Chemical concentrations for exposures were selected based on preliminary exposure data in the wild-type 5D embryos, for an approximate EC_{50} (data not shown). Vials were then placed horizontally on the rocking table in the incubator, until evaluation via fluorescent imaging at 120 hpf.

Results

Initial characterization

Homozygous adult *Tg(l-fabp:CFP-NTR)*^{s891} zebrafish were either incrossed or outcrossed to 5D zebrafish to produce a uniform population of either homozygous or heterozygous eggs, respectively. Exposures to MTZ at 48, 72, and 96 hpf with either the homozygous or heterozygous embryos yielded the same result in terms of visual ablation of the liver at 120 hpf

(Figure 4.1). Fluorescence in the liver was substantially reduced or eliminated in embryos exposed to MTZ, regardless of the time of exposure or the genotype of the embryos. For subsequent experiments, heterozygous embryos were used. Additional characterization of the nitroreductase capability of this line was done using the ROS-ID® Hypoxia/Oxidative Stress Detection Kit (see Appendix C.1), which was developed for use in cell culture and had not been previously adapted to be used in zebrafish. This assay showed nitroreductase activity in the neuromasts and head, but not in the liver as was expected (Appendix C.1).

Liver ablation occurs at lower concentrations of MTZ

Embryos at 96 hpf were exposed to a range of MTZ concentrations, from 1 µM to 10 mM (10,000 µM), and evaluated after 24 hours of exposure, at 120 hpf. Embryos exposed to 1, 10, or 100 µM MTZ had little to no tissue ablation, whereas the embryos exposed to 1,000 or 10,000 µm MTZ had complete ablation, as indicated by the absence of a fluorescent signal in the liver (Figure 4.2a). Low levels of developmental toxicity, primarily jaw malformations and slight truncation of the body axis, were observed in the 10,000 µM exposure animals. Further investigation of MTZ exposure concentrations from 100-1,000 µM showed partial ablation from 700-900 µM, indicated by the decreasing fluorescence in the liver, with complete ablation of fluorescence occurring at 1,000 µM (Figure 4.2b).

Visual progression of liver ablation and recovery

Exposures to 10 mM MTZ on 96 hpf embryos were conducted as previously published with a freshly made MTZ solution (Curado et al., 2008, 2007), and the embryos evaluated following

continuous MTZ exposure. Complete ablation was observed in some fish as early as 5 hours post-exposure (hpe), with consistent ablation occurring by 12 hours post exposure (Figure 4.3a). Recovery of hepatic tissue, indicated by return of the fluorescent signal, in embryos exposed to 10 mM MTZ from 96 to 120 hpf was monitored from 120 to 192 hpf (0 to 72 hpe). Following removal of the MTZ solution and transfer to fresh embryo media, limited liver regeneration was observed by 10-12 hpe, as indicated by a faint fluorescent signal. Significant recovery of the liver had occurred by 24 hpe, and by 48 hpe the liver appeared to be nearly completely recovered (Figure 4.3b).

Windows of exposure

The 120 hpf toxicity profiles elicited by 1-nitropyrene at 6, 24, 48, 72, and 96 hpf in the wild-type zebrafish embryos were nearly identical (Figure 4.4a and Appendix C.2). The primary endpoint observed, circulatory defects marked by pooling of blood in the torso, was observed in a dose-dependent manner for all exposure time points. The malformation profile for 1-aminopyrene (Figure 4.4b) in the wild-type embryos was distinct from 1-nitropyrene, and changed with the different exposure windows. *Tg(l-fabp:CFP-NTR)*^{s891} embryos were exposed to 1-nitropyrene at the same concentration range and time points and evaluated at 120 hpf. The observed toxicity profile was similar to that of the wild-type embryos, indicating that metabolism of 1-nitropyrene was not occurring. To confirm that this line's toxicity profiles can be compared to the wild-type animals, *Tg(l-fabp:CFP-NTR)*^{s891} embryos were also exposed to 1-aminopyrene from 6 to 120 hpf. A similar profile to the wild-type embryo response was observed. Likewise, wild-type and *Tg(l-fabp:CFP-NTR)*^{s891} embryos shared similar developmental toxicity profiles in

response to 9-nitrophenanthrene, which was distinct from the toxicity observed for 9-aminophenanthrene in the wild-type (Figure 4.4c).

Co-exposure experiments

Structures of all chemicals (retene, benzo[a]pyrene, acetaminophen, and flutamide) selected for co-exposure experiments are shown in Figure 4.5a. Exposure to DMSO alone did not result in toxicity to the zebrafish embryos, and the fluorescence in the liver was not impacted following exposure to 1% or 1.5 % DMSO (Figure 1.5b). The concentration of DMSO selected was based on compound solubility. Embryos exposed to MTZ at 48 hpf were phenotypically normal, except for a slight (not statistically significant) increase in jaw malformations and body truncation in some animals. Complete ablation of the liver was observed in all MTZ-treated animals. For compounds where hepatic metabolism is a detoxifying pathway, chemical exposure in the presence of MTZ would result in an increase in observed toxicity. Where hepatic metabolism is a toxicologically activating pathway, co-exposure with MTZ would result in a decrease in observed toxicity.

Retene is known to undergo hepatic metabolism, and therefore elimination of the liver would reduce this metabolic capacity. The toxicity profile of retene would be expected to reflect if hepatic metabolism is primarily a toxifying or detoxifying pathway. Embryos exposed to 10 µM retene were phenotypically normal in the presence or absence of MTZ. Fifty percent of embryos exposed to12 µM retene in the absence of MTZ were malformed, with the common malformations being axial deformities, edema, and craniofacial malformation, and the remaining embryos survived with no malformations. In the presence of MTZ, 80% of embryos died, with

the remaining embryos malformed. Embryos exposed to 14 µM retene without MTZ had almost complete incidence of malformations, with about 20% mortality. Embryos co-exposed with MTZ and 14 µM retene had 100% mortality (Figure 4.5c). Exposure to 16 or 18 µM retene, in the presence or absence of MTZ, resulted in complete mortality.

Thirty-five percent of embryos exposed to 50 or 65 µM B[a]P in the absence of MTZ were phenotypically normal, whereas co-exposure with MTZ increased the incidence of edemas and facial malformations, in particular at 65 µM benzo[a]pyrene (B[a]P), to 60% (Figure 4.5d). Embryos exposed to 100 µM B[a]P in the absence or presence of MTZ showed a 90% incidence of edemas and other malformations, although with greater severity in the presence of MTZ. Significant fluorescence from B[a]P particles in the media and in the yolk sac was also visible, although clearly distinguishable from liver fluorescence.

None of the embryos exposed to 0.125 mM acetaminophen showed any morphological toxicity, either in the presence or absence of MTZ (Figure 4.5e). For increasing concentrations of acetaminophen, a positive correlative relationship was observed with increasing malformation incidence, in the presence and absence of MTZ. Embryos exposed to 2.5 mM acetaminophen without MTZ displayed a 40% incidence of malformations (primarily pericardial edema and craniofacial deformities) and partial hepatic ablation. In the presence of MTZ, the malformation incidence was approximately 65%. Exposure to 5 mM acetaminophen in the absence of MTZ resulted in 70% malformation incidence, as well as partial hepatic ablation. MTZ co-exposure with 5 mM acetaminophen resulted in 100% incidence of malformations. Exposure to 10 mM acetaminophen, with or without MTZ co-exposure, resulted in approximately 80% malformation

incidence, with hepatic ablation also observed in the non-MTZ treated embryos. Treatment with 25 mM acetaminophen, in the presence or absence of MTZ, resulted in complete mortality. Embryos exposed to 5 or 7 µM flutamide were phenotypically normal, with most showing some degree of hepatocyte ablation. (Figure 4.5f). Embryos exposed to MTZ and 5 or 7 µM flutamide showed a similar pattern of malformations and edemas as non-MTZ exposed embryos. Embryos exposed to 10 µM flutamide had a toxicity profile similar to the lower flutamide exposure concentrations. However, embryos exposed to MTZ and 10 µM flutamide experienced 100 percent mortality, as did embryos exposed to 12 µM flutamide, with or without MTZ co-exposure. Wild-type embryos exposed to 5 µM flutamide were phenotypically normal in the absence of MTZ, with a 50% incidence of edemas and craniofacial malformations in the presence of MTZ (Figure 4.5g). Exposure to 7 µM flutamide also resulted in approximately 50% incidence of malformations, and co-treatment with MTZ resulted in complete mortality. Exposure to 10 or 12 µM flutamide, in the presence or absence of MTZ, resulted in 100% mortality.

Discussion

Zebrafish are a powerful model for vertebrate development that can be made more versatile through the use of transgenic lines, in particular when the metabolic capability of zebrafish is made to more closely resemble humans or other model systems. Transgenic lines developed for one purpose can also be useful for others. Tissue ablation has been used to study zebrafish tissue regeneration in a range of organs (Choi et al., 2014; Curado et al., 2007; He et al., 2014; White and Mumm, 2013; White et al., 2011). Using the nitroreductase system for tissue ablation suggests there could be additional uses for the added nitroreductase metabolic capacity. Other

potential uses of these types of transgenic lines include toxicity testing of nitrated environmental contaminants, such as nitro-PAHs, since the addition of nitroreductive capability in the liver specifically increases the similarities between the zebrafish and human metabolic pathways. The ability to completely ablate the liver, and all associated metabolism, would be a powerful tool to interrogate compounds with toxicity mechanisms dependent on their hepatic metabolism or toxicity. These compounds include pharmaceuticals and environmental contaminants, in particular those containing a nitro functional group. This model could be useful for a relatively high-throughput screen investigating the role of nitroreductase or hepatic metabolism in toxicity. In the initial published methods using the *Tg(l-fabp:CFP-NTR)*^{s891} transgenic line (Curado et al., 2008), heterozygous adult animals were incrossed to yield a mixture of genotypes within the offspring. Embryos were visually screened for fluorescence at 96 hpf, immediately prior to exposure with MTZ. Our work confirms that homozygous positive (embryos with two copies of the nitroreductase gene) and heterozygous embryos have the same visual tissue ablation patterns in response to MTZ exposure. We also demonstrated that exposure to MTZ during development of the liver, and before 96 hpf, will result in complete ablation of the liver. This allows for expanded use of the hepatic ablation capabilities of this line during development, such as developmental toxicity testing at earlier stages of development.

Use of a lower MTZ concentration was also effective in causing tissue ablation, which is advantageous as it both reduces the amount of chemical used, and reduces the off-target effects that result from exposure to MTZ. MTZ is generally considered to be non-toxic, with no evidence for developmental toxicity (Roe, 1983), although genotoxicity and neurotoxicity have been reported (Khalil et al., 2007; Kuriyama et al., 2011). The ability to expose embryos to lower

concentrations of MTZ, and at earlier stages of development, expands the possibilities of this model for use in other developmental biology and toxicology studies, in particular at early developmental time points, as well as during normal hepatic development.

The time course of liver ablation following MTZ exposure also showed that visual ablation occurred within 8-12 hours, in contrast to the 24 hours in the previous tissue ablation protocol (Curado et al., 2008, 2007). Visual regeneration of the liver occurred starting at 10-12 hours and was more substantial by 24 hours. This is consistent with other studies on liver regeneration after damage, where the hepatocytes were the first cell population to recover following hepatectomy (Michalopoulos and DeFrances, 1997), although complete recovery of hepatic tissue took several days (He et al., 2014). This expands the potential use of this transgenic line for the investigation of liver recovery and the impacts on recovery which could result from exposure to chemicals or other stressors.

We also attempted to use the ROS-ID® Hypoxia/Oxidative Stress Detection Kit to further characterize the nitroreductase activity in the transgenic and wild-type zebrafish. The kit failed to detect nitroreductase activity in the liver of the transgenic embryos (Appendix C.1). This kit was developed for use in cell culture, and had not been previously published for use in zebrafish or any similar whole-animal systems. The results from this kit was not consistent with the other assays, suggesting that this kit, as provided, may not be suitable for use in zebrafish.

Use of the wild-type 5D line to characterize the toxicity of the nitro-compounds of interest prior to use in the *Tg(l-fabp:CFP-NTR)*^{s891} line provided a background toxicity against which to measure later exposures using the *Tg(l-fabp:CFP-NTR)*^{s891} zebrafish line. The nitro-PAHs studied here had a developmental toxicity profile which was consistent across the range of time

points tested (Chlebowski et al., 2017), allowing for exposures later in development with the *Tg(l-fabp:CFP-NTR)*^{s891} line. While not substantially impacting the overall toxicity profile, the presence of the chorion for the transgenic line exposures could explain the slightly lower incidence of toxicity in the *Tg(l-fabp:CFP-NTR)*^{s891} embryos compared to the dechorionated wild-type exposure (Henn and Braunbeck, 2011).

Had the *Tg(l-fabp:CFP-NTR)*^{s891} fish significantly reduced the nitro-PAHs to the amino-PAHs (or a partially-reduced intermediate), the toxicity profile would have been expected to shift, to be more similar to the amino-PAH. The lack of observable changes in toxicity could be explained by a lack of nitroreduction, potentially due to a low affinity of the nitroreductase enzyme for the nitro-PAHs. It is also possible that other metabolic pathways, such as metabolism by the cytochrome P450s, compete with the nitroreduction pathway, resulting in less substrate available for nitroreduction (Iwanari et al., 2002; Jung et al., 2001). Nitro-PAHs are known to undergo nitroreduction in bacterial systems, and development of a transgenic line similar to *Tg(l-fabp:CFP-NTR)*^{s891} line, but with a different nitroreductase enzyme, would allow for further investigations into the metabolism of nitro-PAHs.

The liver is an important component of metabolism and toxicity for many compounds, and the ability to study toxicity, both in the presence and absence of the liver, would be a useful tool for toxicity testing. The ability to expose a whole-animal system, in the presence and absence of functioning hepatic tissue, can provide powerful insight into the mechanism of toxicity for a variety of compounds. Exposures to the environmental contaminant retene had increased incidence and severity of malformation in the absence of a functioning liver, indicating that hepatic metabolism is a likely a detoxifying pathway for retene. Previous work has implicated

hepatic metabolism, particularly by CYP 450s, as an important component of retene toxicity (Billiard et al., 1999; Fragoso et al., 1999). Exposure to concentrations of retene greater than 14 μM, in the presence or absence of hepatic tissue, resulted in 100% mortality. This suggests other mechanisms of toxicity as well, potentially including CYP 450 metabolism in extrahepatic tissues (Andreasen et al., 2002). B[a]P also had increased developmental toxicity in the absence of the liver, indicating that hepatic metabolism is also a detoxification pathway with regard to the developmental toxicity of this compound. Hepatic metabolism of B[a]P by CYP 450 enzymes is known to generate powerful mutagenic and carcinogenic metabolites (Baird et al., 2005; Shimada and Fujii-Kuriyama, 2004), but the pathway for developmental toxicity is less clear. AHR2 has been implicated as essential for B[a]P induced behavioral endpoints (Knecht et al., 2017). However, AHR2 is primarily located in tissues other than the liver (Goodale et al., 2012), so it would not be expected to change significantly as a result of hepatic ablation. The increased toxicity in the absence of the liver indicates that hepatic metabolism is responsible for detoxification with regards to developmental toxicity, in contrast to the mutagenic activation pathways typically noted for B[a]P.

Flutamide, an anti-androgenic compound used for the treatment of prostate cancer, has been known to cause hepatic injury or necrosis for some human patients (Gomez et al., 1992; Wysowski and Fourcroy, 1996), and was developmentally toxic with and without hepatic ablation in zebrafish. Flutamide exposure alone caused hepatocyte ablation, indicating hepatotoxicity consistent with previous medical reports (Wysowski and Fourcroy, 1996). Flutamide caused effects other than hepatoxicity, including pericardial edema and craniofacial malformations, and is known to undergo metabolism by several subfamilies of CYP 450

enzymes in humans and other model organisms (Berson et al., 1993; Kang et al., 2008; Shet et al., 1997; Tevell et al., 2006). While nitroreduction of the nitro group is possible (Boelsterli et al., 2006), the primary mechanism of metabolism is believed to be through CYP 450 oxidation. Nitroreduction does not appear to be a primary mechanism of hepatotoxicity, because the profile of malformations in the hepatocyte-ablated and hepatocyte-present embryos was similar. To further investigate the role of nitroreductase in developmental toxicity, wild-type embryos were exposed to MTZ and flutamide at the same concentrations as the *Tg(l-fabp:CFP-NTR)*^{s891} embryos. The greater observed toxicity at lower concentrations of flutamide and in the presence of MTZ also indicates that hepatic metabolism is again important in mediating the toxicity of flutamide. As wild-type embryos are not capable of reducing the nitro group, the nitroreduction pathway does not appear to be a driver for toxicity, and instead may play a protective and detoxifying role in the metabolic pathways of flutamide.

In contrast to flutamide, the structurally similar acetaminophen, a known hepatotoxin (Mitchell et al., 1973), required exposure at concentrations orders of magnitude higher than the other compounds tested to cause toxicity (North et al., 2010; Selderslaghs et al., 2012; Weigt et al., 2010). Acetaminophen exposure results in hepatocellular necrosis, caused by metabolites of acetaminophen (Black, 1984). Acetaminophen hepatotoxicity has been previously established to be dependent on oxidative metabolism, by hepatic CYP 450 enzymes, into the reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI). Mice lacking these CYP 450 isoforms were protected against APAP-induced hepatotoxicity (Zaher et al., 1998). Multiple conjugate metabolites are formed as well, although these are relatively non-toxic and readily excreted (Graham et al., 2013). Liver ablation was observed following exposure to acetaminophen

without MTZ, demonstrating the hepatotoxicity of acetaminophen. Toxicity following exposure to acetaminophen in the absence of a liver indicates that acetaminophen can be toxic to other tissues, and that hepatic metabolism is not required for toxicity to occur.

For all four compounds tested, exposure following ablation of the liver increased the observed toxicity. This demonstrates the importance of the liver in metabolic and detoxifying pathways in zebrafish. The ability to selectively ablate hepatic tissue allows for investigation of the role of hepatic metabolism in the toxicity of a compound of interest. The presence of nitroreductase in this zebrafish line increases the relevance of hepatic metabolism to human health and metabolism.

Conclusions

As intended and previously described, treatment with MTZ results in ablation of the hepatic tissue of *Tg(l-fabp:CFP-NTR)*^{s891} zebrafish. We demonstrated that the published methods could be expanded upon, allowing for additional uses of the *Tg(l-fabp:CFP-NTR)*^{s891}, and similar transgenic zebrafish lines, in chemical screening assays. This transgenic line does not appear to be useful in the investigation of nitroreduction as a mechanism for nitro-PAH toxicity, potentially due to the binding affinity of the nitroreductase enzyme used in the development of this line. Development of a similar transgenic line, where the nitroreductase has a higher binding affinity for nitro-PAHs, would be a useful model system in the investigation of the toxicity and metabolism of nitro-PAHs and other nitroaromatic compounds, as well as expanded use in pharmaceutical development and testing. We also demonstrated novel uses for this transgenic zebrafish line, including toxicity testing in the absence of hepatic metabolism which could be used in a high-throughput manner. The ability to determine the role of hepatic metabolism in

compound toxicity is a powerful tool for further mechanistic investigations, in particular for determining the role of the liver in toxicity.

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References

Andreasen, E.A., Spitsbergen, J.M., Tanguay, R.L., Stegeman, J.J., Heideman, W., Peterson, R.E., 2002. Tissue-Specific Expression of AHR2, ARNT2, and CYP1A in Zebrafish Embryos and Larvae: Effects of Developmental Stage and 2,3,7,8-Tetrachlorodibenzo-p-dioxin Exposure. Toxicol. Sci. 68, 403–419. doi:10.1093/toxsci/68.2.403

Baird, W.M., Hooven, L.A., Mahadevan, B., 2005. Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. Environ. Mol. Mutagen. 45, 106–114. doi:10.1002/em.20095

Ball, L.M., Kohan, M.J., Claxton, L.D., Lewtas, J., 1984. Mutagenicity of derivatives and metabolites of 1-nitropyrene: activation by rat liver S9 and bacterial enzymes. Mutat. Res. Toxicol. 138, 113–125. doi:10.1016/0165-1218(84)90033-8

Berson, A., Wolf, C., Chachaty, C., Fisch, C., Fau, D., Eugene, D., Loeper, J., Gauthier, J.C., Beaune, P., Pompon, D., 1993. Metabolic activation of the nitroaromatic antiandrogen flutamide by rat and human cytochromes P-450, including forms belonging to the 3A and 1A subfamilies. J. Pharmacol. Exp. Ther. 265, 366–372.

Billiard, S.M., Querbach, K., Hodson, P.V., 1999. Toxicity of retene to early life stages of two freshwater fish species. Environ. Toxicol. Chem. 18, 2070–2077. doi:10.1002/etc.5620180927

Black, M., 1984. Acetaminophen hepatotoxicity. Annu. Rev. Med. 35, 577–593.

Boelsterli, U.A., Ho, H.K., Zhou, S., Yeow Leow, K., 2006. Bioactivation and Hepatotoxicity of Nitroaromatic Drugs [WWW Document]. (accessed 2.9.17).

Bridgewater, J.A., Springer, C.J., Knox, R.J., Minton, N.P., Michael, N.P., Collins, M.K., 1995. Expression of the bacterial nitroreductase enzyme in mammalian cells renders them selectively sensitive to killing by the prodrug CB1954. Eur. J. Cancer 31, 2362–2370. doi:10.1016/0959-8049(95)00436-X

Bugel, S.M., Tanguay, R.L., Planchart, A., 2014. Zebrafish: A Marvel of High-Throughput Biology for 21st Century Toxicology. Curr. Environ. Health Rep. 1, 341–352. doi:10.1007/s40572-014-0029-5

Chlebowski, A.C., Garcia, G.R., Du, L., K, J., Bisson, W.H., Truong, L., Simonich, M., L, S., Tanguay, R.L., 2017. Mechanistic investigations into the developmental toxicity of nitrated and heterocyclic PAHs. Toxicol. Sci. doi:10.1093/toxsci/kfx035

Choi, T., Ninov, N., Stainier, D.Y.R., Shin, D., 2014. Extensive Conversion of Hepatic Biliary Epithelial Cells to Hepatocytes After Near Total Loss of Hepatocytes in Zebrafish. Gastroenterology 146, 776–788. doi:10.1053/j.gastro.2013.10.019

Curado, S., Anderson, R.M., Jungblut, B., Mumm, J., Schroeter, E., Stainier, D.Y., 2007. Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies. Dev. Dyn. 236, 1025–1035.

Curado, S., Stainier, D.Y., Anderson, R.M., 2008. Nitroreductase-mediated cell/tissue ablation in zebrafish: a spatially and temporally controlled ablation method with applications in developmental and regeneration studies. Nat. Protoc. 3, 948–954.

Fragoso, N.M., Hodson, P.V., Kozin, I.S., Brown, R.S., Parrott, J.L., 1999. Kinetics of mixed function oxygenase induction and retene excretion in retene—exposed rainbow trout (Oncorhynchus mykiss). Environ. Toxicol. Chem. 18, 2268–2274. doi:10.1002/etc.5620181022

Fu, P.P., 1990. Metabolism of nitro-polycyclic aromatic hydrocarbons. Drug Metab. Rev. 22, 209–268.

Fu, P.P., Cerniglia, C.E., Richardson, K.E., Heflich, R.H., 1988. Nitroreduction of 6-nitrobenzo [a] pyrene: a potential activation pathway in humans. Mutat. Res. Lett. 209, 123–129.

Garcia, G.R., Noyes, P.D., Tanguay, R.L., 2016. Advancements in zebrafish applications for 21st century toxicology. Pharmacol. Ther. 161, 11–21. doi:10.1016/j.pharmthera.2016.03.009

Gomez, J.-L., Dupont, A., Cusan, L., Tremblay, M., Suburu, R., Lemay, M., Labrie, F., 1992. Incidence of liver toxicity associated with the use of flutamide in prostate cancer patients. Am. J. Med. 92, 465–470. doi:10.1016/0002-9343(92)90741-S

Goodale, B.C., Du, J.K.L., Bisson, W.H., Janszen, D.B., Waters, K.M., Tanguay, R.L., 2012. AHR2 Mutant Reveals Functional Diversity of Aryl Hydrocarbon Receptors in Zebrafish. PLOS ONE 7, e29346. doi:10.1371/journal.pone.0029346

Graham, G.G., Davies, M.J., Day, R.O., Mohamudally, A., Scott, K.F., 2013. The modern pharmacology of paracetamol: therapeutic actions, mechanism of action, metabolism, toxicity and recent pharmacological findings. Inflammopharmacology 21, 201–232. doi:10.1007/s10787-013-0172-x

Hawliczek, A., Nota, B., Cenijn, P., Kamstra, J., Pieterse, B., Winter, R., Winkens, K., Hollert, H., Segner, H., Legler, J., 2012. Developmental toxicity and endocrine disrupting potency of 4-azapyrene, benzo[b]fluorene and retene in the zebrafish Danio rerio. Reprod. Toxicol., Zebrafish Teratogenesis 33, 213–223. doi:10.1016/j.reprotox.2011.11.001

He, J., Lu, H., Zou, Q., Luo, L., 2014. Regeneration of Liver After Extreme Hepatocyte Loss Occurs Mainly via Biliary Transdifferentiation in Zebrafish. Gastroenterology 146, 789–800.e8. doi:10.1053/j.gastro.2013.11.045

Henn, K., Braunbeck, T., 2011. Dechorionation as a tool to improve the fish embryo toxicity test (FET) with the zebrafish (Danio rerio). Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 153, 91–98. doi:10.1016/j.cbpc.2010.09.003

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S., McLaren, K., Matthews, L., others, 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498–503.

Hsu, C.-C., Hou, M.-F., Hong, J.-R., Wu, J.-L., Her, G.M., 2009. Inducible Male Infertility by Targeted Cell Ablation in Zebrafish Testis. Mar. Biotechnol. 12, 466–478. doi:10.1007/s10126-009-9248-4

Incardona, J.P., Linbo, T.L., Scholz, N.L., 2011. Cardiac toxicity of 5-ring polycyclic aromatic hydrocarbons is differentially dependent on the aryl hydrocarbon receptor 2 isoform during zebrafish development. Toxicol. Appl. Pharmacol. 257, 242–249. doi:10.1016/j.taap.2011.09.010

Iwanari, M., Nakajima, M., Kizu, R., Hayakawa, K., Yokoi, T., 2002. Induction of CYP1A1, CYP1A2, and CYP1B1 mRNAs by nitropolycyclic aromatic hydrocarbons in various human tissue-derived cells: chemical-, cytochrome P450 isoform-, and cell-specific differences. Arch. Toxicol. 76, 287–298. doi:10.1007/s00204-002-0340-z

Jung, D.K.J., Klaus, T., Fent, K., 2001. Cytochrome P450 induction by nitrated polycyclic aromatic hydrocarbons, azaarenes, and binary mixtures in fish hepatoma cell line PLHC-1. Environ. Toxicol. Chem. 20, 149–159. doi:10.1002/etc.5620200117

Kang, P., Dalvie, D., Smith, E., Zhou, S., Deese, A., Nieman, J.A., 2008. Bioactivation of Flutamide Metabolites by Human Liver Microsomes. Drug Metab. Dispos. 36, 1425–1437. doi:10.1124/dmd.108.020370

Khalil, W.K.B., Mahmoud, M.A., Zahran, M.M., Mahrous, K.F., 2007. A sub-acute study of metronidazole toxicity assessed in Egyptian Tilapia zillii. J. Appl. Toxicol. 27, 380–390. doi:10.1002/jat.1217

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., others, 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310.

Knecht, A.L., Goodale, B.C., Truong, L., Simonich, M.T., Swanson, A.J., Matzke, M.M., Anderson, K.A., Waters, K.M., Tanguay, R.L., 2013. Comparative developmental toxicity of environmentally relevant oxygenated PAHs. Toxicol. Appl. Pharmacol. 271, 266–275.

Knecht, A.L., Truong, L., Simonich, M.T., Tanguay, R.L., 2017. Developmental benzo[a]pyrene (B[a]P) exposure impacts larval behavior and impairs adult learning in zebrafish. Neurotoxicol. Teratol. 59, 27–34. doi:10.1016/j.ntt.2016.10.006

Kuriyama, A., Jackson, J.L., Doi, A., Kamiya, T., 2011. Metronidazole-induced central nervous system toxicity: a systematic review. Clin. Neuropharmacol. 34, 241–247.

Lewandoski, M., 2001. Conditional control of gene expression in the mouse. Nat. Rev. Genet. 2, 743–755. doi:10.1038/35093537

Li, Z., He, X., Wang, Z., Yang, R., Shi, W., Ma, H., 2015. in vivo imaging and detection of nitroreductase in zebrafish by a new near-infrared fluorescence off–on probe. Biosens. Bioelectron. 63, 112–116. doi:10.1016/j.bios.2014.07.024

Mandrell, D., Truong, L., Jephson, C., Sarker, M.R., Moore, A., Lang, C., Simonich, M.T., Tanguay, R.L., 2012. Automated Zebrafish Chorion Removal and Single Embryo Placement Optimizing Throughput of Zebrafish Developmental Toxicity Screens. J. Lab. Autom. 17, 66–74.

Mathias, J.R., Zhang, Z., Saxena, M.T., Mumm, J.S., 2014. Enhanced Cell-Specific Ablation in Zebrafish Using a Triple Mutant of Escherichia Coli Nitroreductase. Zebrafish 11, 85–97. doi:10.1089/zeb.2013.0937

McGuire, S.E., Roman, G., Davis, R.L., 2004. Gene expression systems in Drosophila: a synthesis of time and space. Trends Genet. 20, 384–391. doi:10.1016/j.tig.2004.06.012

Michalopoulos, G.K., DeFrances, M.C., 1997. Liver Regeneration. Science 276, 60–66. doi:10.1126/science.276.5309.60

Mitchell, J.R., Jollow, D.J., Potter, W.Z., Davis, D.C., Gillette, J.R., Brodie, B.B., 1973. Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. J. Pharmacol. Exp. Ther. 187, 185–194.

Möller, L., 1994. In vivo metabolism and genotoxic effects of nitrated polycyclic aromatic hydrocarbons. Environ. Health Perspect. 102, 139–146.

North, T.E., Babu, I.R., Vedder, L.M., Lord, A.M., Wishnok, J.S., Tannenbaum, S.R., Zon, L.I., Goessling, W., 2010. PGE2-regulated wnt signaling and N-acetylcysteine are synergistically hepatoprotective in zebrafish acetaminophen injury. Proc. Natl. Acad. Sci. 107, 17315–17320. doi:10.1073/pnas.1008209107

Ober, E.A., Field, H.A., Stainier, D.Y., 2003. From endoderm formation to liver and pancreas development in zebrafish. Mech. Dev. 120, 5–18.

Pisharath, H., Rhee, J.M., Swanson, M.A., Leach, S.D., Parsons, M.J., 2007. Targeted ablation of beta cells in the embryonic zebrafish pancreas using E. coli nitroreductase. Mech. Dev. 124, 218–229. doi:10.1016/j.mod.2006.11.005

Reimers, M.J., La Du, J.K., Periera, C.B., Giovanini, J., Tanguay, R.L., 2006. Ethanol-dependent toxicity in zebrafish is partially attenuated by antioxidants. Neurotoxicol. Teratol. 28, 497–508.

Roe, F.J., 1983. Toxicologic evaluation of metronidazole with particular reference to carcinogenic, mutagenic, and teratogenic potential. Surgery 93, 158–164.

Salem, Z., Murray, T., Yunis, A., 1981. The nitroreduction of chloramphenicol by human liver tissue. J. Lab. Clin. Med. 97, 881–886.

Scott, J.A., Incardona, J.P., Pelkki, K., Shepardson, S., Hodson, P.V., 2011. AhR2-mediated, CYP1A-independent cardiovascular toxicity in zebrafish (Danio rerio) embryos exposed to retene. Aquat. Toxicol. 101, 165–174. doi:10.1016/j.aquatox.2010.09.016

Selderslaghs, I.W.T., Blust, R., Witters, H.E., 2012. Feasibility study of the zebrafish assay as an alternative method to screen for developmental toxicity and embryotoxicity using a training set of 27 compounds. Reprod. Toxicol., Zebrafish Teratogenesis 33, 142–154. doi:10.1016/j.reprotox.2011.08.003

Shet, M.S., McPhaul, M., Fisher, C.W., Stallings, N.R., Estabrook, R.W., 1997. Metabolism of the Antiandrogenic Drug (Flutamide) by Human CYP1A2. Drug Metab. Dispos. 25, 1298–1303.

Shimada, T., Fujii-Kuriyama, Y., 2004. Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and1B1. Cancer Sci. 95, 1–6. doi:10.1111/j.1349-7006.2004.tb03162.x

Tevell, A., Lennernäs, H., Jönsson, M., Norlin, M., Lennernäs, B., Bondesson, U., Hedeland, M., 2006. Flutamide Metabolism in Four Different Species in Vitro and Identification of Flutamide Metabolites in Human Patient Urine by High Performance Liquid Chromatography/Tandem Mass Spectrometry. Drug Metab. Dispos. 34, 984–992. doi:10.1124/dmd.105.008516

Truong, L., Bugel, S.M., Chlebowski, A., Usenko, C.Y., Simonich, M.T., Simonich, S.L.M., Tanguay, R.L., 2016. Optimizing multi-dimensional high throughput screening using zebrafish. Reprod. Toxicol. 65, 139–147. doi:10.1016/j.reprotox.2016.05.015

Truong, L., Harper, S.L., Tanguay, R.L., 2011. Evaluation of embryotoxicity using the zebrafish model. Methods Mol. Biol. Clifton NJ 691, 271–279. doi:10.1007/978-1-60761-849-2_16

Truong, L., Reif, D.M., St Mary, L., Geier, M.C., Truong, H.D., Tanguay, R.L., 2014. Multidimensional in vivo hazard assessment using zebrafish. Toxicol. Sci. 137, 212–233.

Weigt, S., Huebler, N., Braunbeck, T., von Landenberg, F., Broschard, T.H., 2010. Zebrafish teratogenicity test with metabolic activation (mDarT): Effects of phase I activation of acetaminophen on zebrafish Danio rerio embryos. Toxicology 275, 36–49. doi:10.1016/j.tox.2010.05.012

White, D.T., Mumm, J.S., 2013. The nitroreductase system of inducible targeted ablation facilitates cell-specific regenerative studies in zebrafish. Methods, Zebrafish Methods 62, 232–240. doi:10.1016/j.ymeth.2013.03.017

White, Y.A.R., Woods, D.C., Wood, A.W., 2011. A transgenic zebrafish model of targeted oocyte ablation and de novo oogenesis. Dev. Dyn. 240, 1929–1937. doi:10.1002/dvdy.22695

Wysowski, D.K., Fourcroy, J.L., 1996. Flutamide Hepatotoxicity. J. Urol. 155, 209–212. doi:10.1016/S0022-5347(01)66596-0

Zaher, H., Buters, J.T.M., Ward, J.M., Bruno, M.K., Lucas, A.M., Stern, S.T., Cohen, S.D., Gonzalez, F.J., 1998. Protection against Acetaminophen Toxicity in CYP1A2 and CYP2E1 Double-Null Mice. Toxicol. Appl. Pharmacol. 152, 193–199. doi:10.1006/taap.1998.8501

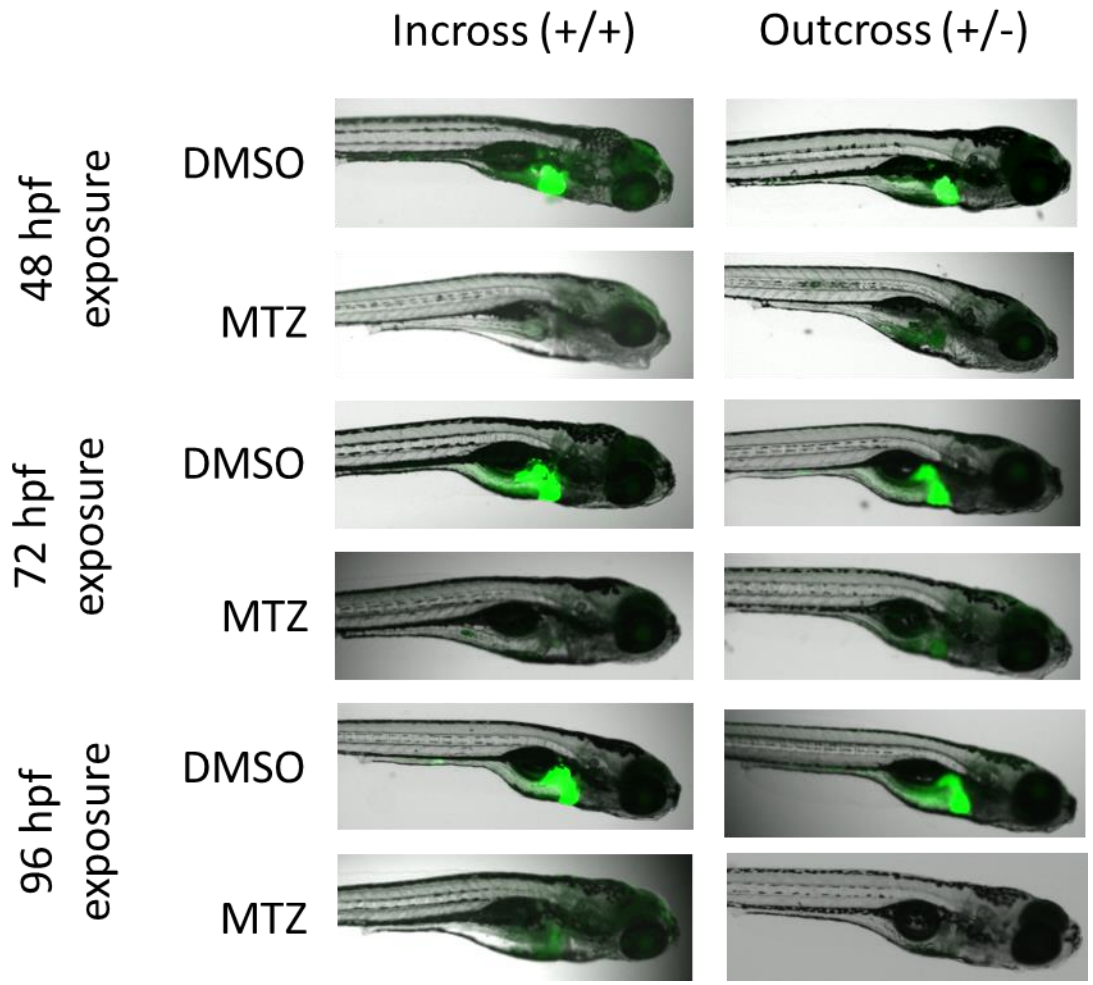

Figure 4.1. Incross and outcross transgenic embryos. Comparison of incrossed (homozygous positive) and outcrossed (heterozygous) *Tg(l-fabp:CFP-NTR)*^{s891} zebrafish. Embryos were exposed to either DMSO or 10 mM metronidazole (MTZ) at 48, 72, or 96 hpf until imaging at 120 hpf. Presence of hepatocytes is indicted by green fluorescence.

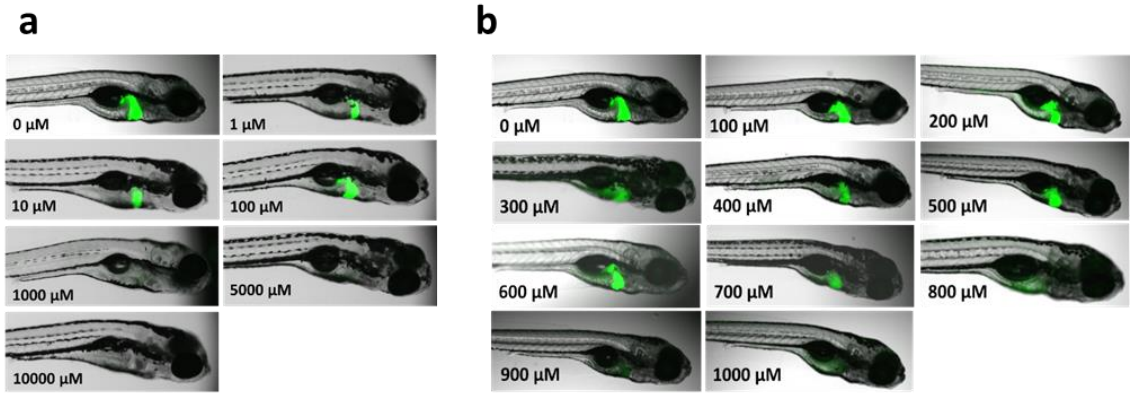

Figure 4.2. MTZ dose-response. Dose-response of *Tg(l-fabp:CFP-NTR)*^{s891} zebrafish embryos exposed to metronidazole (MTZ) at 96 hpf, and imaged at 120 hpf, at a broad (a) and refined (b) range of MTZ concentrations. Ablation of hepatocytes was assessed based on visible green fluorescent signal, where a decreasing fluorescent signal indicates hepatocyte ablation.

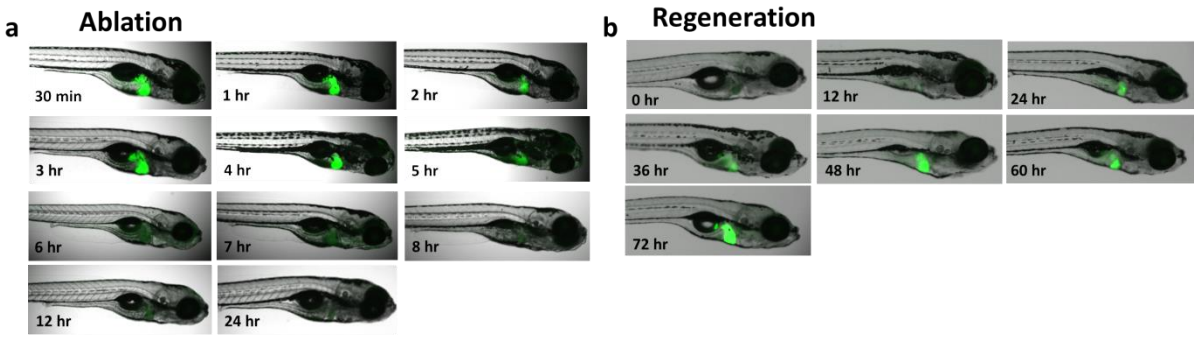

Figure 4.3. Liver ablation and regeneration time courses. Time course of tissue ablation (a) and regeneration (b) using 10 mM metronidazole (MTZ) in the hepatocytes of *Tg(l-fabp:CFP-NTR)*^{s891} zebrafish. For the ablation study, embryos were dosed at 96 hpf with 10 mM MTZ, and ablation was evaluated by visible fluorescence, following continuous exposure until the indicated time point. For the regeneration study, embryos were exposed to 10 mM MTZ from 96-120 hpf, then rinsed and moved to clean media, with imaging at the indicated time points after MTZ removal. The presence of hepatocytes is indicated by green fluorescence.

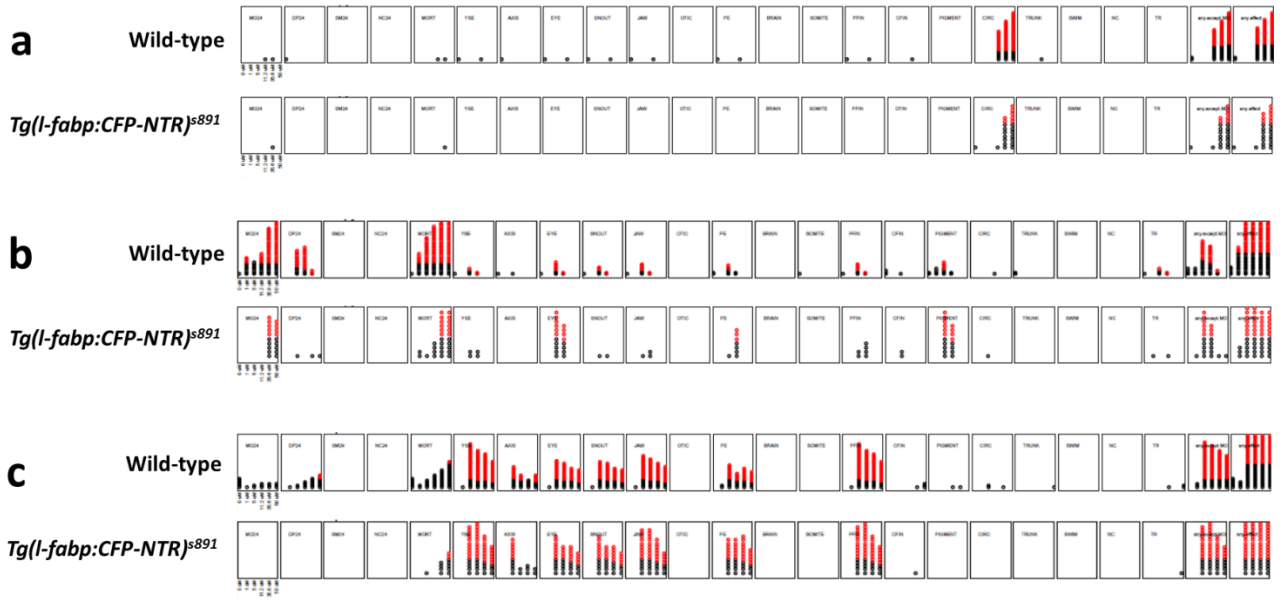

Figure 4.4. Wild-type and transgenic windows of exposure. Windows of exposure in wild-type and *Tg(l-fabp:CFP-NTR)*^{s891} embryos exposed to 1-nitropyrene (a), 1-aminopyrene (b), and 9-nitrophenanthrene (c). Embryos were exposed at 6 hpf with evaluations at 120 hpf. Bar height indicates incidence of each individual endpoint, where red dots indicate statistical significance. Toxicity profiles for exposures at other time points are shown in Appendix C.2.

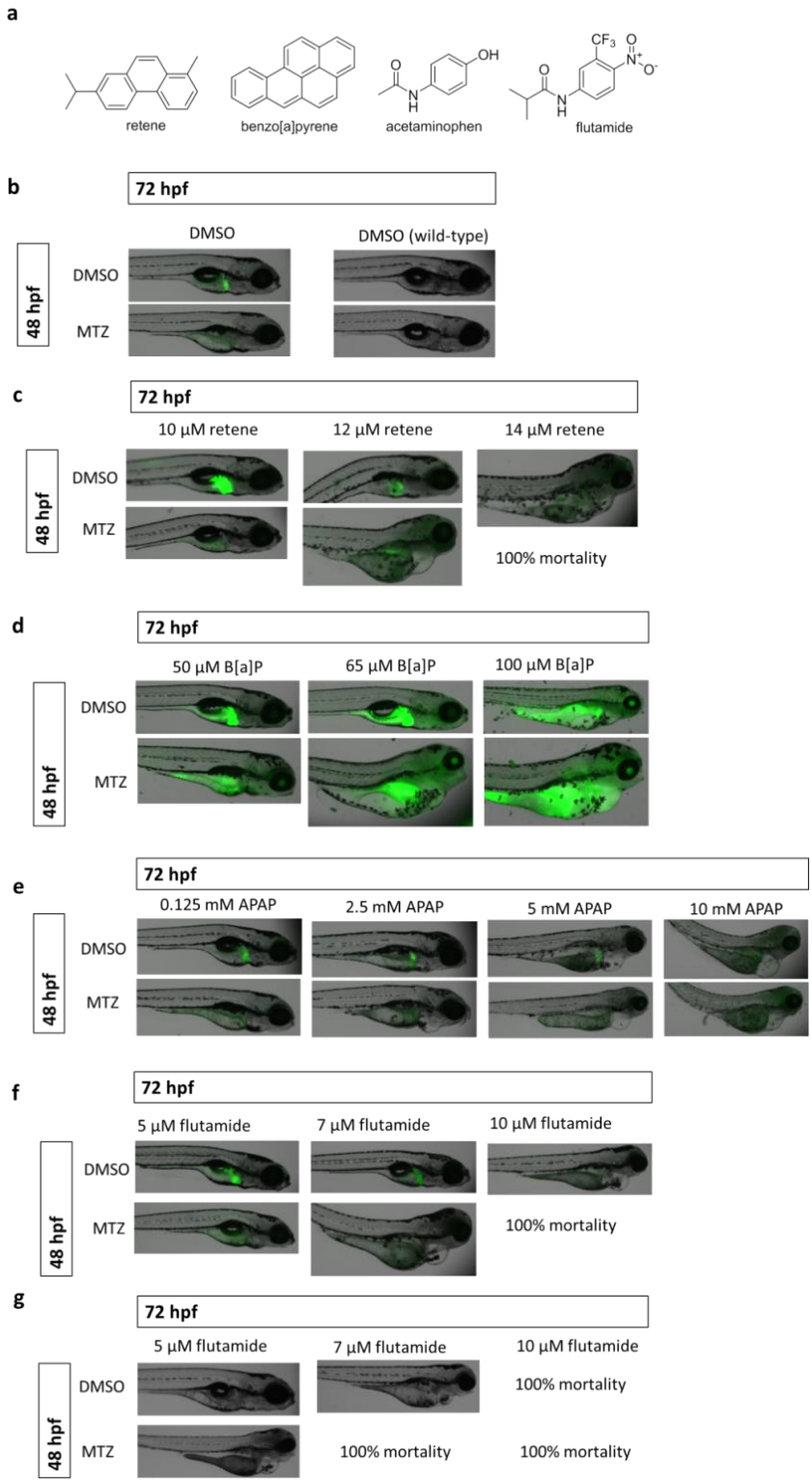
a
retene
benzo[a]pyrene
acetaminophen
flutamide
b
72 hpf
DMSO
DMSO (wild-type)
48 hpf
DMSO
MTZ
c
72 hpf
10 µM retene
12 µM retene
14 µM retene
48 hpf
DMSO
MTZ
100% mortality
d
72 hpf
50 µM B[a]P
65 µM B[a]P
100 µM B[a]P
48 hpf
DMSO
MTZ
e
72 hpf
0.125 mM APAP
2.5 mM APAP
5 mM APAP
10 mM APAP
48 hpf
DMSO
MTZ
f
72 hpf
5 µM flutamide
7 µM flutamide
10 µM flutamide
48 hpf
DMSO
MTZ
100% mortality
g
72 hpf
5 µM flutamide
7 µM flutamide
10 µM flutamide
48 hpf
DMSO
MTZ
100% mortality
100% mortality
100% mortality

Figure 4.5. MTZ co-exposures. Co-exposures with 10 mM metronidazole (MTZ) in *Tg(l-fabp:CFP-NTR)*^{s891} and wild-type zebrafish embryos. Structures of all compounds tested are shown in (a). Embryos were exposed to MTZ at 48 hpf, and to 1% DMSO (1.5% DMSO-exposed animals were phenotypically indistinguishable) (b), retene (c), benzo[a]pyrene (B[a]P) (d), acetaminophen (APAP) (e), or flutamide (f) at 72 hpf. Additionally, wild-type embryos were exposed to flutamide (g). Imaging and evaluations were done for all animals at 120 hpf. Concentrations tested but not shown resulted in 100% mortality both in the presence and absence of MTZ.

Chapter 5 – Discussion and Conclusions

Unsubstituted polycyclic aromatic hydrocarbons (PAHs) are known environmental contaminants resulting from a range of natural and anthropogenic sources, in particular combustion and fossil fuel related processes (Howsam and Jones, 1998). Many PAHs are known mutagens, and several are listed as known or possible human carcinogens (WHO, 2015). Non-cancer endpoints of PAHs and PAH-containing environmental mixtures are also receiving increased attention and awareness, and include impacts on neurobehavior, cardiac development, and respiratory ailments (Cheng et al., 2013; Kim et al., 2011; Knecht et al., 2017).

PAHs are known to occur in the environment as components of complex mixtures, containing both PAHs and PAH derivatives. PAH derivatives have been documented pollutants in a range of environmental matrices alongside unsubstituted PAHs, although remain not as widely studied. Among the PAH derivative classes receiving increased attention are nitrated PAHs (NPAHs) and heterocyclic PAHs (HPAHs). Both NPAHs and HPAHs are known environmental contaminants, and the mechanism of formation of NPAHs in the atmosphere has been well-studied (Jariyasopit et al., 2014).

Despite increasing awareness for the environmental presence of PAH derivatives, they remain understudied with regards to potential health implications from exposure. Some NPAHs are known mutagens, and are classified as probable or possible human carcinogens (RI DEM, 2008; WHO, 2015). *In vitro* and *in vivo* data exists for a limited number of NPAHs, and utilizes an assortment of model systems and organisms. Testing a wide range of NPAHs using a consistent model system, preferably a high-throughput vertebrate model such as zebrafish (*Danio rerio*),

would yield useful and comparable data on the toxicity of this under-studied class of environmental contaminants.

The objective of the research presented here was to explore the use of the zebrafish model for investigating the developmental toxicity of NPAHs. The zebrafish model is useful for the capability to screen the developmental toxicity of chemicals in a high-throughput manner, as well as investigate potential mechanisms of action which are relevant to humans and other vertebrate models. Zebrafish are also amenable to genetic manipulation, making the development and utilization of transgenic lines highly feasible (Garcia et al., 2016).

One of the major challenges in studying PAHs and PAH derivatives is the limited aqueous solubility of many of these chemicals (Riedl and Altenburger, 2007). The hydrophobicity of PAHs is well-established, and leveraged for passive sampling and aqueous extraction techniques. The low water solubility of many PAHs and PAH derivatives presents a challenge for toxicity testing, in particular for systems such as cell culture or zebrafish, where plastic exposure vessels are commonly used. While sorption of PAHs to plastic or glass exposure containers had been previously documented, little had been done to increase the scope of this research beyond individual assay conditions. The concentration of chemical remaining in the exposure media to which the embryo was actually exposed remained unclear and undefined, leading to uncertainty in the interpretation of the resulting toxicity data.

In chapter 2, we investigated the sorptive properties of a set of PAHs and NPAHs to polystyrene plates during the standard high-throughput screening protocol utilized in the Tanguay lab at the time. Sorptive losses were measured directly, and compared to physical-chemical properties with the intent of developing a broadly-applicable predictive model. We demonstrated that for some

compounds, in particular NPAHs, the sorptive losses can be significant, in some cases upwards of 50%. Sorptive losses for NPAHs tended to be higher than for PAHs, suggesting a more complex mechanism of action than is expected based solely on hydrophobicity, potentially related to the charges in the nitro functional group. The sorptive losses observed for PAHs correlated well with the subcooled liquid solubility and molecular weight, whereas the sorption of NPAHs did not correlate well with any of the physical-chemical properties investigated. The modeling efforts with NPAHs were likely inhibited by the lack of experimentally-determined data available for the physical-chemical properties, which required us to rely on estimated values. Had experimentally determined properties been available for NPAHs, the modeling efforts may have been more successful. Properties such as the log K_{ow} are modeled based on chemical structure, resulting in identical values for structural isomers. However, it is known that isomers are not identical, in either physical-chemical behavior or bioactivities. While the modeled values can be a reasonable estimate for some purposes, for our modeling efforts with structurally similar compounds, more accurate, experimentally-determined values are necessary. The power of the high-throughput capability of the zebrafish model was explored in chapter 3, in which we screened a set of nitrated, aminated, and heterocyclic PAHs for developmental toxicity, revealing a range of bioactivities. While several compounds induced malformations and mortality at sub-micromolar exposure concentrations, more than half of the compounds tested resulted in no significant developmental toxicity for the range of concentrations tested. We also utilized a transgenic *cyp1a*/GFP reporter line to investigate induction of *cyp1a*, as an indicator of activation of the aryl hydrocarbon receptor (AHR) pathway. The AHR is a known mechanistic pathway for some PAHs, and our data indicate that the AHR is also a contributing mechanism

for some NPAHs and HPAHs, with distinct expression patterns observed in the liver, vasculature, yolk, skin, and neuromasts. To distinguish the roles of the three zebrafish AHR isoforms in these expression patterns, morpholino oligonucleotides were used to knock down each isoform independently. Consistent with previous data from investigation of oxygenated PAHs (Knecht et al., 2013), *cyp1a*/GFP expression in the liver was related to AHR1A, and expression in the vasculature, skin, and neuromasts was determined to be dependent almost completely on the AHR2, with a low level of expression caused by AHR1B as well. The final *cyp1a*/GFP expression pattern, the yolk sac, was not determined to be dependent on any of the AHR isoforms, and the cause of this fluorescence pattern remains unclear. The *in silico* docking studies to the active sites of the three zebrafish AHR isoforms were fairly successful, with a success rate of approximately 70%. A select set of NPAHs were selected for gene expression studies using qPCR, which yielded further information as to the diversity of potential contributing mechanisms of action. Genes significantly mis-regulated included not only several *cyp* isoforms, which were significantly induced, but also genes involved in cardiac function and the cellular response to oxidative stress, which is consistent with previously reported studies on other PAHs and PAH-containing mixtures (Knecht et al., 2013; McIntyre et al., 2016). While this is strongly suggestive of several contributing mechanisms for NPAHs, investigation of a larger number of compounds and genes would be necessary to more fully elucidate both these as well as other potential mechanisms of action.

The results from chapter 2 demonstrating significant sportive losses complicate the interpretation of the toxicity results from chapter 3. The toxicity data analysis, including the EC50 calculations, is based only on the nominal initial exposure concentrations. Without extensive analytical

measurements, determination of more accurate aqueous concentrations or the dose the embryos received is not possible. Re-determination of toxicity metrics taking into account the dose received by the embryos, or even just sorptive losses, would likely change the results. However, while the concentration or dose which elicits toxicity would shift, the overall result that NPAHs and HPAHs are bioactive, can be acutely toxic, and can induce gene expression changes would not change.

In chapter 4, we characterized and explored the use of a nitroreductase-expressing transgenic zebrafish line for investigating the role of reductive metabolism in NPAH developmental toxicity. Nitroreductases catalyze the reduction of nitro functional groups to amino functional groups, and nitroreduction has been implicated as contributing to the mutagenicity of NPAHs in bacterial and cell culture systems (Rosenkranz and Mermelstein, 1983). In contrast to other model organisms and to humans, zebrafish do not endogenously express nitroreductase. We also demonstrated in the previous chapter that the nitro and amino analogs of the same parent PAH have different developmental toxicity profiles, potentially due to the oxidation status of the nitro/amino functional group.

The genetic manipulability of zebrafish has been previously utilized for the development of transgenic lines which express nitroreductase under the control of a tissue-specific promoter, for use in tissue-specific cell ablation. A non-toxic pro-drug containing a nitro functional group (commonly metronidazole, MTZ) is administered, which upon reduction, becomes acutely cytotoxic and ablates only the tissue in which nitroreductase is expressed.

We sought to use the *Tg(l-fabp:CFP-NTR)*^{s891} transgenic zebrafish line, which expresses nitroreductase using a hepatocyte-specific promoter (Curado et al., 2008), as this most closely

mimics the tissue expression of nitroreductase in humans. The published ablation protocol began at 96 hours post fertilization (hpf), but we demonstrated that the liver can be ablated as it develops, starting at 48 hpf. Ablation was continuous as long as MTZ exposure continued, but upon MTZ removal ablation of the liver was visibly occurring within 12 hours. From the time of MTZ exposure, liver ablation was nearly complete within 12 hours. Unfortunately, exposure to NPAHs of the *Tg(l-fabp:CFP-NTR)*^{s891} embryos resulted in toxicity profiles resembling the NPAH in the wild-type zebrafish, rather than the toxicity profile of the corresponding amino-PAH, suggesting that reductive metabolism was not occurring. As the nitroreductase activity in this line has already been validated, the likely cause is that the concentration of NPAH was too low for effective metabolism by the nitroreductase. The concentration of MTZ required for complete ablation was over an order of magnitude greater than the concentration of NPAHs used, suggesting that the nitroreductase may not be very efficient, in particular for NPAHs.

We then utilized the hepatocyte ablation technique to investigate the role of hepatic metabolism for four compounds which are known hepatotoxins, or to undergo hepatic metabolism. In all cases, the observed toxicity increased in the absence of the liver, indicting the role of the liver for detoxification, or potentially the compounding effects of MTZ co-exposure. Compounds which were hepatotoxic could also be visually identified due to hepatic ablation, indicating loss of hepatic tissue. Compared to similar transgenic lines with only a fluorescent indicator of hepatic tissue, this *Tg(l-fabp:CFP-NTR)*^{s891} transgenic line is advantageous due to the presence of nitroreductase, although further uses would be possible if the affinity of the nitroreductase were greater.

This dissertation demonstrates the utility of zebrafish for the high-throughput developmental toxicity screening and mechanistic investigations of NPAHs. We also demonstrate and characterize challenges in using an aqueous system to study hydrophobic analytes, and the utility of transgenic zebrafish to expand the relevance of the zebrafish model to humans. While zebrafish remain a premiere model for vertebrate development, in particular in a high-throughput manner, numerous challenges still exist in using zebrafish for toxicity testing with human relevance, but when overcome, will continue to unleash the potential for this model system.

References

Cheng, S., Xia, Y., He, J., Liu, X., Chen, X., Ding, Y., Wang, Y., Peng, B., Tu, B., 2013. Neurotoxic effect of subacute benzo(a)pyrene exposure on gene and protein expression in Sprague-Dawley rats. Environ. Toxicol. Pharmacol. 36, 648–658. doi:10.1016/j.etap.2013.06.008

Curado, S., Stainier, D.Y., Anderson, R.M., 2008. Nitroreductase-mediated cell/tissue ablation in zebrafish: a spatially and temporally controlled ablation method with applications in developmental and regeneration studies. Nat. Protoc. 3, 948–954.

Garcia, G.R., Noyes, P.D., Tanguay, R.L., 2016. Advancements in zebrafish applications for 21st century toxicology. Pharmacol. Ther. 161, 11–21. doi:10.1016/j.pharmthera.2016.03.009

Howsam, M., Jones, K.C., 1998. Sources of PAHs in the Environment, in: Neilson, D.A.H. (Ed.), PAHs and Related Compounds, The Handbook of Environmental Chemistry. Springer Berlin Heidelberg, pp. 137–174. doi:10.1007/978-3-540-49697-7_4

Jariyasopit, N., Zimmermann, K., Schrlau, J., Arey, J., Atkinson, R., Yu, T.-W., Dashwood, R.H., Tao, S., Simonich, S.L.M., 2014. Heterogeneous Reactions of Particulate Matter-Bound PAHs and NPAHs with NO3/N2O5, OH Radicals, and O3 under Simulated Long-Range Atmospheric Transport Conditions: Reactivity and Mutagenicity. Environ. Sci. Technol. 48, 10155–10164.

Kim, K.-H., Jahan, S.A., Kabir, E., 2011. A review of diseases associated with household air pollution due to the use of biomass fuels. J. Hazard. Mater. 192, 425–431. doi:10.1016/j.jhazmat.2011.05.087

Knecht, A.L., Goodale, B.C., Truong, L., Simonich, M.T., Swanson, A.J., Matzke, M.M., Anderson, K.A., Waters, K.M., Tanguay, R.L., 2013. Comparative developmental toxicity of environmentally relevant oxygenated PAHs. Toxicol. Appl. Pharmacol. 271, 266–275.

Knecht, A.L., Truong, L., Simonich, M.T., Tanguay, R.L., 2017. Developmental benzo[a]pyrene (B[a]P) exposure impacts larval behavior and impairs adult learning in zebrafish. Neurotoxicol. Teratol. 59, 27–34. doi:10.1016/j.ntt.2016.10.006

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. Environ. Sci. Technol. 50, 1561–1569. doi:10.1021/acs.est.5b04786

RI DEM, 2008. Rhode Island Air Toxics Guideline.

Riedl, J., Altenburger, R., 2007. Physicochemical substance properties as indicators for unreliable exposure in microplate-based bioassays. Chemosphere 67, 2210–2220.

Rosenkranz, H.S., Mermelstein, R., 1983. Mutagenicity and genotoxicity of nitroarenes. Mutat. Res. Genet. Toxicol. 114, 217–267. doi:10.1016/0165-1110(83)90034-9

WHO, 2015. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans [WWW Document]. URL http://monographs.iarc.fr/index.php (accessed 11.2.16).

Chapter 6- Future Directions

In the studies presented in this dissertation, we examined and implemented the zebrafish model for the investigation of NPAHs. We highlighted and characterized sorptive losses which can occur during toxicity testing of NPAHs, and similar hydrophobic compounds. We identified that sorptive losses can be significant, leading to somewhat unpredictable concentrations to which the zebrafish embryos, or any other system using polystyrene plates, is exposed. Future research to further define the sorptive losses and exposure concentrations of PAHs, PAH derivatives such as NPAHs, as well as other hydrophobic contaminants will greatly enhance the accuracy of such toxicity testing. The development of predictive models, allowing for the prediction of sorptive losses for a range of chemicals and concentrations, will allow for a broader applicability of this data, as the tedious testing will not be required for each individual chemical, and the appropriate adjustments to toxicity data can be made. For some classes of compounds, such as NPAHs, models based on physical-chemical properties will require the experimental determination of a greater number of these properties, rather than relying on estimated values.

In addition to more accurate definition of exposure concentrations, quantitative measurements of chemical uptake by the zebrafish embryo (or other model system) will also allow for more thorough toxicity characterization and comparisons across a chemical library. Previous research on a limited number of PAHs indicated that uptake from the aqueous media can be highly variable, but is not currently well-defined. In order to most accurately define toxicity values, an accurate determination of the actual dose of chemical to which the embryo receives is necessary.

There also exists a nearly infinite number of PAHs and PAH derivatives, and while collective efforts have toxicologically evaluated a large number of these, many more exist to be tested. In many cases, including NPAHs, testing is limited by the commercially available standards available, as there are many PAHs for which the nitrated derivatives are not available, and therefore unstudied. Synthesis of a greater number of not only NPAHs, but other PAH derivatives as well, will allow for more comprehensive toxicity evaluations.

PAHs and PAH derivatives do not occur as isolated compounds in the environment. While toxicity testing of these compounds as individual standards is a useful starting point, for true environmental relevance, testing of mixtures is required, although complicated. When investigating complex mixtures, mixture effects need to also be taken into consideration, and need to be defined for PAHs and PAH derivatives.

For the compounds which have been investigated, further elucidation of mechanisms of action, including a greater number of genes and compounds, would allow for further insight into the mechanisms of action of NPAHs and HPAHs. Investigation of select genes is useful, but a more complete picture of NPAH and HPAH toxicity would require a more comprehensive list of genes, or complete RNA sequencing. Comparisons across a wide set of PAHs and PAH derivatives could yield interesting insight with regards to toxicity pathways, and compound classes which should be of greater concern to human health.

The goal with the use of the nitroreductase-expressing transgenic zebrafish line was to investigate the reductive metabolism of NPAHs, and the role of nitoreduction in toxicity. While the transgenic line was determined to be unsuitable for this purpose, other potential uses of this line were explored, including the potential for use in screening hepatotoxic chemicals, and

chemicals for which hepatic metabolism had been implicated as important to toxicity. The development of a similar nitroreductase-expressing transgenic line, which has a nitroreductase with a higher affinity for NPAHs, would be necessary for the investigation of nitroreductase in NPAH metabolism. The use of analytical techniques to quantify metabolites formed, and the ability to test those metabolites for toxicity, would also allow for a more complete investigation of the role nitroreductase has in the toxicity of NPAHs, in particular when other metabolic pathways, such as CYP oxidation are also occurring, as this would most closely replicate the complete metabolic potential of NPAHs in humans.

BIBLIOGRAPHY

Akpambang, V.O.E., Purcaro, G., Lajide, L., Amoo, I.A., Conte, L.S., Moret, S., 2009. Determination of polycyclic aromatic hydrocarbons (PAHs) in commonly consumed Nigerian smoked/grilled fish and meat. Food Addit. Contam. Part A 26, 1096–1103. doi:10.1080/02652030902855406

Albinet, A., Leoz-Garziandia, E., Budzinski, H., ViIlenave, E., 2007. Polycyclic aromatic hydrocarbons (PAHs), nitrated PAHs and oxygenated PAHs in ambient air of the Marseilles area (South of France): Concentrations and sources. Sci. Total Environ. 384, 280–292. doi:10.1016/j.scitotenv.2007.04.028

Alomirah, H., Al-Zenki, S., Al-Hooti, S., Zaghloul, S., Sawaya, W., Ahmed, N., Kannan, K., 2011. Concentrations and dietary exposure to polycyclic aromatic hydrocarbons (PAHs) from grilled and smoked foods. Food Control 22, 2028–2035. doi:10.1016/j.foodcont.2011.05.024

Andreasen, E.A., Mathew, L.K., Tanguay, R.L., 2006. Regenerative Growth Is Impacted by TCDD: Gene Expression Analysis Reveals Extracellular Matrix Modulation. Toxicol. Sci. 92, 254–269. doi:10.1093/toxsci/kfj118

Andreasen, E.A., Spitsbergen, J.M., Tanguay, R.L., Stegeman, J.J., Heideman, W., Peterson, R.E., 2002. Tissue-Specific Expression of AHR2, ARNT2, and CYP1A in Zebrafish Embryos and Larvae: Effects of Developmental Stage and 2,3,7,8-Tetrachlorodibenzo-p-dioxin Exposure. Toxicol. Sci. 68, 403–419. doi:10.1093/toxsci/68.2.403

Antkiewicz, D.S., Peterson, R.E., Heideman, W., 2006. Blocking Expression of AHR2 and ARNT1 in Zebrafish Larvae Protects Against Cardiac Toxicity of 2,3,7,8-Tetrachlorodibenzo-p-dioxin. Toxicol. Sci. 94, 175–182. doi:10.1093/toxsci/kfl093

Arey, J., Zielinska, B., Atkinson, R., Winer, A.M., Ramdahl, T., Pitts, J.N., 1986. The formation of nitro-PAH from the gas-phase reactions of fluoranthene and pyrene with the OH radical in the presence of NO x. Atmospheric Environ. 1967 20, 2339–2345.

Armstrong, B., Hutchinson, E., Unwin, J., Fletcher, T., 2004. Lung Cancer Risk after Exposure to Polycyclic Aromatic Hydrocarbons: A Review and Meta-Analysis. Environ. Health Perspect. 112, 970–978.

Asokan, P., Das, M., Bik, D.P., Howard, P.C., McCoy, G.D., Rosenkranz, H.S., Bickers, D.R., Mukhtar, H., 1986. Comparative effects of topically applied nitrated arenes and their nonnitrated parent arenes on cutaneous and hepatic drug and carcinogen metabolism in neonatal rats. Toxicol. Appl. Pharmacol. 86, 33–43.

Atkinson, R., Arey, J., 2007. Mechanisms of the Gas-Phase Reactions of Aromatic Hydrocarbons and Pahs with Oh and No 3 Radicals. Polycycl. Aromat. Compd. 27, 15–40. doi:10.1080/10406630601134243

Atkinson, R., Arey, J., Zielinska, B., Aschmann, S.M., 1990. Kinetics and nitro-products of the gas-phase OH and NO3 radical-initiated reactions of naphthalene-d8, Fluoranthene-d10, and pyrene. Int. J. Chem. Kinet. 22, 999–1014.

Baek, S.O., Field, R.A., Goldstone, M.E., Kirk, P.W., Lester, J.N., Perry, R., 1991. A review of atmospheric polycyclic aromatic hydrocarbons: Sources, fate and behavior. Water. Air. Soil Pollut. 60, 279–300. doi:10.1007/BF00282628

Baird, W.M., Hooven, L.A., Mahadevan, B., 2005. Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. Environ. Mol. Mutagen. 45, 106–114. doi:10.1002/em.20095

Ball, L.M., Kohan, M.J., Claxton, L.D., Lewtas, J., 1984. Mutagenicity of derivatives and metabolites of 1-nitropyrene: activation by rat liver S9 and bacterial enzymes. Mutat. Res. Toxicol. 138, 113–125. doi:10.1016/0165-1218(84)90033-8

Bamford, H.A., Bezabeh, D.Z., Schantz, M.M., Wise, S.A., Baker, J.E., 2003. Determination and comparison of nitrated-polycyclic aromatic hydrocarbons measured in air and diesel particulate reference materials. Chemosphere 50, 575–587. doi:10.1016/S0045-6535(02)00667-7

Bandowe, B.A.M., Meusel, H., Huang, R., Hoffmann, T., Cao, J., Ho, K., 2016. Azaarenes in fine particulate matter from the atmosphere of a Chinese megacity. Environ. Sci. Pollut. Res. 1–12. doi:10.1007/s11356-016-6740-z

Bandowe, B.A.M., Nkansah, M.A., 2016. Occurrence, distribution and health risk from polycyclic aromatic compounds (PAHs, oxygenated-PAHs and azaarenes) in street dust from a major West African Metropolis. Sci. Total Environ. 553, 439–449. doi:10.1016/j.scitotenv.2016.02.142

Barron, M.G., Heintz, R., Rice, S.D., 2004. Relative potency of PAHs and heterocycles as aryl hydrocarbon receptor agonists in fish. Mar. Environ. Res., Twelfth International Symposium on Pollutant Responses in Marine Organisms 58, 95–100. doi:10.1016/j.marenvres.2004.03.001

Behrendt, L., Jönsson, M.E., Goldstone, J.V., Stegeman, J.J., 2010. Induction of cytochrome P450 1 genes and stress response genes in developing zebrafish exposed to ultraviolet radiation. Aquat. Toxicol. 98, 74–82. doi:10.1016/j.aquatox.2010.01.008

Beischlag, T.V., Morales, J.L., Hollingshead, B.D., Perdew, G.H., 2008. The Aryl Hydrocarbon Receptor Complex and the Control of Gene Expression. Crit. Rev. Eukaryot. Gene Expr. 18. doi:10.1615/CritRevEukarGeneExpr.v18.i3.20

Bernstein, J.A., Alexis, N., Barnes, C., Bernstein, I.L., Nel, A., Peden, D., Diaz-Sanchez, D., Tarlo, S.M., Williams, P.B., Bernstein, J.A., 2004. Health effects of air pollution. J. Allergy Clin. Immunol. 114, 1116–1123. doi:10.1016/j.jaci.2004.08.030

Berson, A., Wolf, C., Chachaty, C., Fisch, C., Fau, D., Eugene, D., Loeper, J., Gauthier, J.C., Beaune, P., Pompon, D., 1993. Metabolic activation of the nitroaromatic antiandrogen flutamide by rat and human cytochromes P-450, including forms belonging to the 3A and 1A subfamilies. J. Pharmacol. Exp. Ther. 265, 366–372.

Billiard, S.M., Querbach, K., Hodson, P.V., 1999. Toxicity of retene to early life stages of two freshwater fish species. Environ. Toxicol. Chem. 18, 2070–2077. doi:10.1002/etc.5620180927

Billiard, S.M., Timme-Laragy, A.R., Wassenberg, D.M., Cockman, C., Giulio, R.T.D., 2006. The Role of the Aryl Hydrocarbon Receptor Pathway in Mediating Synergistic Developmental Toxicity of Polycyclic Aromatic Hydrocarbons to Zebrafish. Toxicol. Sci. 92, 526–536. doi:10.1093/toxsci/kfl011

Bisson, W.H., Koch, D.C., O'Donnell, E.F., Khalil, S.M., Kerkvliet, N.I., Tanguay, R.L., Abagyan, R., Kolluri, S.K., 2009. Modeling of the aryl hydrocarbon receptor (AhR)

ligand binding domain and its utility in virtual ligand screening to predict new AhR ligands. J. Med. Chem. 52, 5635–5641.

Black, M., 1984. Acetaminophen hepatotoxicity. Annu. Rev. Med. 35, 577–593.

Bleeker, E.A., Buckert-de Jong, M.C., van der Geest, H.G., Kraak, M.H., 1996. Toxicity of nitrogencontaining polycyclic aromatic hydrocarbons (NPAH) to the midge Chironomus riparus (Diptera). Proc Exp Appl. Entomol 7, 197–202.

Blum, P., Sagner, A., Tiehm, A., Martus, P., Wendel, T., Grathwohl, P., 2011. Importance of heterocylic aromatic compounds in monitored natural attenuation for coal tar contaminated aquifers: A review. J. Contam. Hydrol. 126, 181–194. doi:10.1016/j.jconhyd.2011.08.004

Boelsterli, U.A., Ho, H.K., Zhou, S., Yeow Leow, K., 2006. Bioactivation and Hepatotoxicity of Nitroaromatic Drugs [WWW Document]. URL http://orst.library.ingentaconnect.com/content/ben/cdm/2006/00000007/00000007/art00003 (accessed 2.9.17).

Bond, J.A., 1983. Bioactivation and biotransformation of 1-nitropyrene in liver, lung and nasal tissue of rats. Mutat. Res. Toxicol. 124, 315–324.

Boom, A., Marsalek, J., 1988. Accumulation of polycyclic aromatic hydrocarbons (PAHs) in an urban snowpack. Sci. Total Environ. 74, 133–148. doi:10.1016/0048-9697(88)90134-9

Bouayed, J., Desor, F., Rammal, H., Kiemer, A.K., Tybl, E., Schroeder, H., Rychen, G., Soulimani, R., 2009. Effects of lactational exposure to benzo[α]pyrene (B[α]P) on postnatal neurodevelopment, neuronal receptor gene expression and behaviour in mice. Toxicology 259, 97–106. doi:10.1016/j.tox.2009.02.010

Brack, W., Schirmer, K., 2003. Effect-Directed Identification of Oxygen and Sulfur Heterocycles as Major Polycyclic Aromatic Cytochrome P4501A-Inducers in a Contaminated Sediment. Environ. Sci. Technol. 37, 3062–3070. doi:10.1021/es020248j

Bridgewater, J.A., Springer, C.J., Knox, R.J., Minton, N.P., Michael, N.P., Collins, M.K., 1995. Expression of the bacterial nitroreductase enzyme in mammalian cells renders them selectively sensitive to killing by the prodrug CB1954. Eur. J. Cancer 31, 2362–2370. doi:10.1016/0959-8049(95)00436-X

Brinkmann, M., Maletz, S., Krauss, M., Bluhm, K., Schiwy, S., Kuckelkorn, J., Tiehm, A., Brack, W., Hollert, H., 2014. Heterocyclic Aromatic Hydrocarbons Show Estrogenic Activity upon Metabolization in a Recombinant Transactivation Assay. Environ. Sci. Technol. 48, 5892–5901. doi:10.1021/es405731j

Brown, D.R., Bailey, J.M., Oliveri, A.N., Levin, E.D., Di Giulio, R.T., 2016. Developmental exposure to a complex PAH mixture causes persistent behavioral effects in naive Fundulus heteroclitus (killifish) but not in a population of PAH-adapted killifish. Neurotoxicol. Teratol. 53, 55–63. doi:10.1016/j.ntt.2015.10.007

Brown, D.R., Clark, B.W., Garner, L.V.T., Giulio, R.T.D., 2014. Zebrafish cardiotoxicity: the effects of CYP1A inhibition and AHR2 knockdown following exposure to weak aryl hydrocarbon receptor agonists. Environ. Sci. Pollut. Res. 22, 8329–8338. doi:10.1007/s11356-014-3969-2

Bugel, S.M., Tanguay, R.L., Planchart, A., 2014. Zebrafish: A Marvel of High-Throughput Biology for 21st Century Toxicology. Curr. Environ. Health Rep. 1, 341–352. doi:10.1007/s40572-014-0029-5

Burstyn, I., Kromhout, H., Partanen, T., Svane, O., Lang\a ard, S., Ahrens, W., Kauppinen, T., Stücker, I., Shaham, J., Heederik, D., others, 2005. Polycyclic aromatic hydrocarbons and fatal ischemic heart disease. Epidemiology 16, 744–750.

Campbell, J., Crumplin, G., Garner, J.V., Garner, R.C., Martin, C.N., Rutter, A., 1981. Nitrated polycyclic aromatic hydrocarbons: potent bacterial mutagens and stimulators of DNA repair synthesis in cultured human cells. Carcinogenesis 2, 559–565.

Carrara, M., Wolf, J.-C., Niessner, R., 2010. Nitro-PAH formation studied by interacting artificially PAH-coated soot aerosol with NO2 in the temperature range of 295–523 K. Atmos. Environ. 44, 3878–3885. doi:10.1016/j.atmosenv.2010.07.032

Cecinato, A., Mabilia, R., Brachetti, A., Filippo, P.D., Liberti, A., 2001. Nitrated-Pah in Urban Air of Italy as Indicators of Motor Vehicle Emission and Light-Induced Reactions. Anal. Lett. 34, 927–936. doi:10.1081/AL-100103603

Cerniglia, C.E., Lambert, K.J., White, G.L., Heflich, R.H., Franklin, W., Fifer, E.K., Beland, F.A., 1988. Metabolism of 1, 8-dinitropyrene by human, rhesus monkey, and rat intestinal microflora. Toxic. Assess. 3, 147–159.

Chan, P., 1996. NTP technical report on the toxicity studies of 1-Nitropyrene (CAS No. 5522-43-0) Administered by Inhalation to F344/N Rats. Toxic. Rep. Ser. 34, 1–D2.

Chandramouli, B., Benskin, J.P., Hamilton, M.C., Cosgrove, J.R., 2015. Sorption of per- and polyfluoroalkyl substances (PFASs) on filter media: Implications for phase partitioning studies. Environ. Toxicol. Chem. 34, 30–36. doi:10.1002/etc.2751

Châtel, A., Faucet-Marquis, V., Pfohl-Leszkowicz, A., Gourlay-Francé, C., Vincent-Hubert, F., 2014. DNA adduct formation and induction of detoxification mechanisms in Dreissena polymorpha exposed to nitro-PAHs. Mutagenesis 29, 457–465. doi:10.1093/mutage/geu040

Chen, Y., Du, W., Shen, G., Zhuo, S., Zhu, X., Shen, H., Huang, Y., Su, S., Lin, N., Pei, L., Zheng, X., Wu, J., Duan, Y., Wang, X., Liu, W., Wong, M., Tao, S., 2016. Household air pollution and personal exposure to nitrated and oxygenated polycyclic aromatics (PAHs) in rural households: Influence of household cooking energies. Indoor Air n/a-n/a. doi:10.1111/ina.12300

Chen, Y., Shen, G., Su, S., Shen, H., Huang, Y., Li, T., Li, W., Zhang, Y., Lu, Y., Chen, H., others, 2014. Contamination and distribution of parent, nitrated, and oxygenated polycyclic aromatic hydrocarbons in smoked meat. Environ. Sci. Pollut. Res. 21, 11521–11530.

Cheng, S., Xia, Y., He, J., Liu, X., Chen, X., Ding, Y., Wang, Y., Peng, B., Tu, B., 2013. Neurotoxic effect of subacute benzo(a)pyrene exposure on gene and protein expression in Sprague-Dawley rats. Environ. Toxicol. Pharmacol. 36, 648–658. doi:10.1016/j.etap.2013.06.008

Chibwe, L., Geier, M.C., Nakamura, J., Tanguay, R.L., Aitken, M.D., Simonich, S.L.M., 2015. Aerobic Bioremediation of PAH Contaminated Soil Results in Increased Genotoxicity and Developmental Toxicity. Environ. Sci. Technol. 49, 13889–13898. doi:10.1021/acs.est.5b00499

Chlebowski, A.C., Garcia, G.R., Du, L., K, J., Bisson, W.H., Truong, L., Simonich, M., L, S., Tanguay, R.L., 2017. Mechanistic investigations into the developmental toxicity of nitrated and heterocyclic PAHs. Toxicol. Sci. doi:10.1093/toxsci/kfx035

Chlebowski, A.C., Tanguay, R.L., Simonich, S.L.M., 2016. Quantitation and prediction of sorptive losses during toxicity testing of polycyclic aromatic hydrocarbon (PAH) and nitrated PAH (NPAH) using polystyrene 96-well plates. Neurotoxicol. Teratol. 57, 30–38. doi:10.1016/j.ntt.2016.05.001

Choi, T., Ninov, N., Stainier, D.Y.R., Shin, D., 2014. Extensive Conversion of Hepatic Biliary Epithelial Cells to Hepatocytes After Near Total Loss of Hepatocytes in Zebrafish. Gastroenterology 146, 776–788. doi:10.1053/j.gastro.2013.10.019

Chou, M.W., Evans, F.E., Yang, S.K., Fu, P.P., 1983. Evidence for a 2, 3-epoxide as an intermediate in the microsomal metabolism of 6-nitrobenzo [a] pyrene. Carcinogenesis 4, 699–702.

Ciganek, M., Neca, J., Adamec, V., Janosek, J., Machala, M., 2004. A combined chemical and bioassay analysis of traffic-emitted polycyclic aromatic hydrocarbons. Sci. Total Environ., Highway and Urban Pollution 334–335, 141–148. doi:10.1016/j.scitotenv.2004.04.034

Clark, A.J., Iwobi, M., Cui, W., Crompton, M., Harold, G., Hobbs, S., Kamalati, T., Knox, R., Neil, C., Yull, F., others, 1997. Selective cell ablation in transgenic mice expressing E. coli nitroreductase. Gene Ther. 4, 101–110.

Cochran, R.E., Jeong, H., Haddadi, S., Fisseha Derseh, R., Gowan, A., Beránek, J., Kubátová, A., 2016a. Identification of products formed during the heterogeneous nitration and ozonation of polycyclic aromatic hydrocarbons. Atmos. Environ. 128, 92–103. doi:10.1016/j.atmosenv.2015.12.036

Collins, J.F., Brown, J.P., Alexeeff, G.V., Salmon, A.G., 1998. Potency Equivalency Factors for Some Polycyclic Aromatic Hydrocarbons and Polycyclic Aromatic Hydrocarbon Derivatives. Regul. Toxicol. Pharmacol. 28, 45–54. doi:10.1006/rtph.1998.1235

Curado, S., Anderson, R.M., Jungblut, B., Mumm, J., Schroeter, E., Stainier, D.Y., 2007. Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies. Dev. Dyn. 236, 1025–1035.

Curado, S., Stainier, D.Y., Anderson, R.M., 2008. Nitroreductase-mediated cell/tissue ablation in zebrafish: a spatially and temporally controlled ablation method with applications in developmental and regeneration studies. Nat. Protoc. 3, 948–954.

Dalton, T.P., Puga, A., Shertzer, H.G., 2002. Induction of cellular oxidative stress by aryl hydrocarbon receptor activation. Chem. Biol. Interact. 141, 77–95. doi:10.1016/S0009-2797(02)00067-4

Debnath, A.K., Lopez Compadre, R.L., Shusterman, A.J., Hansch, C., 1992. Quantitative structure-activity relationship investigation of the role of hydrophobicity in regulating mutagenicity in the Ames test: 2. Mutagenicity of aromatic and heteroaromatic nitro compounds in Salmonella typhimurium TA100. Environ. Mol. Mutagen. 19, 53–70.

Denison, M.S., Nagy, and S.R., 2003. Activation of the Aryl Hydrocarbon Receptor by Structurally Diverse Exogenous and Endogenous Chemicals. Annu. Rev. Pharmacol. Toxicol. 43, 309–334. doi:10.1146/annurev.pharmtox.43.100901.135828

Dimashki, M., Harrad, S., Harrison, R.M., 2000. Measurements of nitro-PAH in the atmospheres of two cities. Atmos. Environ. 34, 2459–2469. doi:10.1016/S1352-2310(99)00417-3

Djomo, J.E., Garrigues, P., Narbonne, J.F., 1996. Uptake and depuration of polycyclic aromatic hydrocarbons from sediment by the zebrafish (Brachydanio Rerio). Environ. Toxicol. Chem. 15, 1177–1181. doi:10.1002/etc.5620150724

Duarte-Salles, T., Mendez, M.A., Morales, E., Bustamante, M., Rodríguez-Vicente, A., Kogevinas, M., Sunyer, J., 2012. Dietary benzo(a)pyrene and fetal growth: Effect modification by vitamin C intake and glutathione S-transferase P1 polymorphism. Environ. Int. 45, 1–8. doi:10.1016/j.envint.2012.04.002

Dumont, J.N., Schultz, T.W., Jones, R.D., 1979. Toxicity and teratogenicity of aromatic amines toXenopus laevis. Bull. Environ. Contam. Toxicol. 22, 159–166. doi:10.1007/BF02026923

Durante, W., Johnson, F.K., Johnson, R.A., 2007. Arginase: a critical regulator of nitric oxide synthesis and vascular function. Clin. Exp. Pharmacol. Physiol. 34, 906–911.

Eastmond, D.A., Booth, G.M., Lee, M.L., 1984. Toxicity, accumulation, and elimination of polycyclic aromatic sulfur heterocycles inDaphnia magna. Arch. Environ. Contam. Toxicol. 13, 105–111. doi:10.1007/BF01055652

Edwards, S.C., Jedrychowski, W., Butscher, M., Camann, D., Kieltyka, A., Mroz, E., Flak, E., Li, Z., Wang, S., Rauh, V., others, 2010. Prenatal exposure to airborne polycyclic aromatic hydrocarbons and children's intelligence at 5 years of age in a prospective cohort study in Poland. Environ. Health Perspect. 118, 1326.

Einistö, P., Watanabe, M., Motoi Ishidate, J., Nohmi, T., 1991. Mutagenicity of 30 chemicals in Salmonella typhimurium strains possessing different nitroreductase or O-acetyltransferase activities. Mutat. Res. Toxicol. 259, 95–102. doi:10.1016/0165-1218(91)90113-Z

Eisentraeger, A., Brinkmann, C., Hollert, H., Sagner, A., Tiehm, A., Neuwoehner, J., 2008. Heterocyclic compounds: Toxic effects using algae, daphnids, and the Salmonella/microsome test taking methodical quantitative aspects into account. Environ. Toxicol. Chem. 27, 1590–1596. doi:10.1897/07-201.1

El-Bayoumy, K., Hecht, S.S., 1982. Identification of Mutagenic Metabolites Formed by C-Hydroxylation and Nitroreduction of 5-Nitroacenaphthene in Rat Liver. Cancer Res. 42, 1243–1248.

El-Bayoumy, K., Sharma, C., Louis, Y.M., Reddy, B., Hecht, S.S., 1983. The role of intestinal microflora in the metabolic reduction of 1-nitropyrene to 1-aminopyrene in conventional and germfree rats and in humans. Cancer Lett. 19, 311–316.

Essumang, D.K., Dodoo, D.K., Adjei, J.K., 2012. Polycyclic aromatic hydrocarbon (PAH) contamination in smoke-cured fish products. J. Food Compos. Anal. 27, 128–138. doi:10.1016/j.jfca.2012.04.007

Esteve, W., Budzinski, H., Villenave, E., 2006. Relative rate constants for the heterogeneous reactions of NO2 and OH radicals with polycyclic aromatic hydrocarbons adsorbed on carbonaceous particles. Part 2: PAHs adsorbed on diesel particulate exhaust SRM 1650a. Atmos. Environ. 40, 201–211. doi:10.1016/j.atmosenv.2005.07.053

Forsberg, N.D., Stone, D., Harding, A., Harper, B., Harris, S., Matzke, M.M., Cardenas, A., Waters, K.M., Anderson, K.A., 2012. Effect of Native American Fish Smoking Methods on Dietary Exposure to Polycyclic Aromatic Hydrocarbons and Possible Risks to Human Health. J. Agric. Food Chem. 60, 6899–6906. doi:10.1021/jf300978m

Fosque, B.F., Sun, Y., Dana, H., Yang, C.-T., Ohyama, T., Tadross, M.R., Patel, R., Zlatic, M., Kim, D.S., Ahrens, M.B., Jayaraman, V., Looger, L.L., Schreiter, E.R., 2015. Labeling of active neural circuits in vivo with designed calcium integrators. Science 347, 755–760. doi:10.1126/science.1260922

Fragoso, N.M., Hodson, P.V., Kozin, I.S., Brown, R.S., Parrott, J.L., 1999. Kinetics of mixed function oxygenase induction and retene excretion in retene—exposed rainbow trout (Oncorhynchus mykiss). Environ. Toxicol. Chem. 18, 2268–2274. doi:10.1002/etc.5620181022

Freeman, D.J., Cattell, F.C., 1990. Woodburning as a source of atmospheric polycyclic aromatic hydrocarbons. Environ. Sci. Technol. 24, 1581–1585.

Fries, E., Zarfl, C., 2012. Sorption of polycyclic aromatic hydrocarbons (PAHs) to low and high density polyethylene (PE). Environ. Sci. Pollut. Res. 19, 1296–1304. doi:10.1007/s11356-011-0655-5

Fu, P.P., 1990. Metabolism of nitro-polycyclic aromatic hydrocarbons. Drug Metab. Rev. 22, 209–268.

Fu, P.P., Cerniglia, C.E., Richardson, K.E., Heflich, R.H., 1988. Nitroreduction of 6-nitrobenzo [a] pyrene: a potential activation pathway in humans. Mutat. Res. Lett. 209, 123–129.

Fu, P.P., Herreno-Saenz, D., 1999. Nitro-polycyclic aromatic hydrocarbons: A class of genotoxic environmental pollutants. J. Environ. Sci. Health Part C 17, 1–43.

Gale, S.L., Noth, E.M., Mann, J., Balmes, J., Hammond, S.K., Tager, I.B., 2012. Polycyclic aromatic hydrocarbon exposure and wheeze in a cohort of children with asthma in Fresno, CA. J. Expo. Sci. Environ. Epidemiol. 22, 386–392. doi:10.1038/jes.2012.29

Garcia, G.R., Noyes, P.D., Tanguay, R.L., 2016. Advancements in zebrafish applications for 21st century toxicology. Pharmacol. Ther. 161, 11–21. doi:10.1016/j.pharmthera.2016.03.009

García-Falcón, M.., Cancho-Grande, B., Simal-Gándara, J., 2004. Stirring bar sorptive extraction in the determination of PAHs in drinking waters. Water Res. 38, 1679–1684. doi:10.1016/j.watres.2003.12.034

Garner, L.V.T., Brown, D.R., Di Giulio, R.T., 2013. Knockdown of AHR1A but not AHR1B exacerbates PAH and PCB-126 toxicity in zebrafish (Danio rerio) embryos. Aquat. Toxicol. Amst. Neth. 142–143, 336–346. doi:10.1016/j.aquatox.2013.09.007

Geerts, C.C., Bots, M.L., Ent, C.K. van der, Grobbee, D.E., Uiterwaal, C.S.P.M., 2012. Parental Smoking and Vascular Damage in Their 5-year-old Children. Pediatrics 129, 45–54. doi:10.1542/peds.2011-0249

Gellert, G., Stommel, A., 1999. Influence of microplate material on the sensitivity of growth inhibition tests with bacteria assessing toxic organic substances in water and waste water. Environ. Toxicol. 14, 424–428. doi:10.1002/(SICI)1522-7278(1999)14:4<424::AID-TOX8>3.0.CO;2-4

George, S.C., Thomas, S., 2001. Transport phenomena through polymeric systems. Prog. Polym. Sci. 26, 985–1017. doi:10.1016/S0079-6700(00)00036-8

Gerlach, C.V., Das, S.R., Volz, D.C., Bisson, W.H., Kolluri, S.K., Tanguay, R.L., 2014. Mono-substituted isopropylated triaryl phosphate, a major component of Firemaster 550, is an AHR agonist that exhibits AHR-independent cardiotoxicity in zebrafish. Aquat. Toxicol. 154, 71–79. doi:10.1016/j.aquatox.2014.05.007

Goldstone, J.V., McArthur, A.G., Kubota, A., Zanette, J., Parente, T., Jönsson, M.E., Nelson, D.R., Stegeman, J.J., 2010a. Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. BMC Genomics 11, 643. doi:10.1186/1471-2164-11-643

Gomez, J.-L., Dupont, A., Cusan, L., Tremblay, M., Suburu, R., Lemay, M., Labrie, F., 1992. Incidence of liver toxicity associated with the use of flutamide in prostate cancer patients. Am. J. Med. 92, 465–470. doi:10.1016/0002-9343(92)90741-S

Goodale, B.C., Du, J.K.L., Bisson, W.H., Janszen, D.B., Waters, K.M., Tanguay, R.L., 2012a. AHR2 Mutant Reveals Functional Diversity of Aryl Hydrocarbon Receptors in Zebrafish. PLOS ONE 7, e29346. doi:10.1371/journal.pone.0029346

Goodale, B.C., Du, J.L., Tilton, S.C., Sullivan, C.M., Bisson, W.H., Waters, K.M., Tanguay, R.L., 2015. Ligand-Specific Transcriptional Mechanisms Underlie Aryl Hydrocarbon Receptor-Mediated Developmental Toxicity of Oxygenated PAHs. Toxicol. Sci. 147, 397–411. doi:10.1093/toxsci/kfv139

Goodale, B.C., Tilton, S.C., Corvi, M.M., Wilson, G.R., Janszen, D.B., Anderson, K.A., Waters, K.M., Tanguay, R.L., 2013. Structurally distinct polycyclic aromatic hydrocarbons induce differential transcriptional responses in developing zebrafish. Toxicol. Appl. Pharmacol. 272, 656–670. doi:10.1016/j.taap.2013.04.024

Graham, G.G., Davies, M.J., Day, R.O., Mohamudally, A., Scott, K.F., 2013. The modern pharmacology of paracetamol: therapeutic actions, mechanism of action, metabolism, toxicity and recent pharmacological findings. Inflammopharmacology 21, 201–232. doi:10.1007/s10787-013-0172-x

Grant, W.B., 2009. Air Pollution in Relation to U.S. Cancer Mortality Rates: An Ecological Study; Likely Role of Carbonaceous Aerosols and Polycyclic Aromatic Hydrocarbons. Anticancer Res. 29, 3537–3545.

Guengerich, F.P., 2001. Common and Uncommon Cytochrome P450 Reactions Related to Metabolism and Chemical Toxicity. Chem. Res. Toxicol. 14, 611–650. doi:10.1021/tx0002583

Hackshaw, A.K., Law, M.R., Wald, N.J., 1997. The accumulated evidence on lung cancer and environmental tobacco smoke. Bmj 315, 980–988.

Haggard, D.E., Noyes, P.D., Waters, K.M., Tanguay, R.L., 2016. Phenotypically anchored transcriptome profiling of developmental exposure to the antimicrobial agent, triclosan, reveals hepatotoxicity in embryonic zebrafish. Toxicol. Appl. Pharmacol. 308, 32–45. doi:10.1016/j.taap.2016.08.013

Hahn, M.E., Karchner, S.I., Merson, R.R., n.d. Diversity as Opportunity: Insights from 600 Million Years of AHR Evolution. Curr. Opin. Toxicol. doi:10.1016/j.cotox.2017.02.003

Hannink, N., Rosser, S.J., French, C.E., Basran, A., Murray, J.A.H., Nicklin, S., Bruce, N.C., 2001. Phytodetoxification of TNT by transgenic plants expressing a bacterial nitroreductase. Nat. Biotechnol. 19, 1168–1172. doi:10.1038/nbt1201-1168

Hardin, J.A., Hinoshita, F., Sherr, D.H., 1992. Mechanisms by which benzo[a]pyrene, an environmental carcinogen, suppresses B cell lymphopoiesis. Toxicol. Appl. Pharmacol. 117, 155–164. doi:10.1016/0041-008X(92)90232-H

Harvey, R.G., 1996. Mechanisms Of Carcinogenesis of Polycyclic Aromatic Hydrocarbons. Polycycl. Aromat. Compd. 9, 1–23. doi:10.1080/10406639608031196

Hattori, T., Tang, N., Tamura, K., Hokoda, A., Yang, X., Igarashi, K., Ohno, M., Okada, Y., Kameda, T., Toriba, A., Hayakawa, K., 2007. Particulate Polycyclic Aromatic Hydrocarbons and Their Nitrated Derivatives in Three Cities in Liaoning Province, China. Environ. Forensics 8, 165–172. doi:10.1080/15275920601180701

Hawliczek, A., Nota, B., Cenijn, P., Kamstra, J., Pieterse, B., Winter, R., Winkens, K., Hollert, H., Segner, H., Legler, J., 2012. Developmental toxicity and endocrine disrupting potency of 4-azapyrene, benzo[b]fluorene and retene in the zebrafish Danio rerio. Reprod. Toxicol., Zebrafish Teratogenesis 33, 213–223. doi:10.1016/j.reprotox.2011.11.001

Hayakawa, K., Kawaguchi, Y., Murahashi, T., Miyazaki, M., 1995. Distribution of nitropyrenes and mutagenicity in airborne particulates collected with an Andersen sampler. Mutat. Res. Lett. 348, 57–61. doi:10.1016/0165-7992(95)00046-1

He, J., Lu, H., Zou, Q., Luo, L., 2014. Regeneration of Liver After Extreme Hepatocyte Loss Occurs Mainly via Biliary Transdifferentiation in Zebrafish. Gastroenterology 146, 789–800.e8. doi:10.1053/j.gastro.2013.11.045

Hecht, S.S., 2003. Tobacco carcinogens, their biomarkers and tobacco-induced cancer. Nat. Rev. Cancer 3, 733–744. doi:10.1038/nrc1190

Henn, K., Braunbeck, T., 2011. Dechorionation as a tool to improve the fish embryo toxicity test (FET) with the zebrafish (Danio rerio). Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 153, 91–98. doi:10.1016/j.cbpc.2010.09.003

Hertz-Picciotto, I., Baker, R.J., Yap, P.-S., Dostá, M., Joad, J.P., Lipsett, M., Greenfield, T., Herr, C.E.W., Beneš, I., Shumway, R.H., Pinkerton, K.E., Šrám, R., 2007. Early Childhood Lower Respiratory Illness and Air Pollution. Environ. Health Perspect. 115, 1510–1518.

Hicken, C.E., Linbo, T.L., Baldwin, D.H., Willis, M.L., Myers, M.S., Holland, L., Larsen, M., Stekoll, M.S., Rice, S.D., Collier, T.K., Scholz, N.L., Incardona, J.P., 2011. Sublethal exposure to crude oil during embryonic development alters cardiac morphology and reduces aerobic capacity in adult fish. Proc. Natl. Acad. Sci. 108, 7086–7090. doi:10.1073/pnas.1019031108

Hill, A.J., Teraoka, H., Heideman, W., Peterson, R.E., 2005. Zebrafish as a Model Vertebrate for Investigating Chemical Toxicity. Toxicol. Sci. 86, 6–19. doi:10.1093/toxsci/kfi110

Hirmann, D., Loibner, A.P., Braun, R., Szolar, O.H.J., 2007. Applicability of the bioluminescence inhibition test in the 96-well microplate format for PAH-solutions and elutriates of PAH-contaminated soils. Chemosphere 67, 1236–1242. doi:10.1016/j.chemosphere.2006.10.047

Hoh, E., Hunt, R.N., Quintana, P.J., Zakarian, J.M., Chatfield, D.A., Wittry, B.C., Rodriguez, E., Matt, G.E., 2012. Environmental tobacco smoke as a source of polycyclic aromatic hydrocarbons in settled household dust. Environ. Sci. Technol. 46, 4174–4183.

Hopfenberg, H.B., 1978. The effect of film thickness and sample history on the parameters describing transport in glassy polymers. J. Membr. Sci. 3, 215–230. doi:10.1016/S0376-7388(00)83023-9

Howard, P.C., Beland, F.A., Cerniglia, C.E., 1983a. Reduction of the carcinogen 1-nitropyrene to 1-aminopyrene by rat intestinal bacteria. Carcinogenesis 4, 985–990. doi:10.1093/carcin/4.8.985

Howard, P.C., Gerrard, J.A., Milo, G.E., Peter, P.F., Beland, F.A., Kadlubar, F.F., 1983b. Transformation of normal human skin fibroblasts by 1-nitropyrene and 6-nitrobenzo [a] pyrene. Carcinogenesis 4, 353–355. doi:10.1093/carcin/4.3.353

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S., McLaren, K., Matthews, L., others, 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498–503.

Howsam, M., Jones, K.C., 1998. Sources of PAHs in the Environment, in: Neilson, D.A.H. (Ed.), PAHs and Related Compounds, The Handbook of Environmental Chemistry. Springer Berlin Heidelberg, pp. 137–174. doi:10.1007/978-3-540-49697-7_4

Hruscha, A., Krawitz, P., Rechenberg, A., Heinrich, V., Hecht, J., Haass, C., Schmid, B., 2013. Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish. Development 140, 4982–4987. doi:10.1242/dev.099085

Hrycay, E.G., Bandiera, S.M., 2010. Cytochrome P450 Enzymes, in: Pharmaceutical Sciences Encyclopedia. John Wiley & Sons, Inc. doi:10.1002/9780470571224.pse048

Hsu, C.-C., Hou, M.-F., Hong, J.-R., Wu, J.-L., Her, G.M., 2009. Inducible Male Infertility by Targeted Cell Ablation in Zebrafish Testis. Mar. Biotechnol. 12, 466–478. doi:10.1007/s10126-009-9248-4

Hsu, H.-J., Hsiao, P., Kuo, M.-W., Chung, B., 2002. Expression of zebrafish cyp11a1 as a maternal transcript and in yolk syncytial layer. Gene Expr. Patterns 2, 219–222. doi:10.1016/S1567-133X(02)00059-5

Hu, S., Herner, J.D., Robertson, W., Kobayashi, R., Chang, M.-C.O., Huang, S., Zielinska, B., Kado, N., Collins, J.F., Rieger, P., others, 2013. Emissions of polycyclic aromatic hydrocarbons (PAHs) and nitro-PAHs from heavy-duty diesel vehicles with DPF and SCR. J. Air Waste Manag. Assoc. 63, 984–996.

Huang, H., Huang, C., Wang, L., Ye, X., Bai, C., Simonich, M.T., Tanguay, R.L., Dong, Q., 2010. Toxicity, uptake kinetics and behavior assessment in zebrafish embryos following exposure to perfluorooctanesulphonicacid (PFOS). Aquat. Toxicol. 98, 139–147. doi:10.1016/j.aquatox.2010.02.003

Huang, L., Wang, C., Zhang, Y., Li, J., Zhong, Y., Zhou, Y., Chen, Y., Zuo, Z., 2012a. Benzo[a]pyrene exposure influences the cardiac development and the expression of cardiovascular relative genes in zebrafish (Danio rerio) embryos. Chemosphere 87, 369–375. doi:10.1016/j.chemosphere.2011.12.026

Hwang, W.Y., Fu, Y., Reyon, D., Maeder, M.L., Tsai, S.Q., Sander, J.D., Peterson, R.T., Yeh, J.-R.J., Joung, J.K., 2013. Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat. Biotechnol. 31, 227–229. doi:10.1038/nbt.2501

Hylland, K., 2006. Polycyclic Aromatic Hydrocarbon (PAH) Ecotoxicology in Marine Ecosystems. J. Toxicol. Environ. Health A 69, 109–123. doi:10.1080/15287390500259327

Hyötyläinen, T., Olkari, A., 1999. The toxicity and concentrations of PAHs in creosote-contaminated lake sediment. Chemosphere 38, 1135–1144. doi:10.1016/S0045-6535(98)00362-2

Incardona, J.P., Collier, T.K., Scholz, N.L., 2004. Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. Toxicol. Appl. Pharmacol. 196, 191–205. doi:10.1016/j.taap.2003.11.026

Incardona, J.P., Day, H.L., Collier, T.K., Scholz, N.L., 2006. Developmental toxicity of 4-ring polycyclic aromatic hydrocarbons in zebrafish is differentially dependent on AH receptor isoforms and hepatic cytochrome P4501A metabolism. Toxicol. Appl. Pharmacol. 217, 308–321. doi:10.1016/j.taap.2006.09.018

Incardona, J.P., Gardner, L.D., Linbo, T.L., Brown, T.L., Esbaugh, A.J., Mager, E.M., Stieglitz, J.D., French, B.L., Labenia, J.S., Laetz, C.A., Tagal, M., Sloan, C.A., Elizur, A., Benetti, D.D., Grosell, M., Block, B.A., Scholz, N.L., 2014. Deepwater Horizon crude oil impacts the developing hearts of large predatory pelagic fish. Proc. Natl. Acad. Sci. 111, E1510–E1518. doi:10.1073/pnas.1320950111

Incardona, J.P., Linbo, T.L., Scholz, N.L., 2011. Cardiac toxicity of 5-ring polycyclic aromatic hydrocarbons is differentially dependent on the aryl hydrocarbon receptor 2 isoform during zebrafish development. Toxicol. Appl. Pharmacol. 257, 242–249. doi:10.1016/j.taap.2011.09.010

Incardona, J.P., Swarts, T.L., Edmunds, R.C., Linbo, T.L., Aquilina-Beck, A., Sloan, C.A., Gardner, L.D., Block, B.A., Scholz, N.L., 2013. Exxon Valdez to Deepwater Horizon: Comparable toxicity of both crude oils to fish early life stages. Aquat. Toxicol. 142–143, 303–316. doi:10.1016/j.aquatox.2013.08.011

Irigaray, P., Newby, J.A., Lacomme, S., Belpomme, D., 2007. Overweight/obesity and cancer genesis: More than a biological link. Biomed. Pharmacother. 61, 665–678. doi:10.1016/j.biopha.2007.10.008

Iwanari, M., Nakajima, M., Kizu, R., Hayakawa, K., Yokoi, T., 2002. Induction of CYP1A1, CYP1A2, and CYP1B1 mRNAs by nitropolycyclic aromatic hydrocarbons in various human tissue-derived cells: chemical-, cytochrome P450 isoform-, and cell-specific differences. Arch. Toxicol. 76, 287–298. doi:10.1007/s00204-002-0340-z

Jaffe, D., Anderson, T., Covert, D., Kotchenruther, R., Trost, B., Danielson, J., Simpson, W., Berntsen, T., Karlsdottir, S., Blake, D., Harris, J., Carmichael, G., Uno, I., 1999. Transport of Asian air pollution to North America. Geophys. Res. Lett. 26, 711–714. doi:10.1029/1999GL900100

Janoszka, B., Warzecha, L., Blaszczyk, U., Bodzek, D., 2004. Organic compounds formed in thermally treated high-protein food. Part II: Azaarenes. Acta Chromatogr. 129–141.

Jarema, K.A., Hunter, D.L., Shaffer, R.M., Behl, M., Padilla, S., 2015. Acute and developmental behavioral effects of flame retardants and related chemicals in zebrafish. Neurotoxicol. Teratol., Investigating the neurotoxicity of past, present, and future flame retardants 52, Part B, 194–209. doi:10.1016/j.ntt.2015.08.010

Jariyasopit, N., McIntosh, M., Zimmermann, K., Arey, J., Atkinson, R., Cheong, P.H.-Y., Carter, R.G., Yu, T.-W., Dashwood, R.H., Massey Simonich, S.L., 2013. Novel nitro-PAH formation from heterogeneous reactions of PAHs with NO2, NO3/N2O5, and OH radicals: prediction, laboratory studies, and mutagenicity. Environ. Sci. Technol. 48, 412–419.

Jariyasopit, N., Zimmermann, K., Schrlau, J., Arey, J., Atkinson, R., Yu, T.-W., Dashwood, R.H., Tao, S., Simonich, S.L.M., 2014a. Heterogeneous Reactions of Particulate Matter-Bound PAHs and NPAHs with NO3/N2O5, OH Radicals, and O3 under Simulated Long-Range Atmospheric Transport Conditions: Reactivity and Mutagenicity. Environ. Sci. Technol. 48, 10155–10164.

Jin, Y., Liu, Z., Liu, F., Ye, Y., Peng, T., Fu, Z., 2015. Embryonic exposure to cadmium (II) and chromium (VI) induce behavioral alterations, oxidative stress and immunotoxicity in zebrafish (Danio rerio). Neurotoxicol. Teratol. 48, 9–17. doi:10.1016/j.ntt.2015.01.002

Joe Jr, F.L., Salemme, J., Fazio, T., 1985. Liquid chromatographic determination of basic nitrogen-containing polynuclear aromatic hydrocarbons in smoked foods. J.-Assoc. Off. Anal. Chem. 69, 218–222.

Johnson, D.E., Riley, M.G., Cornish, H.H., 1984. Acute target organ toxicity of 1-nitronaphthalene in the rat. J. Appl. Toxicol. 4, 253–257.

Jones, H.S., Panter, G.H., Hutchinson, T.H., Chipman, J.K., 2010. Oxidative and Conjugative Xenobiotic Metabolism in Zebrafish Larvae In Vivo. Zebrafish 7, 23–30. doi:10.1089/zeb.2009.0630

Jönsson, M.E., Orrego, R., Woodin, B.R., Goldstone, J.V., Stegeman, J.J., 2007. Basal and 3,3′,4,4′,5-pentachlorobiphenyl-induced expression of cytochrome P450 1A, 1B and 1C genes in zebrafish. Toxicol. Appl. Pharmacol. 221, 29–41. doi:10.1016/j.taap.2007.02.017

Jules, G.E., Pratap, S., Ramesh, A., Hood, D.B., 2012. In utero exposure to benzo(a)pyrene predisposes offspring to cardiovascular dysfunction in later-life. Toxicology 295, 56–67. doi:10.1016/j.tox.2012.01.017

Jung, D., Matson, C.W., Collins, L.B., Laban, G., Stapleton, H.M., Bickham, J.W., Swenberg, J.A., Giulio, R.T.D., 2011. Genotoxicity in Atlantic killifish (Fundulus heteroclitus) from a PAH-contaminated Superfund site on the Elizabeth River, Virginia. Ecotoxicology 20, 1890–1899. doi:10.1007/s10646-011-0727-9

Jung, D.K.J., Klaus, T., Fent, K., 2001. Cytochrome P450 induction by nitrated polycyclic aromatic hydrocarbons, azaarenes, and binary mixtures in fish hepatoma cell line PLHC-1. Environ. Toxicol. Chem. 20, 149–159. doi:10.1002/etc.5620200117

Jung, J.-H., Hicken, C.E., Boyd, D., Anulacion, B.F., Carls, M.G., Shim, W.J., Incardona, J.P., 2013. Geologically distinct crude oils cause a common cardiotoxicity syndrome in developing zebrafish. Chemosphere 91, 1146–1155. doi:10.1016/j.chemosphere.2013.01.019

Jyethi, D.S., Khillare, P.S., Sarkar, S., 2014. Risk assessment of inhalation exposure to polycyclic aromatic hydrocarbons in school children. Environ. Sci. Pollut. Res. 21, 366–378. doi:10.1007/s11356-013-1912-6

Kafafi, S.A., Afeefy, H.Y., Ali, A.H., Said, H.K., Kafafi, A.G., 1993. Binding of polychlorinated biphenyls to the aryl hydrocarbon receptor. Environ. Health Perspect. 101, 422–428.

Kameda, T., Azumi, E., Fukushima, A., Tang, N., Matsuki, A., Kamiya, Y., Toriba, A., Hayakawa, K., 2016. Mineral dust aerosols promote the formation of toxic nitropolycyclic aromatic compounds. Sci. Rep. 6. doi:10.1038/srep24427

Kamens, R.M., Guo, J., Guo, Z., McDow, S.R., 1990. Polynuclear aromatic hydrocarbon degradation by heterogeneous reactions with N2O5 on atmospheric particles. Atmospheric Environ. Part Gen. Top. 24, 1161–1173. doi:10.1016/0960-1686(90)90081-W

Kang, P., Dalvie, D., Smith, E., Zhou, S., Deese, A., Nieman, J.A., 2008. Bioactivation of Flutamide Metabolites by Human Liver Microsomes. Drug Metab. Dispos. 36, 1425–1437. doi:10.1124/dmd.108.020370

Karchner, S.I., Franks, D.G., Hahn, M.E., 2005. AHR1B, a new functional aryl hydrocarbon receptor in zebrafish: tandem arrangement of ahr1b and ahr2 genes. Biochem. J. 392, 153–161. doi:10.1042/BJ20050713

Karickhoff, S.W., Brown, D.S., Scott, T.A., 1979. Sorption of hydrophobic pollutants on natural sediments. Water Res. 13, 241–248. doi:10.1016/0043-1354(79)90201-X

Kewley, R.J., Whitelaw, M.L., Chapman-Smith, A., 2004. The mammalian basic helix–loop–helix/PAS family of transcriptional regulators. Int. J. Biochem. Cell Biol. 36, 189–204. doi:10.1016/S1357-2725(03)00211-5

Khalil, W.K.B., Mahmoud, M.A., Zahran, M.M., Mahrous, K.F., 2007. A sub-acute study of metronidazole toxicity assessed in Egyptian Tilapia zillii. J. Appl. Toxicol. 27, 380–390. doi:10.1002/jat.1217

Khalili, N.R., Scheff, P.A., Holsen, T.M., 1995. PAH source fingerprints for coke ovens, diesel and, gasoline engines, highway tunnels, and wood combustion emissions. Atmos. Environ. 29, 533–542. doi:10.1016/1352-2310(94)00275-P

Kim, K.-H., Jahan, S.A., Kabir, E., 2011. A review of diseases associated with household air pollution due to the use of biomass fuels. J. Hazard. Mater. 192, 425–431. doi:10.1016/j.jhazmat.2011.05.087

Kim, K.-H., Park, H.-J., Kim, J.H., Kim, S., Williams, D.R., Kim, M.-K., Jung, Y.D., Teraoka, H., Park, H.-C., Choy, H.E., Shin, B.A., Choi, S.-Y., 2013. Cyp1a reporter zebrafish reveals target tissues for dioxin. Aquat. Toxicol. Amst. Neth. 134–135, 57–65. doi:10.1016/j.aquatox.2013.03.010

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995a. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310. doi:10.1002/aja.1002030302

Knecht, A.L., Goodale, B.C., Truong, L., Simonich, M.T., Swanson, A.J., Matzke, M.M., Anderson, K.A., Waters, K.M., Tanguay, R.L., 2013b. Comparative developmental toxicity of environmentally relevant oxygenated PAHs. Toxicol. Appl. Pharmacol. 271, 266–275. doi:10.1016/j.taap.2013.05.006

Knecht, A.L., Truong, L., Simonich, M.T., Tanguay, R.L., 2017. Developmental benzo[a]pyrene (B[a]P) exposure impacts larval behavior and impairs adult learning in zebrafish. Neurotoxicol. Teratol. 59, 27–34. doi:10.1016/j.ntt.2016.10.006

Kolahgar, B., Hoffmann, A., Heiden, A.C., 2002. Application of stir bar sorptive extraction to the determination of polycyclic aromatic hydrocarbons in aqueous samples. J. Chromatogr. A 963, 225–230. doi:10.1016/S0021-9673(02)00361-8

Kovacic, P., Somanathan, R., 2014. Nitroaromatic compounds: Environmental toxicity, carcinogenicity, mutagenicity, therapy and mechanism. J. Appl. Toxicol. 34, 810–824.

Kukučka, P., Lammel, G., Dvorská, A., Klánová, J., Möller, A., Fries, E., 2010. Contamination of Antarctic snow by polycyclic aromatic hydrocarbons dominated by combustion sources in the polar region. Environ. Chem. 7, 504–513. doi:10.1071/EN10066

Kuriyama, A., Jackson, J.L., Doi, A., Kamiya, T., 2011. Metronidazole-induced central nervous system toxicity: a systematic review. Clin. Neuropharmacol. 34, 241–247.

Lafontaine, S., Schrlau, J., Butler, J., Jia, Y., Harper, B., Harris, S., Bramer, L.M., Waters, K.M., Harding, A., Simonich, S.L.M., 2015. Relative Influence of Trans-Pacific and Regional

Atmospheric Transport of PAHs in the Pacific Northwest, U.S. Environ. Sci. Technol. 49, 13807–13816. doi:10.1021/acs.est.5b00800

Larsson, B.K., Sahlberg, G.P., Eriksson, A.T., Busk, L.A., 1983. Polycyclic aromatic hydrocarbons in grilled food. J. Agric. Food Chem. 31, 867–873.

Lawson, N.D., Weinstein, B.M., 2002. In Vivo Imaging of Embryonic Vascular Development Using Transgenic Zebrafish. Dev. Biol. 248, 307–318. doi:10.1006/dbio.2002.0711

Lee, M.-S., Magari, S., Christiani, D.C., 2011. Cardiac autonomic dysfunction from occupational exposure to polycyclic aromatic hydrocarbons. Occup. Environ. Med. 68, 474–478. doi:10.1136/oem.2010.055681

Lewandoski, M., 2001. Conditional control of gene expression in the mouse. Nat. Rev. Genet. 2, 743–755. doi:10.1038/35093537

Lewtas, J., 2007. Air pollution combustion emissions: Characterization of causative agents and mechanisms associated with cancer, reproductive, and cardiovascular effects. Mutat. Res. Mutat. Res., The Sources and Potential Hazards of Mutagens in Complex Environmental Matrices - Part II 636, 95–133. doi:10.1016/j.mrrev.2007.08.003

Li, W., Wang, C., Shen, H., Su, S., Shen, G., Huang, Y., Zhang, Y., Chen, Y., Chen, H., Lin, N., Zhuo, S., Zhong, Q., Wang, X., Liu, J., Li, B., Liu, W., Tao, S., 2015. Concentrations and origins of nitro-polycyclic aromatic hydrocarbons and oxy-polycyclic aromatic hydrocarbons in ambient air in urban and rural areas in northern China. Environ. Pollut. 197, 156–164. doi:10.1016/j.envpol.2014.12.019

Li, Z., He, X., Wang, Z., Yang, R., Shi, W., Ma, H., 2015. in vivo imaging and detection of nitroreductase in zebrafish by a new near-infrared fluorescence off–on probe. Biosens. Bioelectron. 63, 112–116. doi:10.1016/j.bios.2014.07.024

Lieschke, G.J., Currie, P.D., 2007. Animal models of human disease: zebrafish swim into view. Nat. Rev. Genet. 8, 353–367. doi:10.1038/nrg2091

Liu, L., Wu, F., Haderlein, S., Grathwohl, P., 2013. Determination of the subcooled liquid solubilities of PAHs in partitioning batch experiments. Geosci. Front. 4, 123–126. doi:10.1016/j.gsf.2012.03.010

Lloyd, A.C., Denton, J.E., 2005. Proposed Identification of Environmental Tobacco Smoke as a Toxic Air Contaminant. Part B Health Eff. Calif. Environ. Prot. Agency Off. Environ. Health Hazard Assess. Httpwww Arb Ca Govregactets2006ets2006 Htm Accès Plus Récent Au Lien 29 Juillet 2009.

Lundstedt, S., Haglund, P., Öberg, L., 2003. Degradation and formation of polycyclic aromatic compounds during bioslurry treatment of an aged gasworks soil. Environ. Toxicol. Chem. 22, 1413–1420. doi:10.1002/etc.5620220701

MacRae, C.A., Peterson, R.T., 2015. Zebrafish as tools for drug discovery. Nat. Rev. Drug Discov. 14, 721–731. doi:10.1038/nrd4627

Maga, J.A., 1986. Polycyclic aromatic hydrocarbon (PAH) composition of mesquite (Prosopis fuliflora) smoke and grilled beef. J. Agric. Food Chem. 34, 249–251.

Mahler, B.J., Van Metre, P.C., Foreman, W.T., 2014. Concentrations of polycyclic aromatic hydrocarbons (PAHs) and azaarenes in runoff from coal-tar- and asphalt-sealcoated pavement. Environ. Pollut. 188, 81–87. doi:10.1016/j.envpol.2014.01.008

Mandrell, D., Truong, L., Jephson, C., Sarker, M.R., Moore, A., Lang, C., Simonich, M.T., Tanguay, R.L., 2012. Automated Zebrafish Chorion Removal and Single Embryo

Placement Optimizing Throughput of Zebrafish Developmental Toxicity Screens. J. Lab. Autom. 17, 66–74. doi:10.1177/2211068211432197

Manzetti, S., 2013. Polycyclic Aromatic Hydrocarbons in the Environment: Environmental Fate and Transformation. Polycycl. Aromat. Compd. 33, 311–330. doi:10.1080/10406638.2013.781042

Marshall, T.C., Royer, R.E., Li, A.P., Kusewitt, D.F., Brooks, A.L., 1982. Acute and genetic toxicity of 1-nitropyrene and its fate after single oral doses to rats. J. Toxicol. Environ. Health Part Curr. Issues 10, 373–384.

Massarsky, A., Jayasundara, N., Bailey, J.M., Oliveri, A.N., Levin, E.D., Prasad, G.L., Di Giulio, R.T., 2015a. Teratogenic, bioenergetic, and behavioral effects of exposure to total particulate matter on early development of zebrafish (Danio rerio) are not mimicked by nicotine. Neurotoxicol. Teratol. 51, 77–88. doi:10.1016/j.ntt.2015.09.006

Mathew, L.K., Andreasen, E.A., Tanguay, R.L., 2006. Aryl hydrocarbon receptor activation inhibits regenerative growth. Mol. Pharmacol. 69, 257–265. doi:10.1124/mol.105.018044

Mathias, J.R., Zhang, Z., Saxena, M.T., Mumm, J.S., 2014. Enhanced Cell-Specific Ablation in Zebrafish Using a Triple Mutant of Escherichia Coli Nitroreductase. Zebrafish 11, 85–97. doi:10.1089/zeb.2013.0937

Mayer, P., Wernsing, J., Tolls, J., de Maagd, P.G.-J., Sijm, D.T.H.M., 1999b. Establishing and Controlling Dissolved Concentrations of Hydrophobic Organics by Partitioning from a Solid Phase. Environ. Sci. Technol. 33, 2284–2290. doi:10.1021/es9808898

McCann, J., Choi, E., Yamasaki, E., Ames, B.N., 1975. Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. 72, 5135–5139.

McCoy, E.C., Rosenkranz, H.S., Mermelstein, R., 1981. Evidence for the existence of a family of bacterial nitroreductases capable of activating nitrated polycyclics to mutagens. Environ. Mutagen. 3, 421–427. doi:10.1002/em.2860030403

McGuire, S.E., Roman, G., Davis, R.L., 2004. Gene expression systems in Drosophila: a synthesis of time and space. Trends Genet. 20, 384–391. doi:10.1016/j.tig.2004.06.012

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. Environ. Sci. Technol. 50, 1561–1569. doi:10.1021/acs.est.5b04786

Menzie, C.A., Potocki, B.B., Santodonato, J., 1992. Exposure to carcinogenic PAHs in the environment. Environ. Sci. Technol. 26, 1278–1284. doi:10.1021/es00031a002

Mesquita, S.R., Dachs, J., van Drooge, B.L., Castro-Jiménez, J., Navarro-Martín, L., Barata, C., Vieira, N., Guimarães, L., Piña, B., 2016. Toxicity assessment of atmospheric particulate matter in the Mediterranean and Black Seas open waters. Sci. Total Environ. 545–546, 163–170. doi:10.1016/j.scitotenv.2015.12.055

Meyer, S., Cartellieri, S., Steinhart, H., 1999. Simultaneous Determination of PAHs, Hetero-PAHs (N, S, O), and Their Degradation Products in Creosote-Contaminated Soils. Method Development, Validation, and Application to Hazardous Waste Sites. Anal. Chem. 71, 4023–4029. doi:10.1021/ac990136j

Michalopoulos, G.K., DeFrances, M.C., 1997. Liver Regeneration. Science 276, 60–66. doi:10.1126/science.276.5309.60

Miller, J.S., Olejnik, D., 2001. Photolysis of polycyclic aromatic hydrocarbons in water. Water Res. 35, 233–243. doi:10.1016/S0043-1354(00)00230-X

Miranda, C.L., Chung, W.G., Wang-Buhler, J.-L., Musafia-Jeknic, T., Baird, W.M., Buhler, D.R., 2006. Comparative in vitro metabolism of benzo[a]pyrene by recombinant zebrafish CYP1A and liver microsomes from β-naphthoflavone-treated rainbow trout. Aquat. Toxicol. 80, 101–108. doi:10.1016/j.aquatox.2006.07.018

Misaki, K., Takamura-Enya, T., Ogawa, H., Takamori, K., Yanagida, M., 2016. Tumour-promoting activity of polycyclic aromatic hydrocarbons and their oxygenated or nitrated derivatives. Mutagenesis 31, 205–213. doi:10.1093/mutage/gev076

Mitchell, J.R., Jollow, D.J., Potter, W.Z., Davis, D.C., Gillette, J.R., Brodie, B.B., 1973. Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. J. Pharmacol. Exp. Ther. 187, 185–194.

Möller, L., 1994. In vivo metabolism and genotoxic effects of nitrated polycyclic aromatic hydrocarbons. Environ. Health Perspect. 102, 139–146.

Mukherji, S., Peters, C.A., Weber, W.J., 1997. Mass Transfer of Polynuclear Aromatic Hydrocarbons from Complex DNAPL Mixtures. Environ. Sci. Technol. 31, 416–423. doi:10.1021/es960227n

Mulero-Navarro, S., Fernandez-Salguero, P.M., 2016. New Trends in Aryl Hydrocarbon Receptor Biology. Signaling 45. doi:10.3389/fcell.2016.00045

Nachtman, J.P., Wei, E.T., 1982. Evidence for enzymatic reduction of 1-nitropyrene by rat liver fractions. Cell. Mol. Life Sci. 38, 837–838.

Nebert, D.W., Dalton, T.P., Okey, A.B., Gonzalez, F.J., 2004. Role of Aryl Hydrocarbon Receptor-mediated Induction of the CYP1 Enzymes in Environmental Toxicity and Cancer. J. Biol. Chem. 279, 23847–23850. doi:10.1074/jbc.R400004200

Neff, J.M., 2002. Bioaccumulation in Marine Organisms: Effect of Contaminants from Oil Well Produced Water. Elsevier.

Ng, G.H.B., Gong, Z., 2013. GFP Transgenic Medaka (Oryzias latipes) under the Inducible cyp1a Promoter Provide a Sensitive and Convenient Biological Indicator for the Presence of TCDD and Other Persistent Organic Chemicals. PLOS ONE 8, e64334. doi:10.1371/journal.pone.0064334

Nič, M., Jirát, J., Košata, B., Jenkins, A., McNaught, A. (Eds.), 2009. IUPAC Compendium of Chemical Terminology: Gold Book, 2.1.0. ed. IUPAC, Research Triagle Park, NC.

Niederer, M., 1998. Determination of polycyclic aromatic hydrocarbons and substitutes (Nitro-, Oxy-PAHs) in urban soil and airborne particulate by GC-MS and NCI-MS/MS. Environ. Sci. Pollut. Res. 5, 209. doi:10.1007/BF02986403

Nielsen, T., Jørgensen, H.E., Larsen, J.C., Poulsen, M., 1996. City air pollution of polycyclic aromatic hydrocarbons and other mutagens: occurrence, sources and health effects. Sci. Total Environ., Highway and Urban Pollution 189, 41–49. doi:10.1016/0048-9697(96)05189-3

Niu, J., Sun, P., Schramm, K.-W., 2007. Photolysis of polycyclic aromatic hydrocarbons associated with fly ash particles under simulated sunlight irradiation. J. Photochem. Photobiol. Chem. 186, 93–98. doi:10.1016/j.jphotochem.2006.07.016

North, T.E., Babu, I.R., Vedder, L.M., Lord, A.M., Wishnok, J.S., Tannenbaum, S.R., Zon, L.I., Goessling, W., 2010. PGE2-regulated wnt signaling and N-acetylcysteine are

synergistically hepatoprotective in zebrafish acetaminophen injury. Proc. Natl. Acad. Sci. 107, 17315–17320. doi:10.1073/pnas.1008209107

Noyes, P.D., Haggard, D.E., Gonnerman, G.D., Tanguay, R.L., 2015. Advanced Morphological — Behavioral Test Platform Reveals Neurodevelopmental Defects in Embryonic Zebrafish Exposed to Comprehensive Suite of Halogenated and Organophosphate Flame Retardants. Toxicol. Sci. 145, 177–195. doi:10.1093/toxsci/kfv044

Nusslein-Volhard, C., Dahm, R., 2002. Zebrafish. OUP Oxford.

Ober, E.A., Field, H.A., Stainier, D.Y.R., 2003. From endoderm formation to liver and pancreas development in zebrafish. Mech. Dev. 120, 5–18. doi:10.1016/S0925-4773(02)00327-1

Ohe, T., Nukaya, H., 1996. Genotoxic activity of 1-nitropyrene in water from the Yodo River, Japan. Sci. Total Environ. 181, 7–12. doi:10.1016/0048-9697(95)04952-5

Ohgaki, H., Matsukura, N., Morino, K., Kawachi, T., Sugimura, T., Morita, K., Tokiwa, H., Hirota, T., 1982. Carcinogenicity in rats of the mutagenic compounds 1-nitropyrene and 3-nitrofluoranthene. Cancer Lett. 15, 1–7. doi:10.1016/0304-3835(82)90068-4

Oliveri, A.N., Bailey, J.M., Levin, E.D., 2015. Developmental exposure to organophosphate flame retardants causes behavioral effects in larval and adult zebrafish. Neurotoxicol. Teratol., Investigating the neurotoxicity of past, present, and future flame retardants 52, Part B, 220–227. doi:10.1016/j.ntt.2015.08.008

Onduka, T., Ojima, D., Ito, K., Mochida, K., Koyama, J., Fujii, K., 2015. Reproductive toxicity of 1-nitronaphthalene and 1-nitropyrene exposure in the mummichog, Fundulus heteroclitus. Ecotoxicology 24, 648–656. doi:10.1007/s10646-014-1412-6

Onduka, T., Ojima, D., Kakuno, A., Ito, K., Koyama, J., Fujii, K., 2012. Nitrated polycyclic aromatic hydrocarbons in the marine environment: acute toxicities for organisms at three trophic levels. Jpn. J. Environ. Toxicol. 15, 1–10. doi:10.11403/jset.15.1

Ozaki, N., Takemoto, N., Kindaichi, T., 2009. Nitro-PAHs and PAHs in Atmospheric Particulate Matters and Sea Sediments in Hiroshima Bay Area, Japan. Water. Air. Soil Pollut. 207, 263–271. doi:10.1007/s11270-009-0134-5

Padilla, S., Corum, D., Padnos, B., Hunter, D.L., Beam, A., Houck, K.A., Sipes, N., Kleinstreuer, N., Knudsen, T., Dix, D.J., Reif, D.M., 2012. Zebrafish developmental screening of the ToxCast™ Phase I chemical library. Reprod. Toxicol., Zebrafish Teratogenesis 33, 174–187. doi:10.1016/j.reprotox.2011.10.018

Pašková, V., Hilscherová, K., Feldmannová, M., Bláha, L., 2006. Toxic effects and oxidative stress in higher plants exposed to polycyclic aromatic hydrocarbons and their N-heterocyclic derivatives. Environ. Toxicol. Chem. 25, 3238–3245. doi:10.1897/06-162R.1

Peddinghaus, S., Brinkmann, M., Bluhm, K., Sagner, A., Hinger, G., Braunbeck, T., Eisenträger, A., Tiehm, A., Hollert, H., Keiter, S.H., 2012. Quantitative assessment of the embryotoxic potential of NSO-heterocyclic compounds using zebrafish (Danio rerio). Reprod. Toxicol., Zebrafish Teratogenesis 33, 224–232. doi:10.1016/j.reprotox.2011.12.005

Pedersen, D.U., Durant, J.L., Taghizadeh, K., Hemond, H.F., Lafleur, A.L., Cass, G.R., 2005. Human cell mutagens in respirable airborne particles from the northeastern United States. 2. Quantification of mutagens and other organic compounds. Environ. Sci. Technol. 39, 9547–9560.

Perera, F.P., Li, Z., Whyatt, R., Hoepner, L., Wang, S., Camann, D., Rauh, V., 2009. Prenatal Airborne Polycyclic Aromatic Hydrocarbon Exposure and Child IQ at Age 5 Years. Pediatrics 124, e195–e202. doi:10.1542/peds.2008-3506

Perera, F.P., Rauh, V., Whyatt, R.M., Tang, D., Tsai, W.Y., Bernert, J.T., Tu, Y.H., Andrews, H., Barr, D.B., Camann, D.E., Diaz, D., Dietrich, J., Reyes, A., Kinney, P.L., 2005a. A Summary of Recent Findings on Birth Outcomes and Developmental Effects of Prenatal ETS, PAH, and Pesticide Exposures. NeuroToxicology, Infant and Child Neurotoxicity StudiesTwenty-first International Neurotoxicology Conference 26, 573–587. doi:10.1016/j.neuro.2004.07.007

Perera, F.P., Whyatt, R.M., Jedrychowski, W., Rauh, V., Manchester, D., Santella, R.M., Ottman, R., 1998. Recent Developments in Molecular Epidemiology: A Study of the Effects of Environmental Polycyclic Aromatic Hydrocarbons on Birth Outcomes in Poland. Am. J. Epidemiol. 147, 309–314.

Perkins, A., Phillips, J., Kerkvliet, N., Tanguay, R., Perdew, G., Kolluri, S., Bisson, W., 2014. A Structural Switch between Agonist and Antagonist Bound Conformations for a Ligand-Optimized Model of the Human Aryl Hydrocarbon Receptor Ligand Binding Domain. Biology 3, 645–669. doi:10.3390/biology3040645

Peters, C.A., Knightes, C.D., Brown, D.G., 1999. Long-Term Composition Dynamics of PAH-Containing NAPLs and Implications for Risk Assessment. Environ. Sci. Technol. 33, 4499–4507. doi:10.1021/es981203e

Peters, C.A., Mukherji, S., Knightes, C.D., Weber, W.J., 1997. Phase Stability of Multicomponent NAPLs Containing PAHs. Environ. Sci. Technol. 31, 2540–2546. doi:10.1021/es960948m

Petersen, K., Fetter, E., Kah, O., Brion, F., Scholz, S., Tollefsen, K.E., 2013. Transgenic (cyp19a1b-GFP) zebrafish embryos as a tool for assessing combined effects of oestrogenic chemicals. Aquat. Toxicol. 138–139, 88–97. doi:10.1016/j.aquatox.2013.05.001

Peterson, F.J., Mason, R.P., Hovsepian, J., Holtzman, J.L., 1979. Oxygen-sensitive and -insensitive nitroreduction by Escherichia coli and rat hepatic microsomes. J. Biol. Chem. 254, 4009–4014.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 29, e45–e45. doi:10.1093/nar/29.9.e45

Philibert, D.A., Philibert, C.P., Lewis, C., Tierney, K.B., 2016. Comparison of Diluted Bitumen (Dilbit) and Conventional Crude Oil Toxicity to Developing Zebrafish. Environ. Sci. Technol. doi:10.1021/acs.est.6b00949

Pisharath, H., Parsons, M., 2009. Nitroreductase-Mediated Cell Ablation in Transgenic Zebrafish Embryos, in: Lieschke, G.J., Oates, A.C., Kawakami, K. (Eds.), Zebrafish, Methods in Molecular Biology. Humana Press, pp. 133–143. doi:10.1007/978-1-60327-977-2_9

Pisharath, H., Rhee, J.M., Swanson, M.A., Leach, S.D., Parsons, M.J., 2007. Targeted ablation of beta cells in the embryonic zebrafish pancreas using E. coli nitroreductase. Mech. Dev. 124, 218–229. doi:10.1016/j.mod.2006.11.005

P.J. Lindstrom and W.G. Mallard (Ed.), 2015. NIST Chemistry WebBook, NIST Standard Reference Database Number 69. National Institute of Standards and Technology, Gaithersburg, MD.

Polidori, A., Kwon, J., Turpin, B.J., Weisel, C., 2010. Source proximity and residential outdoor concentrations of PM2.5, OC, EC, and PAHs. J. Expo. Sci. Environ. Epidemiol. 20, 457–468. doi:10.1038/jes.2009.39

Prasch, A.L., Teraoka, H., Carney, S.A., Dong, W., Hiraga, T., Stegeman, J.J., Heideman, W., Peterson, R.E., 2003a. Aryl Hydrocarbon Receptor 2 Mediates 2,3,7,8-Tetrachlorodibenzo-p-dioxin Developmental Toxicity in Zebrafish. Toxicol. Sci. 76, 138–150. doi:10.1093/toxsci/kfg202

Puga, A., Ma, C., Marlowe, J.L., 2009. The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways. Biochem. Pharmacol., Biological Functions of the Ah Receptor: Beyond Induction of Cytochrome P450s 77, 713–722. doi:10.1016/j.bcp.2008.08.031

Purohit, V., Basu, A.K., 2000. Mutagenicity of Nitroaromatic Compounds. Chem. Res. Toxicol. 13, 673–692. doi:10.1021/tx000002x

Qian, Y., Posch, T., Schmidt, T.C., 2011. Sorption of polycyclic aromatic hydrocarbons (PAHs) on glass surfaces. Chemosphere 82, 859–865. doi:10.1016/j.chemosphere.2010.11.002

R Core Team, 2013. R: A language and environment for statistical computing. R Found. Stat. Comput. Vienna Austria.

Ramesh, A., Walker, S.A., Hood, D.B., Guillén, M.D., Schneider, K., Weyand, E.H., 2004. Bioavailability and Risk Assessment of Orally Ingested Polycyclic Aromatic Hydrocarbons. Int. J. Toxicol. 23, 301–333. doi:10.1080/10915810490517063

Ramírez, N., Cuadras, A., Rovira, E., Marcé, R.M., Borrull, F., 2011. Risk assessment related to atmospheric polycyclic aromatic hydrocarbons in gas and particle phases near industrial sites. Environ. Health Perspect. 119, 1110.

Rauh, V.A., Whyatt, R.M., Garfinkel, R., Andrews, H., Hoepner, L., Reyes, A., Diaz, D., Camann, D., Perera, F.P., 2004. Developmental effects of exposure to environmental tobacco smoke and material hardship among inner-city children. Neurotoxicol. Teratol. 26, 373–385. doi:10.1016/j.ntt.2004.01.002

Reif, D.M., Truong, L., Mandrell, D., Marvel, S., Zhang, G., Tanguay, R.L., 2015. High-throughput characterization of chemical-associated embryonic behavioral changes predicts teratogenic outcomes. Arch. Toxicol. 1–12. doi:10.1007/s00204-015-1554-1

Reimers, M.J., La Du, J.K., Periera, C.B., Giovanini, J., Tanguay, R.L., 2006. Ethanol-dependent toxicity in zebrafish is partially attenuated by antioxidants. Neurotoxicol. Teratol. 28, 497–508.

Reisen, F., Arey, J., 2005. Atmospheric Reactions Influence Seasonal PAH and Nitro-PAH Concentrations in the Los Angeles Basin. Environ. Sci. Technol. 39, 64–73. doi:10.1021/es035454l

Ren, A., Qiu, X., Jin, L., Ma, J., Li, Z., Zhang, L., Zhu, H., Finnell, R.H., Zhu, T., 2011. Association of selected persistent organic pollutants in the placenta with the risk of neural tube defects. Proc. Natl. Acad. Sci. 108, 12770–12775. doi:10.1073/pnas.1105209108

Rennie, M.Y., Detmar, J., Whiteley, K.J., Yang, J., Jurisicova, A., Adamson, S.L., Sled, J.G., 2011. Vessel tortuousity and reduced vascularization in the fetoplacental arterial tree after maternal exposure to polycyclic aromatic hydrocarbons. Am. J. Physiol. - Heart Circ. Physiol. 300, H675–H684. doi:10.1152/ajpheart.00510.2010

RI DEM, 2008. Rhode Island Air Toxics Guideline.

Riedl, J., Altenburger, R., 2007b. Physicochemical substance properties as indicators for unreliable exposure in microplate-based bioassays. Chemosphere 67, 2210–2220. doi:10.1016/j.chemosphere.2006.12.022

Rochman, C.M., Manzano, C., Hentschel, B.T., Simonich, S.L.M., Hoh, E., 2013. Polystyrene Plastic: A Source and Sink for Polycyclic Aromatic Hydrocarbons in the Marine Environment. Environ. Sci. Technol. 47, 13976–13984. doi:10.1021/es403605f

Roe, F.J., 1983. Toxicologic evaluation of metronidazole with particular reference to carcinogenic, mutagenic, and teratogenic potential. Surgery 93, 158–164.

Rogge, W.F., Hildemann, L.M., Mazurek, M.A., Cass, G.R., Simoneit, B.R.T., 1993. Sources of fine organic aerosol. 2. Noncatalyst and catalyst-equipped automobiles and heavy-duty diesel trucks. Environ. Sci. Technol. 27, 636–651. doi:10.1021/es00041a007

Rosenkranz, H.S., Mermelstein, R., 1983. Mutagenicity and genotoxicity of nitroarenes. Mutat. Res. Genet. Toxicol. 114, 217–267. doi:10.1016/0165-1110(83)90034-9

Sabaté, J., Bayona, J.M., Solanas, A.M., 2001. Photolysis of PAHs in aqueous phase by UV irradiation. Chemosphere 44, 119–124. doi:10.1016/S0045-6535(00)00208-3

Salem, Z., Murray, T., Yunis, A., 1981. The nitroreduction of chloramphenicol by human liver tissue. J. Lab. Clin. Med. 97, 881–886.

Satcher, D., Zeise, L., Dunn, A.J., 2000. Health Effects of Exposure to Environmental Tobacco Smoke: The Report of the California Environmental Protection Agency. DIANE Publishing.

Sauer, J.-M., Eversole, R.R., Lehmann, C.L., Johnson, D.E., Beuving, L.J., 1997. An ultrastructural evaluation of acute 1-nitronaphthalene induced hepatic and pulmonary toxicity in the rat. Toxicol. Lett. 90, 19–27.

Schauer, C., Niessner, R., Pöschl, U., 2003. Polycyclic Aromatic Hydrocarbons in Urban Air Particulate Matter: Decadal and Seasonal Trends, Chemical Degradation, and Sampling Artifacts. Environ. Sci. Technol. 37, 2861–2868. doi:10.1021/es034059s

Schlemitz, S., Pfannhauser, W., 1996. Analysis of nitro-PAHs in food matrices by on-line reduction and high performance liquid chromatography. Food Addit. Contam. 13, 969–977.

Schmidt, J.V., Bradfield, C.A., 1996. Ah Receptor Signaling Pathways. Annu. Rev. Cell Dev. Biol. 12, 55–89. doi:10.1146/annurev.cellbio.12.1.55

Scholz, S., Fischer, S., Gündel, U., Küster, E., Luckenbach, T., Voelker, D., 2008. The zebrafish embryo model in environmental risk assessment—applications beyond acute toxicity testing. Environ. Sci. Pollut. Res. 15, 394–404. doi:10.1007/s11356-008-0018-z

Schreiber, R., Altenburger, R., Paschke, A., Küster, E., 2008. How to deal with lipophilic and volatile organic substances in microtiter plate assays. Environ. Toxicol. Chem. 27, 1676–1682. doi:10.1897/07-504.1

Schuetzle, D., Riley, T.J., Prater, T.J., Harvey, T.M., Hunt, D.F., 1982. Analysis of nitrated polycyclic aromatic hydrocarbons in diesel particulates. Anal. Chem. 54, 265–271. doi:10.1021/ac00239a028

Schultz, T.W., Cajina-Quezada, M., Dumont, J.N., 1980. Structure-toxicity relationships of selected nitrogenous heterocyclic compounds. Arch. Environ. Contam. Toxicol. 9, 591–598. doi:10.1007/BF01056938

Scornaienchi, M.L., Thornton, C., Willett, K.L., Wilson, J.Y., 2010. Functional differences in the cytochrome P450 1 family enzymes from Zebrafish (Danio rerio) using heterologously expressed proteins. Arch. Biochem. Biophys. 502, 17–22. doi:10.1016/j.abb.2010.06.018

Scott, J.A., Incardona, J.P., Pelkki, K., Shepardson, S., Hodson, P.V., 2011. AhR2-mediated, CYP1A-independent cardiovascular toxicity in zebrafish (Danio rerio) embryos exposed to retene. Aquat. Toxicol. 101, 165–174. doi:10.1016/j.aquatox.2010.09.016

Sehnert, A.J., Stainier, D.Y.R., 2002. A window to the heart: can zebrafish mutants help us understand heart disease in humans? Trends Genet. 18, 491–494. doi:10.1016/S0168-9525(02)02766-X

Selderslaghs, I.W.T., Blust, R., Witters, H.E., 2012. Feasibility study of the zebrafish assay as an alternative method to screen for developmental toxicity and embryotoxicity using a training set of 27 compounds. Reprod. Toxicol., Zebrafish Teratogenesis 33, 142–154. doi:10.1016/j.reprotox.2011.08.003

Senft, A.P., Dalton, T.P., Nebert, D.W., Genter, M.B., Puga, A., Hutchinson, R.J., Kerzee, J.K., Uno, S., Shertzer, H.G., 2002. Mitochondrial reactive oxygen production is dependent on the aromatic hydrocarbon receptor. Free Radic. Biol. Med. 33, 1268–1278. doi:10.1016/S0891-5849(02)01014-6

Shailaja, M.S., Rajamanickam, R., Wahidulla, S., 2006. Formation of Genotoxic Nitro-PAH Compounds in Fish Exposed to Ambient Nitrite and PAH. Toxicol. Sci. 91, 440–447. doi:10.1093/toxsci/kfj151

Shen, G., Xue, M., Wei, S., Chen, Y., Wang, B., Wang, R., Lv, Y., Shen, H., Li, W., Zhang, Y., Huang, Y., Chen, H., Wei, W., Zhao, Q., Li, B., Wu, H., Tao, S., 2013. Emissions of parent, nitrated, and oxygenated polycyclic aromatic hydrocarbons from indoor corn straw burning in normal and controlled combustion conditions. J. Environ. Sci. 25, 2072–2080. doi:10.1016/S1001-0742(12)60249-6

Shet, M.S., McPhaul, M., Fisher, C.W., Stallings, N.R., Estabrook, R.W., 1997. Metabolism of the Antiandrogenic Drug (Flutamide) by Human CYP1A2. Drug Metab. Dispos. 25, 1298–1303.

Shimada, T., Fujii-Kuriyama, Y., 2004. Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and1B1. Cancer Sci. 95, 1–6. doi:10.1111/j.1349-7006.2004.tb03162.x

Shimizu, Y., Nakatsuru, Y., Ichinose, M., Takahashi, Y., Kume, H., Mimura, J., Fujii-Kuriyama, Y., Ishikawa, T., 2000. Benzo [a] pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor. Proc. Natl. Acad. Sci. 97, 779–782.

Smith, K.E.C., Oostingh, G.J., Mayer, P., 2009. Passive Dosing for Producing Defined and Constant Exposure of Hydrophobic Organic Compounds during in Vitro Toxicity Tests. Chem. Res. Toxicol. 23, 55–65. doi:10.1021/tx900274j

Sogbanmu, T.O., Nagy, E., Phillips, D.H., Arlt, V.M., Otitoloju, A.A., Bury, N.R., 2016. Lagos lagoon sediment organic extracts and polycyclic aromatic hydrocarbons induce embryotoxic, teratogenic and genotoxic effects in Danio rerio (zebrafish) embryos. Environ. Sci. Pollut. Res. 1–13. doi:10.1007/s11356-016-6490-y

Sonnack, L., Kampe, S., Muth-Köhne, E., Erdinger, L., Henny, N., Hollert, H., Schäfers, C., Fenske, M., 2015. Effects of metal exposure on motor neuron development, neuromasts

and the escape response of zebrafish embryos. Neurotoxicol. Teratol. 50, 33–42. doi:10.1016/j.ntt.2015.05.006

Sousa, M.S.A., Latini, F.R.M., Monteiro, H.P., Cerutti, J.M., 2010. Arginase 2 and nitric oxide synthase: Pathways associated with the pathogenesis of thyroid tumors. Free Radic. Biol. Med. 49, 997–1007. doi:10.1016/j.freeradbiomed.2010.06.006

Stanley, K.A., Curtis, L.R., Massey Simonich, S.L., Tanguay, R.L., 2009. Endosulfan I and endosulfan sulfate disrupts zebrafish embryonic development. Aquat. Toxicol., Zebrafish Issue 95, 355–361. doi:10.1016/j.aquatox.2009.10.008

Su, Y.-H., Zhu, Y.-G., Sheng, G., Chiou, C.T., 2006. Linear Adsorption of Nonionic Organic Compounds from Water onto Hydrophilic Minerals: Silica and Alumina. Environ. Sci. Technol. 40, 6949–6954. doi:10.1021/es0609809

Swanson, H.I., 2002. DNA binding and protein interactions of the AHR/ARNT heterodimer that facilitate gene activation. Chem. Biol. Interact. 141, 63–76. doi:10.1016/S0009-2797(02)00066-2

Sydbom, A., Blomberg, A., Parnia, S., Stenfors, N., Sandström, T., Dahlén, S.-E., 2001. Health effects of diesel exhaust emissions. Eur. Respir. J. 17, 733–746.

Taga, R., Tang, N., Hattori, T., Tamura, K., Sakai, S., Toriba, A., Kizu, R., Hayakawa, K., 2005. Direct-acting mutagenicity of extracts of coal burning-derived particulates and contribution of nitropolycyclic aromatic hydrocarbons. Mutat. Res. Toxicol. Environ. Mutagen. 581, 91–95. doi:10.1016/j.mrgentox.2004.11.013

Tang, D., Li, T., Liu, J.J., Chen, Y., Qu, L., Perera, F., 2006. PAH-DNA Adducts in Cord Blood and Fetal and Child Development in a Chinese Cohort. Environ. Health Perspect. 114, 1297–1300.

Teraoka, H., Dong, W., Tsujimoto, Y., Iwasa, H., Endoh, D., Ueno, N., Stegeman, J.J., Peterson, R.E., Hiraga, T., 2003. Induction of cytochrome P450 1A is required for circulation failure and edema by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin in zebrafish. Biochem. Biophys. Res. Commun. 304, 223–228.

Tevell, A., Lennernäs, H., Jönsson, M., Norlin, M., Lennernäs, B., Bondesson, U., Hedeland, M., 2006. Flutamide Metabolism in Four Different Species in Vitro and Identification of Flutamide Metabolites in Human Patient Urine by High Performance Liquid Chromatography/Tandem Mass Spectrometry. Drug Metab. Dispos. 34, 984–992. doi:10.1124/dmd.105.008516

Titaley, I.A., Chlebowski, A., Truong, L., Tanguay, R.L., Massey Simonich, S.L., 2016. Identification and Toxicological Evaluation of Unsubstituted PAHs and Novel PAH Derivatives in Pavement Sealcoat Products. Environ. Sci. Technol. Lett. doi:10.1021/acs.estlett.6b00116

Tobiszewski, M., Namieśnik, J., 2012. PAH diagnostic ratios for the identification of pollution emission sources. Environ. Pollut. 162, 110–119. doi:10.1016/j.envpol.2011.10.025

Toftgård, R., Franzén, B., Gustafsson, J.-Å., Löfroth, G., 1985. Characterization of TCDD-receptor ligands present in extracts of urban particulate matter. Environ. Int., Genotoxic Air Pollutants 11, 369–374. doi:10.1016/0160-4120(85)90031-5

Truong, L., Bugel, S.M., Chlebowski, A., Usenko, C.Y., Simonich, M.T., Simonich, S.L.M., Tanguay, R.L., 2016. Optimizing multi-dimensional high throughput screening using zebrafish. Reprod. Toxicol. 65, 139–147. doi:10.1016/j.reprotox.2016.05.015

Truong, L., Harper, S.L., Tanguay, R.L., 2011. Evaluation of embryotoxicity using the zebrafish model. Methods Mol. Biol. Clifton NJ 691, 271–279. doi:10.1007/978-1-60761-849-2_16

Truong, L., Moody, I.S., Stankus, D.P., Nason, J.A., Lonergan, M.C., Tanguay, R.L., 2010. Differential stability of lead sulfide nanoparticles influences biological responses in embryonic zebrafish. Arch. Toxicol. 85, 787–798. doi:10.1007/s00204-010-0627-4

Truong, L., Reif, D.M., Mary, L.S., Geier, M.C., Truong, H.D., Tanguay, R.L., 2014. Multidimensional In Vivo Hazard Assessment Using Zebrafish. Toxicol. Sci. 137, 212–233. doi:10.1093/toxsci/kft235

Uno, S., Tanaka, H., Miki, S., Kokushi, E., Ito, K., Yamamoto, M., Koyama, J., 2011. Bioaccumulation of nitroarenes in bivalves at Osaka Bay, Japan. Mar. Pollut. Bull., 6th International Conference on Marine Pollution and Ecotoxicology 63, 477–481. doi:10.1016/j.marpolbul.2011.02.044

US EPA, 2015. Estimation Programs Interface Suite [TM] for Microsoft Windows. United States Environmental Protection Agency, Washington, DC, USA.

US EPA, 2014. Priority Pollutant List [WWW Document]. URL https://www.epa.gov/sites/production/files/2015-09/documents/priority-pollutant-list-epa.pdf (accessed 11.2.16).

US EPA, n.d. Method 8280A [WWW Document]. Anal. Polychlorinated Dibenzo-P-Dioxins Polychlorinated Dibenzofurans High-Resolut. Gas Chromatogr. Resolut. Mass Spectrom. HRGCLRMS. URL http://www.accustandard.com/assets/8280a.pdf (accessed 3.8.16a).

US EPA, n.d. Estimation Programs Interface Suite [TM] for Microsoft Windows. United States Environmental Protection Agency, Washington DC, USA.

US EPA National Center for Environmental Assessment, I.O., Jones, S., n.d. Development of a Relative Potency Factor (RPF) Approach for Polycyclic Aromatic Hydrocarbon (PAH) Mixtures (External Review Draft) [WWW Document]. URL https://cfpub.epa.gov/ncea/iris_drafts/recordisplay.cfm?deid=194584 (accessed 2.3.17).

Usenko, S., Landers, D.H., Appleby, P.G., Simonich, S.L., 2007. Current and Historical Deposition of PBDEs, Pesticides, PCBs, and PAHs to Rocky Mountain National Park. Environ. Sci. Technol. 41, 7235–7241. doi:10.1021/es0710003

Van de Wiele, T.R., Peru, K.M., Verstraete, W., Siciliano, S.D., Headley, J.V., 2004. Liquid chromatography–mass spectrometry analysis of hydroxylated polycyclic aromatic hydrocarbons, formed in a simulator of the human gastrointestinal tract. J. Chromatogr. B 806, 245–253. doi:10.1016/j.jchromb.2004.04.001

Van Metre, P.C., Mahler, B.J., 2010. Contribution of PAHs from coal–tar pavement sealcoat and other sources to 40 U.S. lakes. Sci. Total Environ. 409, 334–344. doi:10.1016/j.scitotenv.2010.08.014

Van Tiem, L.A., Di Giulio, R.T., 2011. AHR2 knockdown prevents PAH-mediated cardiac toxicity and XRE- and ARE-associated gene induction in zebrafish (Danio rerio). Toxicol. Appl. Pharmacol. 254, 280–287. doi:10.1016/j.taap.2011.05.002

Vergauwen, L., Schmidt, S.N., Stinckens, E., Maho, W., Blust, R., Mayer, P., Covaci, A., Knapen, D., 2015b. A high throughput passive dosing format for the Fish Embryo Acute Toxicity test. Chemosphere 139, 9–17. doi:10.1016/j.chemosphere.2015.05.041

Veyrand, B., Sirot, V., Durand, S., Pollono, C., Marchand, P., Dervilly-Pinel, G., Tard, A., Leblanc, J.-C., Le Bizec, B., 2013. Human dietary exposure to polycyclic aromatic

hydrocarbons: Results of the second French Total Diet Study. Environ. Int. 54, 11–17. doi:10.1016/j.envint.2012.12.011

Vicente, E.D., Vicente, A.M., Bandowe, B.A.M., Alves, C.A., 2015. Particulate phase emission of parent polycyclic aromatic hydrocarbons (PAHs) and their derivatives (alkyl-PAHs, oxygenated-PAHs, azaarenes and nitrated PAHs) from manually and automatically fired combustion appliances. Air Qual. Atmosphere Health 1–16. doi:10.1007/s11869-015-0364-1

Wakeham, S.G., 1979. Azaarenes in recent lake sediments. Environ. Sci. Technol. 13, 1118–1123.

Walsh, C.D., Schrlau, J., Simonich, S.M., 2015. Development and Use of a Method for the Determination of Polycyclic Aromatic Hydrocarbon and Organochlorine Pesticide Concentrations in Freshly Fallen Snow. Polycycl. Aromat. Compd. 35, 57–73. doi:10.1080/10406638.2014.910239

Watanabe, M., Ishidate Jr., M., Nohmi, T., 1989. A sensitive method for the detection of mutagenic nitroarenes: construction of nitroreductase-overproducing derivatives of Salmonella typhimurium strains TA98 and TA100. Mutat. Res. Mutagen. Relat. Subj. 216, 211–220. doi:10.1016/0165-1161(89)90007-1

Wei, C., Bandowe, B.A.M., Han, Y., Cao, J., Zhan, C., Wilcke, W., 2015. Polycyclic aromatic hydrocarbons (PAHs) and their derivatives (alkyl-PAHs, oxygenated-PAHs, nitrated-PAHs and azaarenes) in urban road dusts from Xi'an, Central China. Chemosphere 134, 512–520. doi:10.1016/j.chemosphere.2014.11.052

Weigt, S., Huebler, N., Braunbeck, T., von Landenberg, F., Broschard, T.H., 2010. Zebrafish teratogenicity test with metabolic activation (mDarT): Effects of phase I activation of acetaminophen on zebrafish Danio rerio embryos. Toxicology 275, 36–49. doi:10.1016/j.tox.2010.05.012

White, D.T., Mumm, J.S., 2013. The nitroreductase system of inducible targeted ablation facilitates cell-specific regenerative studies in zebrafish. Methods, Zebrafish Methods 62, 232–240. doi:10.1016/j.ymeth.2013.03.017

White, Y.A.R., Woods, D.C., Wood, A.W., 2011. A transgenic zebrafish model of targeted oocyte ablation and de novo oogenesis. Dev. Dyn. 240, 1929–1937. doi:10.1002/dvdy.22695

Whitlock Jr, J.P., 1999. Induction of cytochrome P4501A1. Annu. Rev. Pharmacol. Toxicol. 39, 103–125.

WHO, 2015. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans [WWW Document]. URL http://monographs.iarc.fr/index.php (accessed 11.2.16).

Wickström, K., Pyysalo, H., Plaami-Heikkillä, S., Tuominen, J., 1986. Polycyclic aromatic compounds (PAC) in leaf lettuce. Z. Für Lebensm.-Unters. Forsch. 183, 182–185. doi:10.1007/BF01027443

Wilhelm, M., Ghosh, J.K., Su, J., Cockburn, M., Jerrett, M., Ritz, B., 2012. Traffic-related air toxics and term low birth weight in Los Angeles County, California. Environ. Health Perspect. 120, 132.

Wills, L.P., Matson, C.W., Landon, C.D., Di Giulio, R.T., 2010. Characterization of the recalcitrant CYP1 phenotype found in Atlantic killifish (Fundulus heteroclitus) inhabiting

a Superfund site on the Elizabeth River, VA. Aquat. Toxicol. 99, 33–41. doi:10.1016/j.aquatox.2010.03.015

Wincent, E., Jönsson, M.E., Bottai, M., Lundstedt, S., Dreij, K., 2015. Aryl Hydrocarbon Receptor Activation and Developmental Toxicity in Zebrafish in Response to Soil Extracts Containing Unsubstituted and Oxygenated PAHs. Environ. Sci. Technol. 49, 3869–3877. doi:10.1021/es505588s

Wislocki, P.G., Bagan, E.S., Lu, A.Y.H., Dooley, K.L., Fu, P., Han-Hsu, H., Beland, F.A., Kadlubar, F.F., 1986. Tumorigenicity of nitrated derivatives of pyrene, benz[a]anthracene, chrysene and benzo[a]pyrene in the newborn mouse assay. Carcinogenesis 7, 1317–1322. doi:10.1093/carcin/7.8.1317

Wolska, L., Rawa-Adkonis, M., Namieśnik, J., 2005. Determining PAHs and PCBs in aqueous samples: finding and evaluating sources of error. Anal. Bioanal. Chem. 382, 1389–1397. doi:10.1007/s00216-005-3280-7

Wretling, S., Eriksson, A., Eskhult, G.A., Larsson, B., 2010. Polycyclic aromatic hydrocarbons (PAHs) in Swedish smoked meat and fish. J. Food Compos. Anal. 23, 264–272.

Wu, M.-T., Lee, T.-C., Wu, I.-C., Su, H.-J., Huang, J.-L., Peng, C.-Y., Wang, W., Chou, T.-Y., Lin, M.-Y., Lin, W.-Y., Huang, C.-T., Pan, C.-H., Ho, C.-K., 2011. Whole Genome Expression in Peripheral-Blood Samples of Workers Professionally Exposed to Polycyclic Aromatic Hydrocarbons. Chem. Res. Toxicol. 24, 1636–1643. doi:10.1021/tx200181q

Wysowski, D.K., Fourcroy, J.L., 1996. Flutamide Hepatotoxicity. J. Urol. 155, 209–212. doi:10.1016/S0022-5347(01)66596-0

Xiong, K.M., Peterson, R.E., Heideman, W., 2008. Aryl Hydrocarbon Receptor-Mediated Down-Regulation of Sox9b Causes Jaw Malformation in Zebrafish Embryos. Mol. Pharmacol. 74, 1544–1553. doi:10.1124/mol.108.050435

Xu, X., Hu, H., Kearney, G.D., Kan, H., Sheps, D.S., 2013. Studying the effects of polycyclic aromatic hydrocarbons on peripheral arterial disease in the United States. Sci. Total Environ. 461–462, 341–347. doi:10.1016/j.scitotenv.2013.04.089

Yang, L., Ho, N.Y., Alshut, R., Legradi, J., Weiss, C., Reischl, M., Mikut, R., Liebel, U., Müller, F., Strähle, U., 2009. Zebrafish embryos as models for embryotoxic and teratological effects of chemicals. Reprod. Toxicol., 37th Annual Conference of the European Teratology Society 28, 245–253. doi:10.1016/j.reprotox.2009.04.013

Yang, X.-Y., Igarashi, K., Tang, N., Lin, J.-M., Wang, W., Kameda, T., Toriba, A., Hayakawa, K., 2010. Indirect- and direct-acting mutagenicity of diesel, coal and wood burning-derived particulates and contribution of polycyclic aromatic hydrocarbons and nitropolycyclic aromatic hydrocarbons. Mutat. Res. Toxicol. Environ. Mutagen. 695, 29–34. doi:10.1016/j.mrgentox.2009.10.010

Yeager, R.L., Reisman, S.A., Aleksunes, L.M., Klaassen, C.D., 2009. Introducing the "TCDD-Inducible AhR-Nrf2 Gene Battery." Toxicol. Sci. 111, 238–246. doi:10.1093/toxsci/kfp115

Yoshikawa, T., Giamalva, D.H., Flory, W., Ruhr, L.P., Church, D.F., Pryor, W.A., 1988. Toxicity of aromatic hydrocarbons. VII. Hepatotoxicity of 9-nitrophenanthrene, and protection against it by beta-naphthoflavone. Biochem. Int. 16, 935–940.

Yunker, M.B., Macdonald, R.W., Vingarzan, R., Mitchell, R.H., Goyette, D., Sylvestre, S., 2002. PAHs in the Fraser River basin: a critical appraisal of PAH ratios as indicators of PAH source and composition. Org. Geochem. 33, 489–515. doi:10.1016/S0146-6380(02)00002-5

Zaher, H., Buters, J.T.M., Ward, J.M., Bruno, M.K., Lucas, A.M., Stern, S.T., Cohen, S.D., Gonzalez, F.J., 1998. Protection against Acetaminophen Toxicity in CYP1A2 and CYP2E1 Double-Null Mice. Toxicol. Appl. Pharmacol. 152, 193–199. doi:10.1006/taap.1998.8501

Zhang, Y., Tao, S., 2009. Global atmospheric emission inventory of polycyclic aromatic hydrocarbons (PAHs) for 2004. Atmos. Environ. 43, 812–819. doi:10.1016/j.atmosenv.2008.10.050

Zimmermann, K., Jariyasopit, N., Massey Simonich, S.L., Tao, S., Atkinson, R., Arey, J., 2013. Formation of nitro-PAHs from the heterogeneous reaction of ambient particle-bound PAHs with N2O5/NO3/NO2. Environ. Sci. Technol. 47, 8434–8442.

Zok, S., Görge, G., Kalsch, W., Nagel, R., 1991. Bioconcentration, metabolism and toxicity of substituted anilines in the zebrafish (Brachydanio rerio). Sci. Total Environ., QSAR in Environmental Toxicology 109, 411–421. doi:10.1016/0048-9697(91)90196-L

APPENDICES

Appendix A

Supplemental Data for Chapter Two

Quantitation and prediction of sorptive losses during toxicity testing of polycyclic aromatic hydrocarbons (PAHs) and nitrated PAHs using polystyrene plates

Appendix A1. Exposure concentrations

Appendix A2. Glass plates

Appendix A3. Analytical detail

Appendix A4. Subcooled liquid solubility calculations

Appendix A1. Exposure concentrations

Table A1.1. Nominal concentrations of each analyte in the two exposure solutions.

	High exposure concentration		Low exposure concentration	
Analyte	µM	µg/L (ppb)	µM	µg/L (ppb)
FLA	0.59	120	0.059	12
3NF	0.25	62	0.025	6.2
PYR	0.73	150	0.073	15
1NP	0.40	98	0.04	9.8
1,6DNP	0.20	58	0.02	5.8
CHR	0.27	62	0.027	6.2
6NC	0.24	65	0.024	6.5
BaP	0.23	59	0.023	5.9
6NBaP	0.11	32	0.011	3.2

Appendix A2: Glass plates

Methods

Glass plates were purchased from Cayman Chemical (Ann Arbor, MI). Prior to use, the glass plates were cleaned using a plate cleaner, then rinsed with ethanol and reverse osmosis water. Plates were exposed to PAHs as a mixture, with individual analyte concentrations of 2 µM in 1% DMSO, as is standard for zebrafish toxicity testing in the Tanguay laboratory. Following the 5-day exposure in the 28°C incubator in the dark, the exposure solution was removed and the plates rinsed three times with 50:50 acetone:hexane as the extraction solvent. Surrogate standards were added to the 9.6 mL plate extract, the volume reduced to 300 µL, and solvent exchanged into ethyl acetate. Internal surrogate standards were added and the samples analyzed using GC/MS.

Results

Sorption of PAHs to the plastic plates was 1.6-43%, whereas sorption to the glass plates was 0.096-94% (Appendix 1). The high sorptive losses in the glass plates were likely due to the roughness of the inside surface of the wells, which did not appear to be smoothed following manufacture. Due to the high sorptive losses to the glass plates compared to the plastic plates, we decided to focus the plastic plates for further investigation.

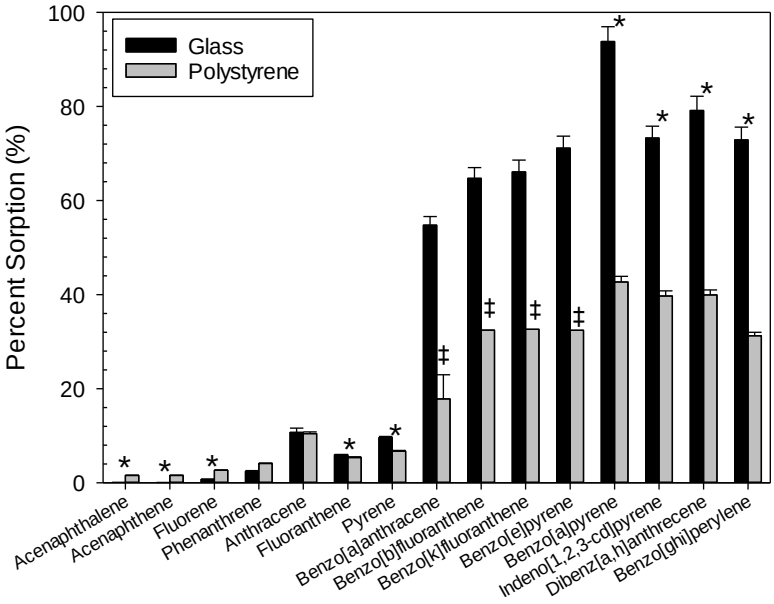

Fig A2.1 Comparison of percent sorption between glass and polystyrene plates. *n*=3, except as indicated with ‡, where *n*=1. Error bars show ± 1 standard error, * indicates statistical significance, with $p<0.05$

Appendix A3: Analytical Detail.

Table A3.2. Ions for identification of analytes and estimated detection limits (EDLs) for GC/MS analysis.

		Quantification ion (*m/z*)	Qualifier ion 1 (*m/z*)	Qualifier ion 2 (*m/z*)	EDLs (pg/µL)
Surrogate and internal standards	Acenaphthene-d_{10}	164	162	NA	NA
	Fluoranthene-d_{10}	212	213	NA	NA
	Pyrene-d_{10}	212	213	NA	NA
	Benzo[a]pyrene-d_{12}	264	260	265	NA
	2-nitrofluorene-d_9	220	221	219	NA
	9-nitroanthracene-d_9	232	223	231	NA
	3-nitrofluoranthene-d_9	256	257	255	NA
	1-nitropyrene-d_9	256	257	258	NA
	6-nitrochrysene-d_9	284	285	283	NA
Analytes	Fluoranthene	202	200	203	0.37
	3-nitrofluoranthene	247	248	NA	0.73
	Pyrene	202	203	200	0.06
	1-nitropyrene	247	248	256	0.30
	1,6-dinitropyrene	292	293	NA	3.0
	Chrysene	228	226	229	0.19
	6-nitrochrysene	273	274	275	0.30
	Benzo[a]pyrene	252	250	253	0.30
	6-nitrobenzo[a]pyrene	297	298	252	2.5

Surrogate and internal standards were added so that the final concentration injected into the instrument was 500 pg/µL. Internal standards were used for the quantification of surrogate recoveries, which ranged from approximately 70 to 105%. Surrogate standards were used to account for analyte losses during sample preparation and quantify the analyte concentrations. Calibration curves were established for each analyte, with an operational concentration range of 5 to 1000 pg/µL, so that concentrations to be measured were within the calibrated concentration range. A calibration check standard was run every 6-8 samples to ensure ongoing accuracy. The estimated detection limits (EDLs of the analytes was calculated using USEPA method 8280 A (Table A3.2). Measured concentrations were reported only in the linear region of the calibration curve and when the concentration was greater than the LOD.

Polystyrene 96-well plates, exposed only to DMSO, were extracted alongside the plates exposed to analytes of interest as a laboratory blank. Levels of PAHs, but not NPAHs, were detected in the blank plates at levels one-third or lower than the analyte concentrations in the PAH and NPAH exposed plates, and were subtracted out from the measured concentrations.

Appendix A4: Subcooled liquid solubility calculations

Subcooled liquid solubility was calculated as previously described (Mukherji et al., 1997; Peters et al., 1999). The fugacity ratio ($\frac{f_s}{f_l}$) can be calculated using thermodynamic properties (P.J. Lindstrom and W.G. Mallard 2015):

$$\ln\left(\frac{f_s}{f_l}\right) = -\frac{\Delta H_f}{RT}\left(1 - \frac{T}{T_f}\right)$$

Where ΔH_f is the enthalpy of fusion (kJ/mol), R is the universal gas constant (0.008314 kJ/mol K), T is the system temperature (K) and T_f is the temperature of fusion (K). Subcooled liquid solubility (S_{ls}) can then be calculated from the fugacity ratio:

$$S_{ls} = \frac{C_{H_2O}}{f_s/f_l}$$

Where S_{ls} is the subcooled liquid solubility and $C_{H_2O}$is the aqueous water solubility (mg/L). Experimental data was used when possible, but for the NPAHs estimated values from EPI Suite were used (US EPA 2015). Where ΔH_f was not available, it was estimated as $\Delta H_f = \Delta S_f * T_f$, where ΔS_f is the entropy of fusion, which can be reasonably estimated as 0.0564 kJ/mol for rigid, planar molecules such as PAHs (Peters et al., 1997). The enthalpies of fusion, fugacity ratios, and subcooled liquid solubilites for the compounds used in this study are listed in Table A4.4.

Table A4.4. Enthalpy of fusion, fugacity ratio, and calculated subcooled liquid water solubilities (S_{ls}) for the compounds used in this study.

	ΔH_f (kJ/mol)	f_s/f_l	S_{ls} (mg/L)
FLA	18.7	0.186	1.40
3NF	22.6	0.0601	1.13
PYR	17.4	0.125	1.08
1NP	18.9	0.103	0.361
1,6DNP	16.8[a]	0.0365	1.48
CHR	26.2	0.0101	0.198
6NC	28.4	0.0118	1.29
BaP	17.3	0.0926	0.0175
6NBaP	30.2	0.00466	0.751

[a] indicates value was calculated using an estimated entropy of fusion value

Appendix B

Supplemental Data for Chapter Three

Mechanistic investigations into the developmental toxicity of nitrated and heterocyclic PAHs

Appendix B1. Chemical properties of compounds investigated in this study

Appendix B2. Immunohistochemistry

Appendix B3. Primer sequences used for qPCR

Appendix B4. Summary table of all data, including EC_{50} values, *cyp1a*/GFP expression at 120 hpf, predicted docking to the zebrafish AHR, and genes with significantly altered expression

Appendix B1. Chemical properties of compounds investigated in this study

Table B1.1. List of all 39 chemicals tested, including the concentration of the DMSO stock solution, the concentration used for cyp1a/GFP screening using the *Tg(cyp1a:nls-egfp)* transgenic zebrafish, as well as qPCR for selected compounds. Additional physical-chemical properties listed include the molecular weight (MW, g/mol), water solubility (µM), and logK_{ow}. Values were compiled from EPI Suite (US EPA, n.d.), where †indicates experimentally derived value, otherwise values were estimated by the EPI Suite software.

	DMSO Stock Concentration (mM)	CYP/PCR conc (µmol)	MW	water solubility (µmol, 25C)	log Kow
NPAHs					
1-nitronaphthalene	10	50	173.17	53.0†	3.19†
2-nitronaphthalene	10	5	173.17	53.4†	3.24†
2-nitrobiphenyl	10	50	199.21	80	3.57
3-nitrobiphenyl	10	50	199.21	44.7	3.87†
4-nitrobiphenyl	10	50	199.21	6.17†	3.82†
3-nitrodibenzofuran	10	5	213.19	2.76	3.53
5-nitroacenaphthalene	10	50	197.19	4.51	3.85†
2-nitrofluorene	10	3.56	211.22	1.02†	3.37†
9-nitroanthracene	10	50	223.23	0.645†	4.78†
9-nitrophenanthrene	10	50	223.23	1.31	4.16
2-nitrodibenzothiophene	10	5	229.25	1.31	3.98
3-nitrophenanthrene	10	50	223.23	1.31	4.16
2-nitroanthracene	10	50	223.23	1.31	4.16
2-nitrofluoranthene	10	50	247.25	8.58	3.98
3-nitrofluoranthene	10	50	247.25	0.0789†	4.75
1-nitropyrene	10	50	247.25	0.0477†	5.06†
2-nitropyrene	10	50	247.25	0.275	4.75
7-nitrobenz[a]anthracene	10	50	273.29	0.0559	5.34
2,8-dinitrodibenzothiophene	1	5	274.25	0.885	3.8
6-nitrochrysene	10	50	273.29	0.0559	5.34
3-nitrobenzanthrone	1	5	275.26	0.247	4.54
1,3-dinitropyrene	1	3.56	292.25	0.185	4.57
1,6-dinitropyrene	1	5	292.25	0.185	4.57
1,8-dinitropyrene	1	5	292.25	0.185	4.57
6-nitrobenzo[a]pyrene	10	50	297.31	0.0118	5.93
7-nitrobenzo[k]fluoranthene	10	35.6	297.31	0.0118	5.93
3,7-dinitrobenzo[k]fluoranthene	10	50	342.31	0.00791	5.93
Amino PAHs					
1-aminopyrene	10	5	217.27	2.65	4.31†
9-aminophenanthrene	10	50	193.25	17.3	3.56†
HPAHs					
Indole	10	50	117.15	30400†	2.14†
Quinoline	10	50	129.16	47300†	2.03†
2-Methylbenzofuran	10	50	132.16	1214	3.22†
Thianaphthene	10	50	134.2	968.7†	3.12†
8-Methylquinoline	10	50	143.19	3410	2.6†
Carbazole	10	36.5	167.21	10.8†	3.72†
Dibenzofuran	10	50	168.19	18.4†	4.12†
5,6-Benzoquinoline	10	11.2	179.22	439†	3.43†
Acridine	10	50	179.22	214†	3.4†
Xanthene	10	50	182.22	5.59	4.23†

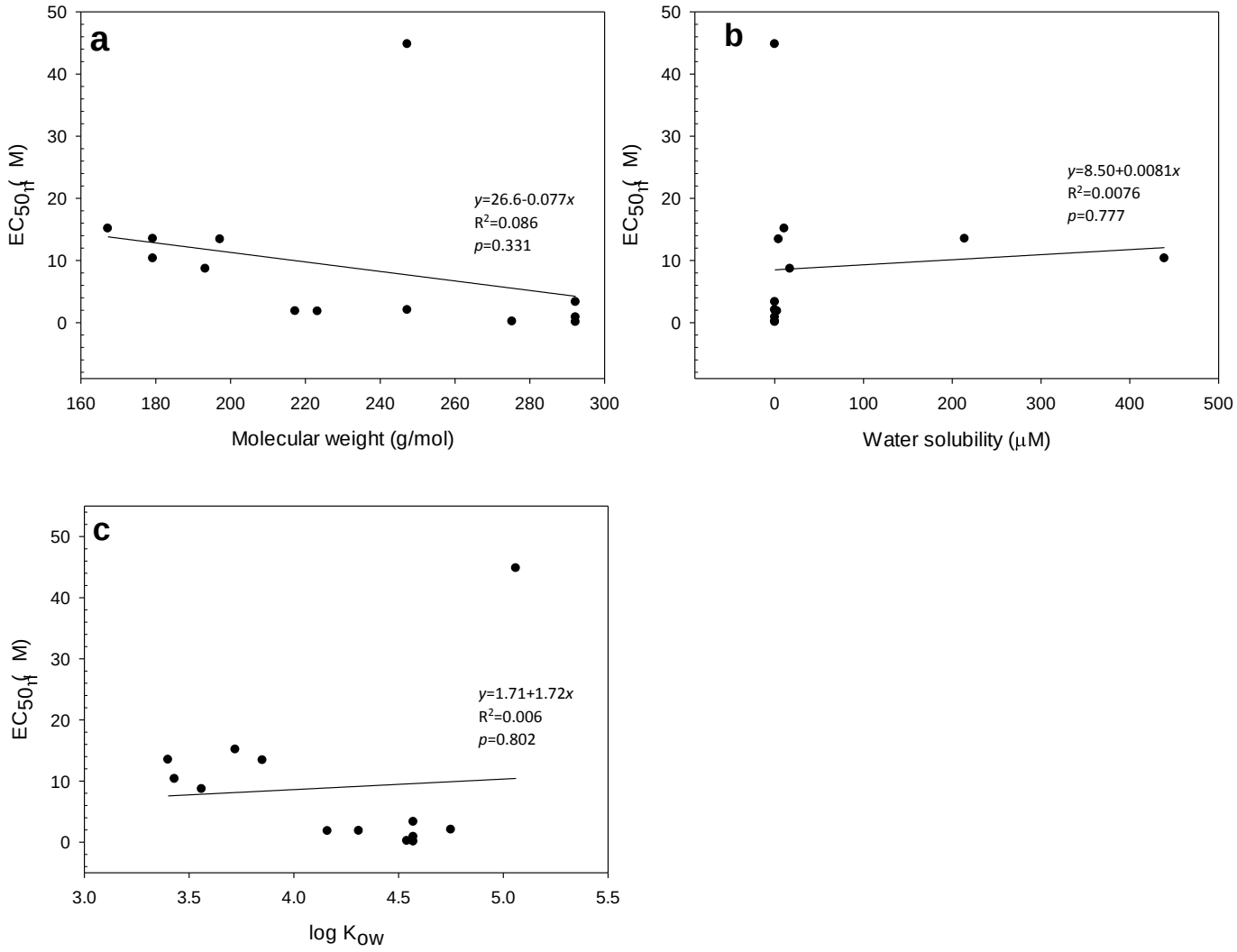

Figure B1.1. Plots of calculable EC_{50} values (values calculated to be within the range of tested concentrations) plotted against (a) molecular weight, (b) water solubility (μM), and (c) log K_{ow}. Linear regressions were performed, and the resulting R^2 and *p*-values are indicated.

Appendix B2. Immunohistochemistry

Methods

For immunohistochemistry (IHC), dechorionated zebrafish embryos were exposed to individual chemicals as described in section 2.2, but without the overnight shaking. Embryos were exposed to individual compounds at concentrations sufficient to induce morphological changes without resulting in significant mortality. At 120hpf, embryos were fixed overnight at 4°C in 4% paraformaldehyde, rinsed in PBS and stored at 4 °C in PBS-NaAzide for IHC. The primary mouse α fish CYP1A monoclonal antibody (Biosense Laboratories) and the secondary antibody Alexafluor 546 rabbit α mouse IgG (H+L) (Molecular Probes, Eugene, OR) were used to assess CYP1A protein localization. The CYP1A immunohistochemistry method has been previously described (Knecht et al., 2013; Mathew et al., 2006). Briefly, fixed embryos were permeabilized with 0.005% trypsin at 4°C for 10 minutes, rinsed with PBS + Tween 20 (PBST) and post-fixed in 4% paraformaldehyde for 10 minutes. Samples were blocked for 1h in 10% normal goat serum and incubated with primary antibody (1:500) in 1% normal goat serum-PBS + 0.5% Triton X-100 (PBSTx) overnight at 4°C. Samples were then washed four times in PBST and incubated with the secondary antibody (1:1000) for two hours. Embryos were imaged by epi-fluorescence microscopy using a Zeiss Axiovert 200 M microscope with 5x and 10x objectives and assessed for CYP1A expression.

Table B2.2. Immunohistochemistry (IHC) results for all compounds tested following exposure to individual compounds from 6-120 hpf.

NPAHs	Liver	Vasculature	Yolk	Skin	Neuromast	None
1-nitronaphthalene						x
2-nitronaphthalene						x
2-nitrobiphenyl						x
3-nitrobiphenyl						x
4-nitrobiphenyl						x
3-nitrodibenzofuran						x
5-nitroacenaphthalene	x					
2-nitrofluorene	x					
9-nitroanthracene						x
9-nitrophenanthrene	x					
2-nitrodibenzothiophene	x					
3-nitrophenanthrene	x					x
2-nitroanthracene						x
2-nitrofluoranthene						x
3-nitrofluoranthene			x			
1-nitropyrene			x			
2-nitropyrene						x
7-nitrobenz[a]anthracene		x				
2,8-dinitrodibenzothiophene	x		x			
6-nitrochrysene		x				
3-nitrobenzanthrone	x		x			
1,3-dinitropyrene						x
1,6-dinitropyrene		x		x		
1,8-dinitropyrene						x
6-nitrobenzo[a]pyrene						x
7-nitrobenzo[k]fluoranthene		x		x	x	
3,7-dinitrobenzo[k]fluoranthene						x
Amino PAHs						
1-aminopyrene		x	x			
9-aminophenanthrene						x
HPAHs						
Indole			x			
Quinoline						x
2-Methylbenzofuran						x
Thianaphthene						x
8-Methylquinoline			x			
Carbazole	x					
Dibenzofuran						x
5,6-Benzoquinoline						x
Acridine	x					
Xanthene						x

Appendix B3. Primer sequences used for qPCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
CYP1A	TGCCGATTTCATCCCTTTCC	AGAGCCGTGCTGATAGTGTC
CYP1B1	CTGCATTGATTTCCGAGACGTG	CACACTCCGTGTTGACAGC
CYP1C1	AGTGGCACAGTCTACTTTGAGAG	TCGTCCATCAGCACTCAG
CYP1C2	GTGGTGGAGCACAGACTAAG	TTCAGTATGAGCCTCAGTCAAAC
gst p1	TTCAGTCCAACGCCATGC	ATGAGATCTGATCGCCAACC
gst p2	TCTGGACTCTTTCCCGTCTCTCAA	ATTCACTGTTTGCCGTTGCCGT
SOD1 (CuZn)	CTAGCCCGCTGACATTACATC	TTGCCCACATAGAAATGCAC
SOD2 (Mn)	CGCATGTTCCCAGACATCTA	GAGCGGAAGATTGAGGATTG
SOD L	AACCTGCGTGGATTTCCTGAAACTG	TGAGGCTTGGTGATGCCAATAACAC
gclc	CTATCTGGAGAACATGGAGG	CATTTTCCTCTGTTGACCGG
gclm	TGGCTTCGTCAGCACACTAAAGTG	TCACGGGAACATTAAAACAGGCCC
gpx 1a	AGATGTCATTCCTGCACACG	AAGGAGAAGCTTCCTCAGCC
gpx 1b	TCTTGGAGAAAGTGGACGTGAACG	TGCTATGCTAAGCAAGAACGGGAC
gpx 7	TTGTGGGTATTGGGGCAAACAATGC	TAATCTCACGCTGCGCTGTTTGAAG
nqo1	AGCACAAGGTGGAGCAGGCG	CGCAGCACTCCATTCTGTAAGGGC
myl6 (cmlc1)	GGAAACGGAACAGTGATGGGTGCT	ACCCGGAGAGGATGTGCTTGATGA
myl7	AGGAGTTTAAGGAGGCTTTTGG	CCTTTTCCTTCTGTTAGCATGG
nppb	CATGGGTGTTTTAAAGTTTCTCC	CTTCAATATTTGCCGCCTTTAC
arg2	AACGGCGGACTGACCTAC	CCAGAGCGGATGCAACTA
ctgfb	TGTAACCAATGACAATGAGC	CATCCAGACAACTCGAAACG
p53	CTCTCCCACCAACATCCACT	ACGTCCACCACCATTTGAAC
β-actin (actb2)	AAGCAGGAGTACGATGAGTC	TGGAGTCCTCAGATGCATTG

Appendix B4. Summary table of all data, including EC_{50} values, *cyp1a*/GFP expression at 120 hpf, predicted docking to the zebrafish AHR, and genes with significantly altered expression

		120 hpf CYP Expression						Predicted High	
	EC_{50} (µM)	Liver	Vasculature	Yolk	Skin	Neuromast	None	**Priority Docking**	**Genes significantly altered (48 or 120 hpf)**
NPAHs									
1-nitronaphthalene							x		
2-nitronaphthalene							x		
2-nitrobiphenyl							x		
3-nitrobiphenyl		x							
4-nitrobiphenyl							x		
3-nitrodibenzofuran							x	†	
5-nitroacenaphthalene	13.41	x						†	*SOD 2 , arg2 , gpx7 , nqo1*
2-nitrofluorene							x	†	
9-nitroanthracene	222.13‡						x	†	
9-nitrophenanthrene	1.81	x						†	
2-nitrodibenzothiophene		x						†	
3-nitrophenanthrene		x						†	
2-nitroanthracene				x				†	*arg2 , SOD 2 , gclc , gst p1 , gpx 1a , myl7*
2-nitrofluoranthene		x	x	x					
3-nitrofluoranthene	2.04		x	x				†	
1-nitropyrene	44.82			x				†	
2-nitropyrene							x		*arg2 , SOD L , gpx 1a , p53*
7-nitrobenz[a]anthracene	237.6‡		x		x			†	
2,8-dinitrodibenzothiophene							x	†	
6-nitrochrysene			x		x			†	
3-nitrobenzanthrone	0.2			x				†	
1,3-dinitropyrene	0.1			x					*gst p1 , gst p2 , arg2 , cgtgfp , myl6 , nppb*
1,6-dinitropyrene	0.88		x		x				*gpx 1a , arg2 , gst p2 , cyp1A , cyp1B1 , cyp1C1 , cyp1C2*
1,8-dinitropyrene	3.33						x		*arg2 , nppb , myl7*
6-nitrobenzo[a]pyrene		x	x		x				
7-nitrobenzo[k]fluoranthene		x	x		x	x			*gst p1 , arg2 , p53 , cyp1A , cyp1B1 , cyp1C1*
3,7-dinitrobenzo[k]fluoranthene			x		x	x			
Amino PAHs									
1-aminopyrene	1.85		x	x				†	
9-aminophenanthrene	8.68	x						†	
HPAHs									
Indole			x		x	x		†	
Quinoline							x		
2-Methylbenzofuran							x		
Thianaphthene							x		
8-Methylquinoline							x		
Carbazole	15.15	x	x					†	
Dibenzofuran	228.3‡						x	†	
5,6-Benzoquinoline	10.35	x			x			†	
Acridine	13.49	x					x	†	
Xanthene							x	†	

Appendix C

Supplemental Data for Chapter Four

Investigating the application of a nitroreductase-expressing transgenic zebrafish line for high-throughput toxicity testing

Appendix C1. The ROS-ID® Hypoxia/Oxidative Stress Detection Kit

Appendix C2. Exposure Windows

Appendix C1: The ROS-ID® Hypoxia/Oxidative Stress Detection Kit

The ROS-ID ® Kit was developed for the detection of hypoxic conditions in cultured cells, as indicated by the activity of nitroreductase enzymes. Production of the red fluorophore from the colorless precursor is an indication of nitroreductase activity. No developmental toxicity was observed in the wild-type or *Tg(l-fabp:CFP-NTR)*^{s891} embryos following treatment with the kit reagents at the suggested concentrations. For both the wild-type and *Tg(l-fabp:CFP-NTR)*^{s891} zebrafish, red fluorescence was observed prominently in the head, neuromasts, and intestine of embryos exposed to the red fluorescent probe (Figure SI 1). Fluorescence was observed following as little as one hour of exposure to the reagents, and was consistent with all other time points tested (data not shown). No red fluorescence was observed in the livers of the wild-type or *Tg(l-fabp:CFP-NTR)*^{s891} embryos, indicating that nitroreductase activity was not occurring in the liver. Exposure to only embryo media in either the wild-type or transgenic fish resulted in only minimal background red fluorescence. Exposure to both the fluorescent probe and DFO positive control did not impact the observed fluorescence in the liver or other tissues of the *Tg(l-fabp:CFP-NTR)*^{s891} embryos, compared to embryos exposed only to the red fluorescent probe.

The ROS-ID® Hypoxia/Oxidative Stress Detection Kit, which has not been previously utilized in the zebrafish model for the detection of nitroreductase activity (used in the kit as an indicator of hypoxia) was meant to further characterize and confirm the activity of nitroreductase in the liver, and potential expression in tissues other than the liver. Nitroreductase activity, indicated by red fluorescence, was expected only in the liver of the *Tg(l-fabp:CFP-NTR)*^{s891} animals, and in no tissues in the wild-type 5D zebrafish. The lack

of fluorescent signal in the liver, in both the presence and absence of the positive control DFO, indicates a lack of nitroreductase activity occurring in the liver. As we had already established that MTZ could undergo nitroreduction in the liver of this transgenic fish line, it is likely that the concentration of the probe was not adequate to visualize the nitroreduction, possibly due to the low affinity of the nitroreductase enzyme for the fluorescent probe. Another plausible explanation for the lack of red fluorescence in the liver would be that that following reduction, the probe was transported to other tissues and excreted. However, complete removal of the fluorescent probe from the liver seems unlikely. The fluorescent signal observed in the intestine after all exposure durations suggests that the embryos ingested the probe, which was then reduced by intestinal microbiota. The presence of nitroreducing intestinal microbiota has been established in other animals, including humans, but had not been previously observed in zebrafish (Fu et al., 1988; Möller, 1994). The fluorescence indicating nitroreductase activity in the neuromasts means either that the neuromasts have a previously uncharacterized nitroreductase activity, or that these highly active cells are taking up and retaining fluorescent probe that had already been reduced and excreted into the aqueous media (Dambly-Chaudière et al., 2003).

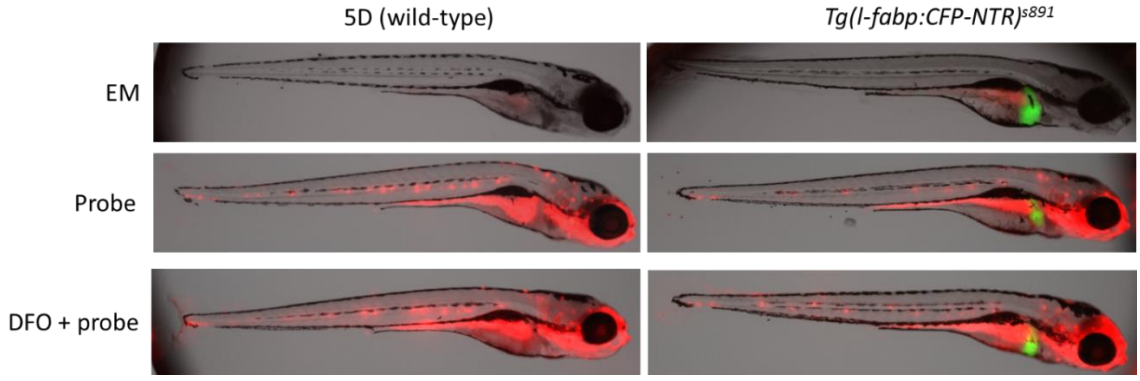

Figure Appendix C.1. Fluorescent patterns observed in *Tg(l-fabp:CFP-NTR)*^{s891} and wild-type embryos following treatment with the Enzo Hypoxia Detection Kit, with exposure to embryo media (EM), the red fluorescent probe included in the kit, or the probe coupled with deferoxamine (DFO), a hypoxia inducer. Embryos were exposed at 96 hpf for 24 hours until 120 hpf. Red fluorescence indicates nitroreductase activity, Green fluorescence indicates the livers of the *Tg(l-fabp:CFP-NTR)*^{s891} zebrafish.

Appendix C2: Exposure Windows

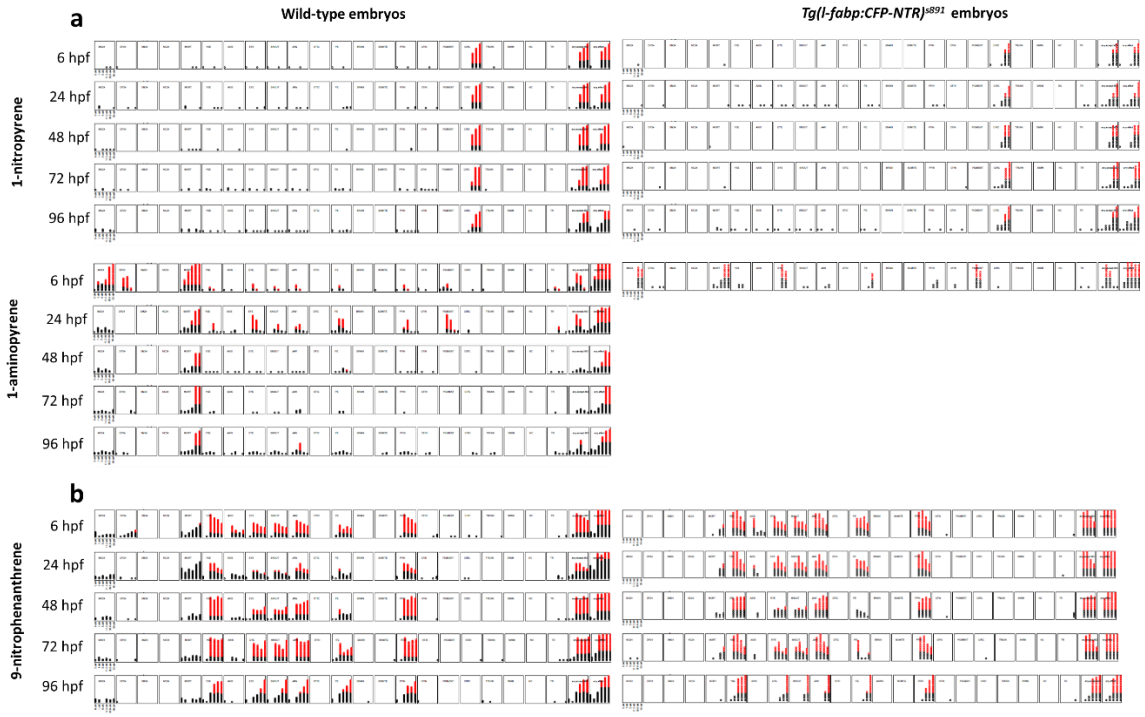

Figure SI C.2. Windows of exposure in wild-type and *Tg(l-fabp:CFP-NTR)*^{s891} embryos exposed to 1-nitropyrene and 1-aminopyrene (a) and 9-nitrophenanthrene (b). Embryos were exposed at 6, 24, 48, 72, or 96 hpf, with morphological evaluations at 24 and 120 hpf.