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THE APPLICATION OF A PASSIVE DOSING SYSTEM FOR DETERMINING ZEBRAFISH EARLY LIFE STAGE TOXICITY OF HYDROCARBONS FOR USE IN CALIBRATING A PREDICTIVE MODEL TO ACUTE AND CHRONIC ENDPOINTS

by

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ABSTRACT OF THE DISSERTATION

THE APPLICATION OF A PASSIVE DOSING SYSTEM FOR DETERMINING ZEBRAFISH EARLY LIFE STAGE TOXICITY OF HYDROCARBONS FOR USE IN CALIBRATING A PREDICTIVE MODEL TO ACUTE AND CHRONIC ENDPOINTS

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The target lipid model has been used to derive water quality objectives that are intended to be protective of chronic effects posed by hydrocarbons. However, experimental Early Life Stage toxicity data for fish are limited and further data are needed to confirm model predictions. Efforts are underway to develop a zebrafish embryo toxicity test guideline to reduce, refine and replace the use of vertebrates in animal testing. An adaptation of this method which includes embryo lethal and sub-lethal developmental endpoints after a 5 day exposure as well as larval survival and growth endpoints during a subsequent 25 day test period is described. To deliver well controlled exposure concentrations, a passive dosing system consisting of silicone coated vials and silicone O-rings was employed. This research consisted of three phases. During the first phase, phenanthrene was used as a reference substance to test efficacy of a passive dosing design as well as compare observed effect data with published effect data. Concentrations shown to cause adverse effects in this phase of the research were in the range of previous studies that have

investigated the chronic effects of phenanthrene on fish. In the second phase, separate exposures to five aromatic hydrocarbons (AHs) including 1-methylnaphthalene, phenanthrene, 1, 2, 3, 4, 5, 6, 7, 8, octahydrophenanthrene, benzo(a)pyrene and chrysene utilizing the passive dosing system and ELS design were investigated. Subsequently, during phase two, six short-term (120 hour) exposures for naphthalene, 1-methylnaphthalene, biphenyl, phenanthrene, pyrene and 1, 2, 3, 4, 5, 6, 7, 8, -octahydrophenanthrene were also employed. Data collected from acute and chronic exposures were used to estimate the acute and chronic critical target lipid body burdens for zebrafish using the TLM framework. During the third phase a simple mixture of ten saturated, unsaturated, and aromatic hydrocarbons with a K_{OW} range of ca. 4 - 7.5 was tested during a 30 day ELS test. Three treatment levels were selected to provide concentrations that corresponded to a toxic unit range above and below the predicted effect concentration assuming concentration addition. Observed acute and chronic effects with the mixture were consistent with the assumption of additive toxicity.

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ABBREVIATIONS

ACR	Acute to Chronic Ratio
AhR	Aryl hydrocarbon Receptor
ATSDR	Agency for Toxic Substances and Disease Registry
CBB	Critical Body Burdon
CTLBB	Critical Target Lipid Body Burdon
DO	Dissolved Oxygen
ELS	Early Life Stage
EPA	Environmental Protection Agency
FET	Fish Embryo Test
GC-MSD	Gas Chromatography-Mass Selection Detector
HC5	Hazard Concentration 5^{th} Percentile
HOC	Hydrophobic Organic Compound
HPLC	High Performance Liquid Chromatography
HS-GC-FID	Head Space-Gas Chromatography with Flame Ionization Detector
K_{OW}	Octanol-water Partition Coefficient
K_{TLW}	Target Lipid-Water Partition Coefficient
MAH	Mono-Aromatic Hydrocarbons
MATC	Maximum Allowable Threshold Concentration
NOEC	No Observed Effect Concentration
OECD	Organization for Economic and Cooperative Development
PAH	Polycyclic Aromatic Hydrocarbon

PDMS	Polydimethylsiloxane
PF	Post Fertilization
PH	Petroleum Hydrocarbon
PNEC	Predicted No Effect Concentration
SPARC	Sparc Performs Automated Reasoning in Chemistry
SPME	Solid Phase MicroExtraction
TCDD	2, 3, 7, 8, - Tetrachlorodibenzo-*p*-dioxin
TLM	Target Lipid Model
TU	Toxic Unit
USEPA	United States Environmental Protection Agency
VOA	Volatile Organic Acid

Chapter 1

General Introduction

1.0 Petroleum Hydrocarbons

Petroleum products are ubiquitous environmental pollutants, with well-documented toxic, mutagenic, and carcinogenic properties (Yu, H. 2002). As such they receive a great deal of attention from regulatory agencies (EPA). They are a family of compounds that contain primarily hydrocarbons (compounds containing only carbon and hydrogen), heteroatom compounds (compounds containing carbon, hydrogen and one or more heteroatoms such as sulfur, nitrogen, or oxygen) and small amounts of trace elements such as metallic components (ATSDR, 1999). Petroleum compounds can be classified into two categories, hydrocarbons and non-hydrocarbons. Hydrocarbons are of interest in this research and make up the majority of the components in petroleum products. They can be grouped into saturated, unsaturated and aromatic hydrocarbons (Prince et al., 2007).

Saturated, aliphatic (straight chain or branched) and alicyclic (cyclic compounds) hydrocarbons are primary components of petroleum products. In the petroleum industry aliphatics are generally referred to as parrafins and isoparrafins respectively (Table 1.1). Alicyclics are often referred to as napthenes or cycloparaffins. Regardless of their molecular configuration they have C-C single bonds and are completely saturated with H atoms (Table 1.1).

Unsaturates, generally form as part of the "cracking" process during the synthesis of other heavier molecules. "Cracking" is a process by which catalysts, solvents and heat are used to split heavy complex molecules (heavy hydrocarbon chains) into lighter, simpler, more useful ones (alkenes and alkynes) (Gary and Handwerk., 2001; Speight., 2006). When there is not enough hydrogen available to saturate the molecule the molecules crack and form smaller molecules with a greater number of double and triple C-C bonds. These unsaturates are referred to as alkenes/olefins (straight chain, branched or cyclic) with a formula of C_nH_{2n} or alkynes/acetylenes (straight chain or branched) with a formula of CnH_{2n-2} (Table 1.1).

Aromatics are also unsaturates based on a single benzene ring structure which can be referred to as monocyclic aromatic hydrocarbons (MAHs) (Table 1.1). Every carbon on the ring bonds with a hydrogen. There may be two or more fused rings in which case they are considered to be polycyclic aromatic hydrocarbons (PAHs) (Table 1.1). The hydrogens associated with the MAH and PAH compounds may also be substituted with side chains making them an alkyl-MAH or alkyl-PAH respectively.

Petroleum hydrocarbons (PHs) are released into the US environment (atmosphere, surface water, soil, and groundwater) through accidents, as releases from industry or as byproducts from commercial or private use (ATSDR 1999; National Research Council 2003; Incardona et al., 2006). Sources include, but are not limited to, municipal and industrial waste incineration, vehicle exhausts, leakages from hazardous waste sites and underground storage tanks, wildfires and asphalt roads, (Eisler, 1987; ATSDR 1999; Daler, D., 2004; Neff et al., 2005). If released into the atmosphere they may be found as gases or bound to organic particulate. In the latter circumstance they can be transported

long distances until they fall from the sky as wet or dry deposition (Latimer et al. 1990; ATSDR 1995; ATSDR 1999; Azimi et al., 2005). If released into the water, the lighter molecular weight fractions can be found floating on the surface, forming surface films while heavier fractions are likely to be found bound to organic matter in the water column or in the sediment (Kan et al., 2009). PHs released into the soil may become bound to organic matter and remain in the soil for a long period of time, while some may become dissolved into groundwater. Their movement throughout the environment is a function of their individual physical-chemical properties.

1.1 Background of Fish Early Life Stage Testing

While many studies have explored the sensitivity of fish Early Life Stages (ELSs) to chemical and/or mechanical stimulus (McKim, 1977; Hutchinson et al. 2006; Korwin-Kossakowski 2008), few have explored the developmental effects as a result of hydrocarbon exposure. In the past, chronic lifecycle tests were used to assess Maximum Allowable Threshold Concentrations (Ward et al. 1980; McKim, 1985). During the 1970s and part of the 1980s, chronic testing was performed with a variety of toxicants to a variety of fish species (Schimmel et al. 1974;McKim, 1977;Woltering, 1984; Elonen et al., 1998). It was observed during this time that there were some stages of development which were more sensitive than others. This observation led to the development of sub-chronic testing and ultimately the Fish Embryo Toxicity test (FET) where the toxicant was only exposed during embryonic stages (Ward et al. 1980; OECD, 2006). Historically larger salmonid and freshwater species were used as model test organisms. However,

since the late 1980s, research has shifted toward shorter, more efficient ELS toxicity tests, which provide similar, fast and reliable effects data. This has substantially reduced the time and effort expended, and ultimately the cost of generating toxicological results. ELS tests encompass some of, if not the most sensitive life stages in fish (McKim 1977; Woltering 1984; Korwin-Kossakowski 2008; Carls et al., 2008; Belanger et al., 2010; MacIntosh et al., 2010; Incardona et al., 2011). The rapidity of growth and number of morphological changes during early fish development are important factors in the usefulness of ELS tests (McKim 1985; Antkiewicz et al., 2005; Augustine-Rauch et al., 2010). It is accepted that chronic lifecycle toxicity tests give a better reflection of the real toxic effects, but because of cost and time consumption they are often replaced with ELS tests (Roex 2002). In most cases ELS tests reflect the effects that would be obtained from full chronic life cycle tests (Van Leween et al. 1985; Miracle et al., 2005; Raimondo et al., 2007).

1.2 Passive Dosing

Conventional dosing (static, semi-static renewals, etc.,) of hydrophobic organic compounds (HOCs) in toxicity tests using nominal concentrations tends to overestimate the freely dissolved concentrations of test compounds due to losses including sorption, volatilization and degradation (Smith, K. et al., 2010; Butler et al., 2013) (Figure 1.1A). Introduction of co-solvents to increase the solubility of a HOC into test systems was also found to be problematic due to their own toxic effects (Hutchinson, T. et al., 2006). Recently, passive dosing techniques have been developed to overcome these limitations

(Table 1.2). They provide continuous partitioning of sparingly soluble compounds from a loaded biocompatible polymer reservoir such as polydimethylsiloxane (PDMS), therefore maintaining defined and constant aqueous exposure concentrations for the duration of toxicity tests (Figure 1.1B) (Butler et al., 2013). Studies have been performed investigating the performance of passive dosing and have successfully applied it to toxicity testing of HOCs (Table 1.2). However, most of the testing previously performed using a passive dosing approach has been done acutely and largely to invertebrates. These initial experiments provided the basis for our testing where we extended the passive dosing approach to include chronic fish ELS testing.

1.3 Hydrocarbon metabolism

Hydrocarbons are ubiquitous environmental contaminants and when they are present in the aqueous phase fish, among other aquatic organisms can become affected. HOCs such as hydrocarbons are readily absorbed across the hydrophobic lipid membranes of the gills and skin primarily by passive diffusion (Tuvikene, 1995; Elskus et al., 2005; Arnot et al., 2010). It has been shown that fish readily transform HOCs to more polar compounds through phase I and II biotransformation mechanisms (Livingstone et al., 1998; Ferriera et al., 2006). The metabolites formed through these mechanisms are generally more water soluble than parent compounds and are generally more easily eliminated from the body. Phase I biotransformation of PAHs in fish are generally associated with the cytochrome P450 (CYP450) enzyme family. CYP450 enzymes are located primarily in the liver, but can also be found in other organs and cells through which foreign substances

(xenobiotics) enter the body (Boelsterli, U., 2002; Chen, 2012). The CYP450 1A1 (CYP1A1) enzyme, catalyzes the addition of a hydroxyl group to the parent compound by the NADPH reaction process (Figure 1.2). After this reaction takes place the PAH parent compound will likely become one of the following; phenol, diol, quinone or an epoxide. Many of these metabolites do not possess sufficient hydrophilicity to be easily excreted, and need to undergo further transformation in order to be eliminated from the body. The formation of the reactive intermediates can cause DNA and protein adducts which result in additional toxicity that can lead to acute and chronic disease. Phase II conjugation provides a means by which further transformation to more water soluble metabolites can be completed. The metabolites synthesized during the phase I catalysis (phenols, quinones, diols, etc,) act as a reactive site to which endogenous ligands such as glucuronide, sulfate, glutathione are attached. The result is a conjugated metabolite which normally has sufficient water solubility to be easily eliminated (NLM., 2010).

1.4 Hydrocarbon Modes of Toxic Action

PHs in their many molecular forms have been shown to be associated with varying levels of toxicity (ATSDR 1999; Smith et al., 2009; Incardona et al., 2011). ELSs of fish have been shown to be sensitive to xenobiotics particularly PHs (McKim et al., 1985; ;Norcross et al., 1996; Billiard et al., 2008;Carls et al., 2008; Foekema et al., 2012;). It is accepted that most hydrocarbon components due to their lipophilicity are readily absorbed into the lipids of an organism.

There are many mechanisms by which hydrocarbons cause toxicity to fish, some specific while others are non-specific. Three primary mechanisms based on embryo/larval toxicity include narcosis (membrane disruption), metabolic activation to form reactive intermediates and Aryl Hydrocarbon Receptor (AhR) mediated, dioxin-like toxicity. Narcosis is a result in disruption of the central and peripheral nervous system and is likely to occur when the lipophilic parent hydrocarbon dissolves into nerve cell membranes and disrupts the function of membrane proteins and lipids. This disruption of the membrane structure and fluidity can disrupt normal cell migration and membrane charge distribution. Passive absorption is a non-specific, reversible effect that is driven by the physical chemical properties of the compound (ATSDR, 1999; Incardona et al., 2004).

In the majority of cases, metabolic biotransformation transforms the parent compounds into less toxic, more water soluble metabolites that are more easily eliminated through the organism, while eliciting little to no adverse health effect. However, in some instances the metabolism of the parent compound, mediated by pathways involving CYP450 enzymes, can result in a more toxic metabolite (Carls et al., 1999; Neilson., 2000; Incardona et al., 2011).

AhR mediated dioxin – like toxicity was observed after the Exxon Valdez oil spill in Prince William Sound, Alaska. Shortly after the spill developing fish embryos presented symptoms such as edema, hemorrhaging and spinal curvature (Billiard et al., 1999). The cardiovascular system is one of the most sensitive systems in the developing embryo because of the complex nature of its formation and development. The general effects observed in developing fish embryos with pericardial edema and dismorphogenesis of the

heart have been call “Blue Sac Disease”. These same symptoms were also observed in previous fish embryo exposures studies involving 2, 3, 7, 8 tetrachlorodibenzo-*p*-dioxin (TCDD) and were found to be mediated by the AhR in developing fish, hence the name”dioxin-like toxicity (Marty et al., 1997; Billiard et al., 1999). PAHs and TCDD affect a similar cytosolic receptor that disrupts vascular endothelial development. Once absorbed, compounds eliciting dioxin like toxicity bind to a ligand-dependent transcription factor called the AhR, resulting in the activation of CYP1A1 among other genes involved in xenobiotic metabolism (Rowlands and Gustafsson, 1997; Scott et al., 2011). Once activated, the AhR has been shown to translocate into the nucleus and heterodimerize with the AhR specific nuclear translocator (Mimura & Kuriyama et al 2003; Mitchell et al., 2009). The resulting complex binds to response elements in the promotor region of the AhR genes and initiates transcription of a battery of genes involved in cellular homeostasis.

1.5 Predicting Hydrocarbon Toxicity

In many cases hydrocarbons are thought to exert their toxic effect through either of two modes of action (1) “Blue sac like” toxicity mediated by activation of the AhR, which controls a battery of genes involved in PAH metabolism, such as CYP4501A1 or, (2) “Nonpolar narcosis”, in which tissue uptake is dependent solely on hydrophobicity where toxicity is mediated through the disruption of cell membrane fluidity by interacting with polar lipids including membrane-associated lipids such as phospholipids, free fatty acids, and lipoproteins (Incardona et al., 2006). Models based on these theories have been

created in an attempt to predict toxicity (McCarty and MacKay 1993; Ditoro et al., 2000; McGrath et al., 2004; Kipka et al 2009; McGrath & DiToro et al., 2009; Parkerton et al., In Press).

The Target Lipid Model (TLM) in which PAHs are thought to bioconcentrate according to their log K_{OW} value (partition coefficient between octanol-water) is based on the Critical Body Burdon (CBB) approach proposed by McCarty and McKay (1993), where the basic hypothesis, is that toxic effects are not observed unless the chemical reaches the site of action (eqn., 1.1).

$$CBB = BCF \times LC50 \qquad (1.1)$$

Where CBB (umol/g), is the Critical Body Burdon of the organism, LC50 is the lethal concentration that causes 50% mortality, and BCF is the Bio-Concentration Factor. However, this approach did not take into account the lipid content of the organism tissue. Nevertheless, the lipid fraction (target lipid) was later recognized as an important parameter influencing residue toxicity relationships (Ditoro et al., 2000). The CBB then became the Critical Target Lipid Body Burdon (CTLBB) which normalized the CBB to the lipid fraction of the organism (eqn., 1.2).

$$CTLBB = \frac{CBB}{f_{lipid}} \qquad (1.2)$$

Where CTLBB (umol/g), is the Critical Target Lipid Body Burdon and f_{lipid} is the lipid fraction of the organism to which the CBB is normalized.

The TLM takes the CTLBB and divides it by a partition coefficient between target lipid to water; K_{TLW} (L/Kg) (eqn., 1.3).

$$LC50 = \frac{CTLBB}{K_{TLW}} \qquad (1.3)$$

TLM predicted toxicity can be represented by a linear relationship between log (LC50) and the log K_{OW} which suggests that toxicity may be caused by a species specific constant and universal body burden (McGrath and DiToro, 2009).

The TLM has been calibrated to 47 individual organisms endpoints (LC50s) including fish, daphnid and algae which are described elsewhere (DiToro et al. 2000; McGrath et al. 2006; McGrath and DiToro, 2009). However, because model parameters are species- and -endpoint specific, further calibration using zebrafish ELS toxicity data would be useful.

The uncertainties associated with predictive models, in particular the TLM have been identified (McGrath and DiToro 2009). A method that adapts the Hazard Concentration (HC)-5 initially proposed by McGrath et al., (2004) has been presented (McGrath and DiToro 2009). This method is used to compute the concentration that protects 95% of the tested species by considering the variance in all the available CTLBBs. Based on an extensive compilation and analysis of acute and chronic toxicity data. Parkerton et al., (In Press) shows that the TLM derived HC-5 is protective of 95% of the organisms exposed.

1.6 Compounds of Interest

Currently there is a need for reliable zebrafish ELS toxicity testing to cyclic saturated, unsaturated and aromatic hydrocarbons. Water solubilities of compounds tested ranged from 0.001 – 25 mg/L with $\log K_{ow}$ values from ca. 3.9-7.5 and molecular weights between 142.2-218.4 g/mol. The structures of these compounds as well as pertinent physico-chemical properties are shown within figure 1.3.

1.9 Objectives

The overall objectives of this dissertation were to generate a sufficient data set for a variety of hydrocarbon compounds that could be used to calibrate the TLM for ELSs of zebrafish. The hypothesis was that regardless of the mode of action by which hydrocarbons exert their toxic effects, the TLM could accurately predict effect concentrations as well as provide a framework for No Observed Effect Concentrations (NOEC) to be predicted (PNEC). The specific aims of this dissertation are broken into bullt points by chapter.

Chapter 2:

- Develop zebrafish culture and test system for assessing acute and chronic ELS effects.
- Evaluate performance of passive dosing technique for delivering consistent aqueous exposure concentrations of single test compounds in zebrafish toxicity tests.

- Compare chronic results obtained for reference compound (phenanthrene) using PD to previously reported fish early life stage toxicity tests.

Chapter 3

- Develop acute and chronic effects data for zebrafish with selected aromatic hydrocarbons using PD approach.
- Assess relative sensitivity of ELS endpoints.
- Investigate whether acute toxicity data are consistent with predictions derived using the target lipid model.
- Determine acute to chronic ratios for zebrafish ELS effects and compare to historical ACRs for other species.
- Assess if HC5 predictions obtained using statistical extrapolation procedure reported by McGrath & Di Toro (2009) are protective of chronic zebrafish ELS effects for the test substances investigated.

Chapter 4

- Evaluate performance of passive dosing technique for delivering consistent aqueous exposure concentrations of multiple test compounds in zebrafish toxicity tests.
- Assess if acute and chronic effects to a mixture of ten cyclic hydrocarbons are consistent with the assumption of concentration addition.

Chapter 5

- Summarize key findings and future research needs.

1.7 Experimental Design

D. rerio embryo/larvae were acutely and chronically exposed to various single hydrocarbons as well as a moderately complex mixture. Acute exposures were performed for 120 hours post-fertilization (PF) while chronic exposures lasted 30 days PF. Including two different exposure periods afforded the opportunity to select exposure concentrations which targeted different developmental life stages. Further, this design allowed empirical acute to chronic ratios (ACRs) to be calculated and compared to earlier compilations across hydrocarbons and test species (McGrath and DiToro 2009). Inclusion of chronic tests also allowed direct comparison of chronic endpoints to TLM-derived HC5 predictions to determine if this framework was protective for chronic effects on fish ELSs.

1.7.1 Acute tests

Embryos were exposed to 5 concentrations and a control per exposure. Concentrations were selected with the use of literature values as well as TLM predictions. Embryos were exposed in 20 mL headspace vials that had 0.13 gram O-rings resting on the bottom. Vials were then capped with teflon coated screw caps and placed into an environmental chamber at 28±1°C. Twenty replicates with one embryo per vial were tested for each treatment. Microscopic observations were performed at 24-hour intervals. Lethal (coagulation, lack of heartbeat) and sub-lethal (pericardial and yolk sac edemas, tail curvature, hatching time) endpoints included those defined in the OECD (2006) FET

draft guideline up to 120 hours post fertilization. The test media for the acute phase was prepared using Instant Ocean® sea salt, 60ug/mL stock salts as described in Westerfield, (2001).

1.7.2 Chronic tests

Larvae were exposed to single compound exposures as well as a mixture of hydrocarbons for 30-days post fertilization each exposure having 3 concentrations and a control. For two of the single exposure compounds (chrysene and benzo a pyrene) which exhibit low aqueous solubility, only a single test concentration and control was investigated at the highest aqueous test concentration that could be achieved in the test system. During the chronic exposures, embryos were initially exposed in vials for 120 hours, similar to the acute experiments discussed above. Upon 120 hours PF, embryos that hatched into larval fish as well as any unhatched embryos exposed in vials were divided and transferred into four different 300 mL flow-through chambers, each with up to 5 fish per replicate chamber (Figure 1.4B, 1 and Figure 1.4C). Four-liter mixing vessels containing 30, 1.0g O-rings for each concentration were prepared (Figure 1.4B, 1). Three, 1.0g O-rings were also added to each customized flow through chamber (Figure 1.4C). This process is shown conceptually in figure 1.4A. The flow through each test chamber was supplied by a peristaltic pump (Figure 1.4B, 2) and was set to a rate of 5 mL/min. Mixing vessels supersaturated with oxygen, were exchanged every 5 days ± 24 hours with a new set of O-rings to ensure optimal water quality, avoid biofouling and limit possible PDMS depletion.

The customized flow through chambers contained three individual ports; two ports were located on top, one of which served as an out-flow port and the other strictly for feeding purposes. The third port was located on bottom and served as the in-flow port. This design allowed for continuous flow with optimum mixing. The out-flow port was partially blocked with steel mesh to prevent larvae from swimming out of the exposure chamber (Figure 1.5). Test media for the chronic phase (days 5-30) was prepared according to *Standard Methods for the Examination of Water and Wastewater* 21st Edition method 8010E (2005), moderately hard reconstituted water ($NaHCO_3$, 12 mg/L; $CaSO_4$ $2H_2O$, 60 mg/L; $MgSO_4$, 60 mg/L; KCl, 4 mg/L; water hardness, 80-100 mg $CaCO_3$/L; Alkalinity, 60 mg $CaCO_3$/L; pH 7.4-7.8).

Upon transfer to the flow through chambers, fish were considered to be exogenously feeding (Belanger et al., 2010) and were fed *Paramecium multimicronucleatumn.* Larval fish were fed *Artemia nauplii* starting at 12 days. Paramecium and brine shrimp were fed *ad libitum*, twice daily throughout the experiment. During the feeding period, flow was turned off. After approximately 20 minutes of feeding, the flow-through system was re-started and flushed into a drainage basin for 10 minutes. After 10 minutes of flushing with test media all mixing vessels were filled and supersaturated with oxygen before starting flow recirculation. Fish were observed daily for immobility, lack of respiratory movement and lack of reaction to mechanical stimulus (OECD, 210). To evaluate potential larval growth effects, surviving fish were measured (total length) at study termination.

1.8 Chemical Analysis

The chemical composition of petroleum products is complex and may change over time. These factors make it challenging to select the most appropriate analytical methods for evaluating environmentally relevant concentrations. During the initial single compound exposures the analysis for phenanthrene in water was performed using static headspace gas chromatography with flame ionization detection (HS-GC-FID) using a Perkin Elmer Autosystem XL gas chromatograph. Similarly during the multiple individual compound exposures analysis of naphthalene, 1-methylnaphthalene, biphenyl, and phenanthrene in water was performed using static HS-GC-FID using a Perkin Elmer Autosystem XL gas chromatograph. Whereas, analysis of 1, 2, 3, 4, 5, 6, 7, 8 octahydrophenanthrene was performed by HS-GC-FID modified with a trap accessory to provide additional sensitivity. Pyrene, B(a)P and chrysene along with all mixture components were analyzed by automated direct immersion DI solid phase microextraction (SPME) coupled with gas chromatography with mass selective detection (GC-MSD). Chemical analysis for the complex hydrocarbon mixture experiment was also performed by automated direct immersion SPME coupled with GC-MSD.

Table 1.1: Examples of common saturated, unsaturated and aromatic hydrocarbons with their associated molecular formula.

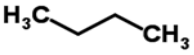
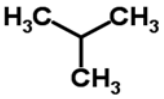
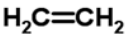
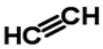
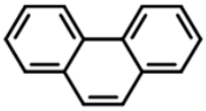
Industry Name	Formal Name	Molecular Formula	Molecular Structure
Paraffins	Butane	C4H10	
Isoparaffins	Isobutane	C4H10	
Cycloparaffins	Cyclohexane	C6H12	
Alkenes/Olefins	Ethylene	C2H4	
Alkynes/Acetylenes	Acetylene	C2H2	
MAHs	Benzene	C6H6	
PAHs	Phenanthrene	C14H10	

Table 1.2: Literature review of sentinel publications revealing the passive dosing approach, class of compound tested, organisms used, passive dosing device used and test duration.

Test Substance Investigated	Organism	Test Duration	Passive dosing device used	Reference
Halogenated aromatics	*	N/A	PDMS film	Mayer et al., 1999
PAHs, alkyl PAHs	*	N/A	silicone tubing	Brown et al., 2001
PAHs	*Folsomia candida*	7 days	PDMS film	Mayer et al., 2008
PAHs	*Daphnia magna*	2 days	PDMS film	Smith et al., 2010
Alkyl PAHs	*Orzias latipes*	17 days	PDMS film	Turcotte et al., 2008
PAHs	*Danio rerio*	30 days	silicone rings	Butler et al., 2013

*Analytical method development studies only. No organisms were used.

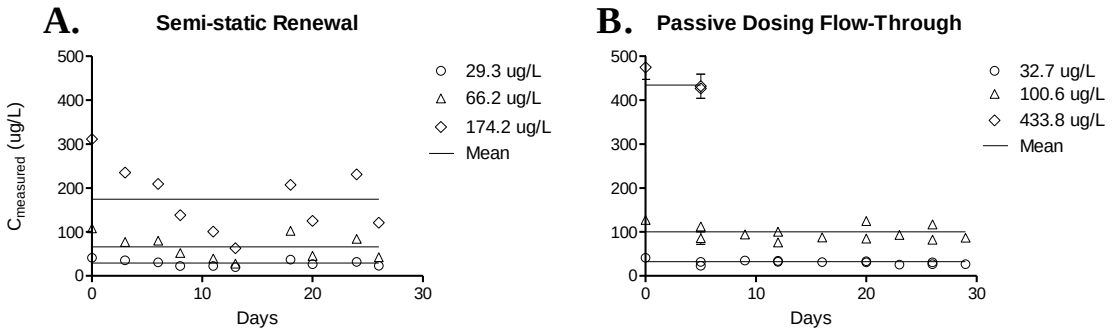


Figure 1.1: Comparison of traditional semi-static renewal versus passive dosing flow-through exposure concentration during fish ELS test. Nominal concentrations for both exposures were 35,122 and 405 µg/L. Mean measured concentrations are shown in the legends. Note the variability of the semistatic renewal exposure compared to the passive dosing flow-through exposure.

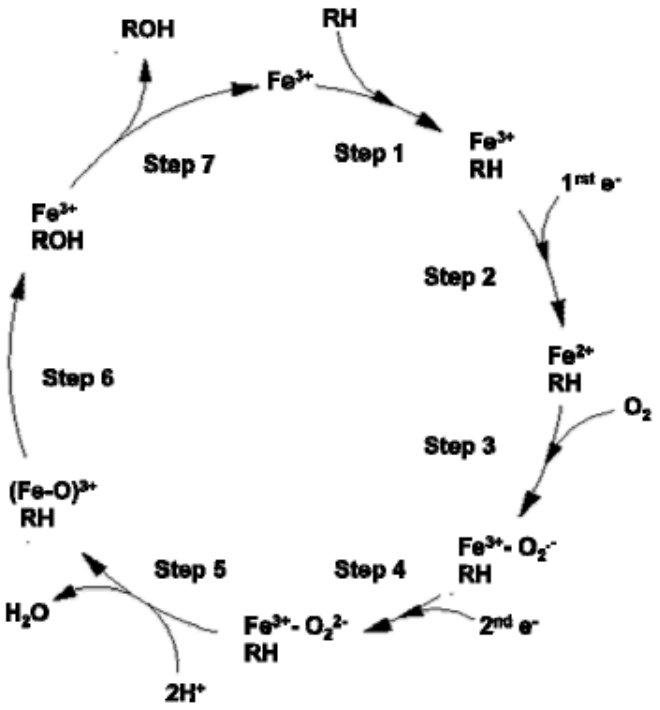


Figure 1.2: CYP 450 catalytic cycle. Step 1- Substrate binding; Step 2- First reduction by NADPH; Step 3- Oxygen binding; Step 4- Second reduction by NADPH; Step 5- Cleavage of O2; Step 6- Product formation; Step 7- Product release. The overall reaction is $RH + O_2 + 2H^+ + 2e^- \longrightarrow ROH + H2O$ (Turcott et al., 2008).

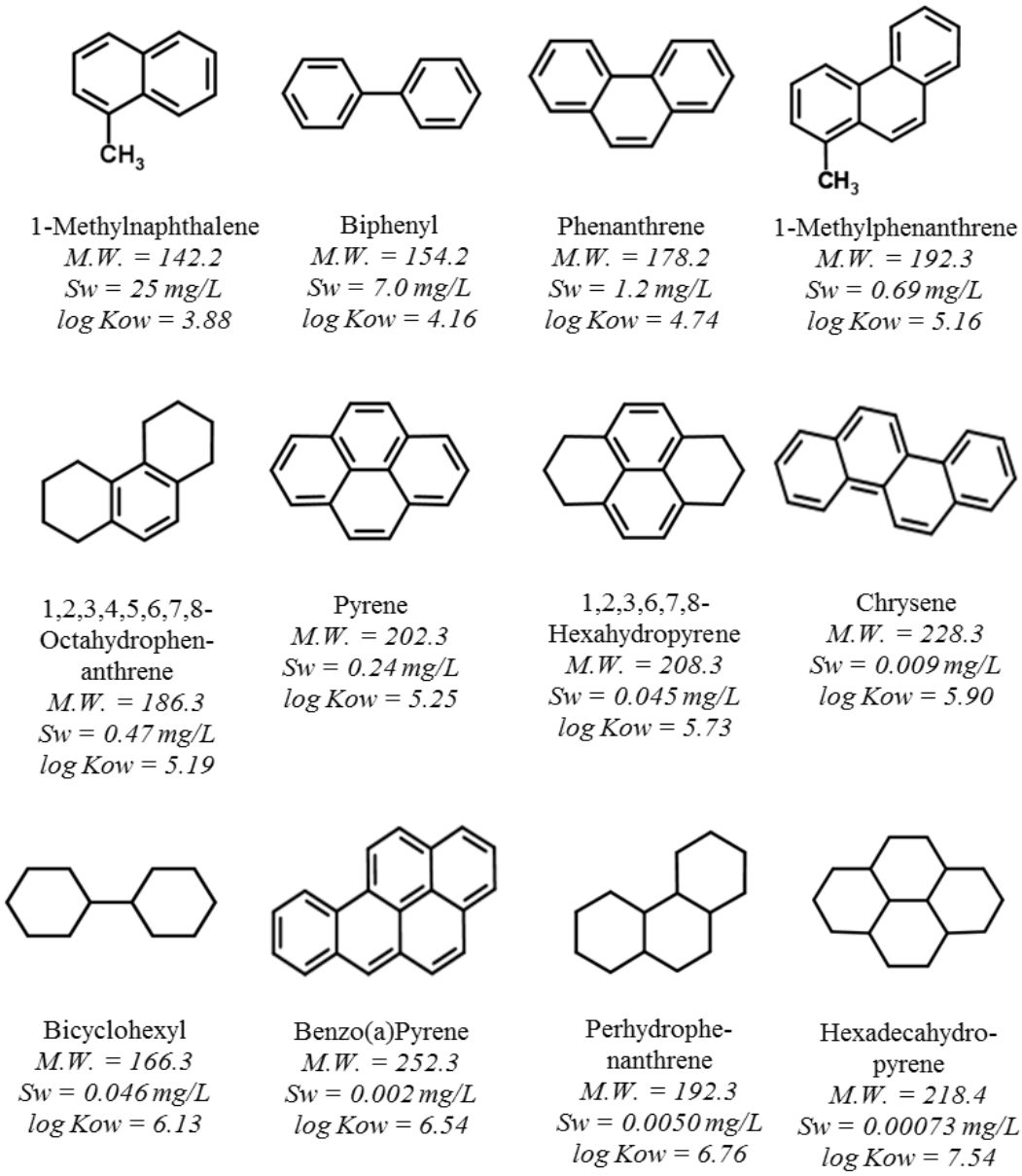


Figure 1.3: Compounds of interest. Within this figure fully saturated aromatic, partially saturated, unsaturated aromatic and partially unsaturated hydrocarbons used in this research are shown.

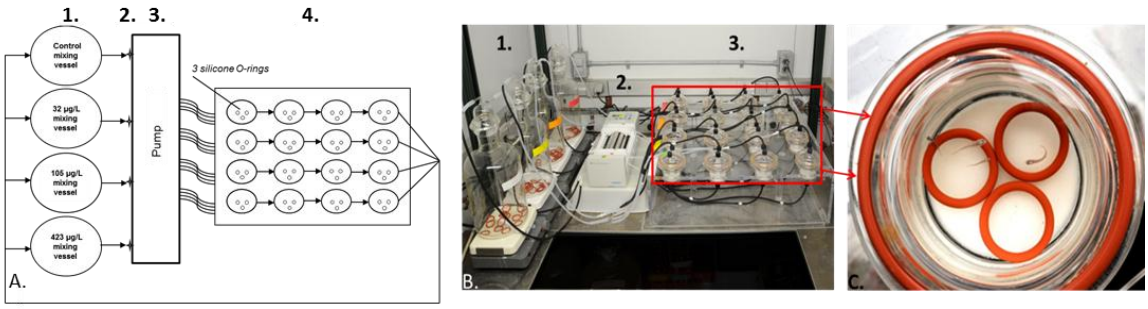


Figure 1.4: Conceptual and experimental design of recirculating flow-through test system used for larval zebrafish exposures. Figure 1.4A. Conceptual design of recirculating flow-through test system used for larval zebrafish toxicity tests: (1) 4L mixing vessels containing phenanthrene-dosed O-rings serve as the main dosing reservoir; aqueous concentrations obtained from these vessels are split into 4 different channels with a glass manifold (2) and pumped (3) through 4 replicates (300 mL customized, glass flow through exposure chambers) each containing three supplemental passive dosing O-rings (4). After flowing sequentially through each chamber each effluent is re-circulated back to the respective mixing vessel. Arrows indicate flow direction. Figure 1.4B. Experimental Photo-representation of recirculating flow through system used for larval toxicity tests: (1) 4L mixing vessels containing phenanthrene-dosed O-rings serve as the main dosing reservoir; aqueous concentrations obtained from these vessels are split into 4 different channels with a glass manifold and pumped (2) through 4 replicates (300 mL customized, glass flow through exposure chambers) (3) each containing three supplemental passive dosing O-rings (figure 1.4.C).

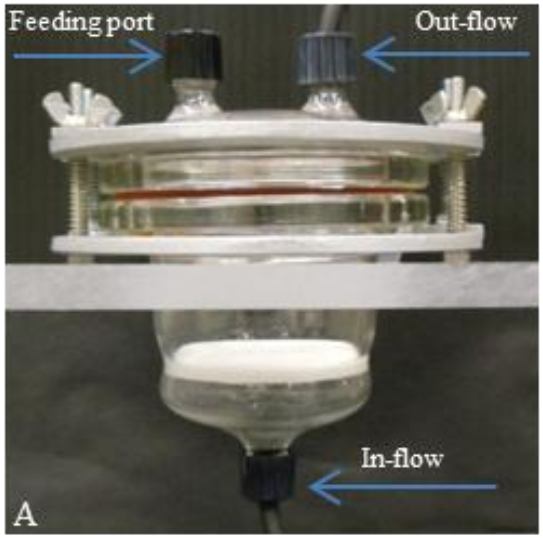


Figure 1.5: 300mL customized flow-through chamber labeled with port functionality.

Chapter 2

A Novel Passive Dosing System for Determining the Toxicity of Phenanthrene to Early Life Stages of Zebrafish

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Abstract

The target lipid model (TLM) has been used to derive water quality objectives that are intended to be protective of chronic effects posed by hydrocarbons. However, reliable experimental early life stage chronic toxicity data for fish are limited and further data are needed to confirm model predictions. Efforts are underway to develop a zebrafish embryo toxicity test guideline to reduce, refine and replace the use of vertebrates in animal testing. An adaptation of this method which includes embryo lethal and sub-lethal developmental endpoints after a 5-d exposure as well as larval survival and growth endpoints during a subsequent 25-d test period is described using phenanthrene as a test substance. To deliver well-controlled exposure concentrations, a passive dosing system consisting of silicone coated vials and silicone O-rings was employed. Acute results

indicated that edema and spinal curvature were the most sensitive sub-lethal effects observed and in many cases preceded observed mortality. The 30-day LC/EC_{10} for larval survival and growth was 40 and 67 µg/L respectively. Concentrations shown to cause adverse effects in this study are in the range of previous studies that have investigated the chronic effects of phenanthrene on fish. Based on these results the predicted water quality objectives for phenanthrene derived using the target lipid model (10.4 µg/L) would be protective of early life stage effects on zebrafish.

2.1. Introduction

An environmental assessment of complex petroleum substances, (e.g. kerosene, fuel oil) often begins with the evaluation of the fate and effects of constituent hydrocarbons (King et al., 1996; Foster et al., 2005; Van De Meent et al., 2010). These evaluations are conducted to support decision-making in the event of a chemical release into the environment. One important class of hydrocarbons that are known to be important contributors for predicting the aquatic toxicity of petroleum substances are polyaromatic hydrocarbons (French-McCay, 2002; McGrath & DiToro, 2009; Redman et al., 2012) Recently, the Target Lipid Model (TLM) has been used to predict water quality objectives, i.e. Predicted No Effect Concentrations (PNECs), for hydrocarbons including polyaromatic hydrocarbons using the HC5 statistical extrapolation method (McGrath & DiToro et al., 2009). Briefly, the TLM is used to characterize the species sensitivity distribution of hydrocarbon acute effects using the critical body residue concept (McElroy et al., 2011). Chronic protection is then predicted via extrapolation using an empirically derived distribution of acute to chronic ratios (ACRs). While an initial validation of this framework has been provided (McGrath & DiToro et al., 2009), reliable data on chronic effects to fish Early Life Stages (ELS) were limited. It has been previously reported that PAHs may cause toxicity to fish via specific modes of action (i.e. narcosis, receptor-mediated, AhR agonism) (Barron et al., 2004; Incardona et al., 2004; Incardona et al., 2006). Regardless of the mode of action data are needed to confirm whether TLM predictions are protective of chronic effects to potentially sensitive embryo and larval fish life stages.

Achieving constant concentrations of poorly water soluble compounds can be challenging to investigate in longer term toxicity tests because of the likelihood of compound losses due to loss mechanisms such as; sorption, degradation, and volatilization. Traditional static/semi-static exposure methods used in the past often exhibit significant losses due to these loss processes. This tends to complicate data interpretation (Basu et al., 2001; Smith et al., 2010). Providing a reliable means to chronically expose aquatic organisms with less chemical concentration fluctuation and loss would improve the reliability of toxicity test data and better facilitate comparison with TLM model predictions.

Over the last several years, novel passive dosing techniques are increasingly being applied with Hydrophobic Organic Compounds (HOCs) to deliver and maintain concentrations in aquatic tests (Brown et al., 2001; Kiparissis et al., 2003; Mayer et al., 2008; Smith et al., 2010; Turcotte et al., 2011). Chemical partitioning from a solvent solution into a Polydimethylsiloxane (PDMS) reservoir has been conveniently used to "load" the test system. Subsequent partitioning from the loaded PDMS phase into the aqueous test media enables test organisms to be constantly exposed despite potential loss processes (e,g, sorption, biodegradation, biotransformation) that occur in the test system. In addition to improving control of test chemical exposure, this technique has the ability to decrease chemical use and costs associated with test design.

In this study, the toxicity of phenanthrene to zebrafish (*Danio rerio*) early life stages (ELS) was investigated for 30 days post fertilization (pf). The objectives of this study were to: 1) demonstrate the ability of passive dosing to maintain constant, exposure concentrations, 2) apply the passive dosing technique to investigate the chronic toxicity of phenanthrene as a model polyaromatic hydrocarbon to ELSs of zebrafish, 3) assess

relative sensitivity of embryo and larval chronic effect endpoints, and 4) compare chronic effect concentrations for phenanthrene obtained in this study to other fish ELS studies reported in literature as well as TLM predicted water quality objectives that are intended to be protective for chronic effects.

2.2. Methods and Materials

2.2.1. Chemicals and materials

Twenty-two mL GC vials were purchased from MicroLiter Analytical Supplies, Inc. (Suwanee, Georgia). Silicone O-rings were obtained from O-rings West (Seattle, WA). Low VOC silicone resin conformal coating 1-2620 was purchased from Dow Corning (Midland MI). Phenanthrene (purity >97%), was obtained from TCI America (Portland OR). Stock phenanthrene solutions were made in methanol (HPLC grade) obtained from J.T. Baker (Phillipsburg, NJ). Hexane (HPLC grade) used in the preparation of PDMS vial coatings was purchased from J.T. Baker (Phillipsburg, NJ). Paramecium and brine shrimp (*Artemia nauplii*) used for feeding larval zebrafish were purchased from Z-FIN (Portland, OR) and Argent Foods (Salt Lake City, UT), respectively.

2.2.2. Loading PDMS coated silicone vials

Passive dosing vials were prepared using a method similar to Smith et al. (2010) for acute phase exposures (days 0-5). A 0.5 mL aliquot of PDMS was pipetted into 22-mL GC vials with a positive displacement pipette and mixed with hexane at a 1:3 ratio. Vials were placed on a vial roller for 24 hours with no cap then placed in an oven at 110°C for 2 hours. The silicone was then washed 3 times with 2-mLs methanol in a closed vial to remove impurities from the silicone. The silicone was then washed 3 times with 2-mLs deionized water in closed vials. All vials were then dried at 110°C for one hour and then kept at room temperature. A stock solution of a phenanthrene in methanol was prepared prior to dilution into 3 test concentrations ranging from 600 to 6900 mg/L. Approximately 10 mLs of each dosing solution was added to each vial. This was repeated for each test concentration. Clean methanol was transferred to control vials. Vials were closed with teflon coated airtight screw caps to prevent evaporation. Phenanthrene was allowed to partition from the methanol solution into the silicone coated vial for 72 hours. The dosed silicone was then cleaned 3 times with 3-5 mL deionized water and wiped with lint-free tissue to remove residual methanol/free material from the silicone coating. Vials were then filled with no headspace using test media and allowed to shake on an orbital shaker at 400 rpm for at least 16 hours. The test media for the acute phase was prepared using Instant Ocean® sea salt, 60ug/mL stock salts as described in (Westerfield et al., 2001).

2.2.3. Loading PDMS silicone O-rings

Similar to the method above, silicone O-rings (1 gram each) were loaded with phenanthrene as a dosing source for larval exposures. Two hundred silicone O-rings were placed into an Erlenmeyer flask and rinsed once overnight with ethylacetate. O-rings were then rinsed 3 times with methanol for a total of 24 hours contact time per rinse. Finally, the O-rings were rinsed three times with deionized water with a total contact time per rinse of 24 hours. O-rings were dried at 110°C for one hour and then kept at room temperature until needed. A stock of methanol/phenanthrene solution was prepared in a 2-L volumetric flask prior to dilution into appropriate test concentrations using 250-mL volumetric flasks. Each test solution was then transferred into a 250-mL erlenmeyer flask. Clean methanol was transferred to a control flask. Forty-two O-rings were added to each concentration and control solution. Phenanthrene was allowed to partition from the methanol solution into the silicone O-rings for 72 hours. O-rings were removed from the flask and cleaned as previously described for vials. Each concentration of O-rings were then activated by inserting into a 1 liter Erlenmeyer flask filled with test water and allowed to shake on an orbital shaker at 400 rpm for at least 16 hours. O-rings of each concentration were then inserted into their respective mixing vessel and customized flow through test chambers. Flow through chambers (300 mLs) were used during chronic exposure. Chambers contained three individual ports (Figure 2.1); two ports were located on top, one which served as an out-flow port and the other strictly for feeding purposes. One port was located on bottom and served as the in-flow port. This design allowed for continuous flow with optimum mixing. The out-flow port was partially blocked with steel

mesh to prevent larvae from swimming out of exposure chamber. Test media (moderately hard reconstituted water) for the chronic phase (days 5-30) was prepared according to (American Public Health Association, Method for the Examination of Water and Wastewater, 21st ed. 2005) ($NaHCO_3$, 12 mg/L; $CaSO_4$ $2H_2O$, 60 mg/L; $MgSO_4$, 60 mg/L; KCl, 4 mg/L; water hardness, 80-100 mg $CaCO_3$/L; Alkalinity, 60 mg $CaCO_3$/L; pH 7.4-7.8).

2.2.4. PDMS coated vial/silicone O-ring loading calculations

The release of phenanthrene from a methanol solution to PDMS coated vials/O-rings and then to the aqueous phase was predicted by applying Eqn. (2.1) to (2.2):

$$C_{PDMS} = \frac{C_{MeOH}}{K_{MeOH:PDMS}} \qquad (2.1)$$

$$C_{water} = \frac{C_{PDMS}}{K_{PDMS:water}} \qquad (2.2)$$

where C_{MeOH}, C_{PDMS} and C_{water} is the phenanthrene concentration in the methanol stock solution, PDMS and water, respectively and $K_{MeOH:PDMS}$ and $K_{PDMS:water}$ are the corresponding partition coefficients between these phases.

Substituting Eqn. (2.1) into (2.2) yields:

$$C_{water} = \frac{C_{MeOH}}{K_{MeOH:water}} \qquad (2.3)$$

where $K_{MeOH:water}$ is the partition coefficient of phenanthrene between methanol and water and is estimated by SPARC Performs Automated Reasoning in Chemistry (SPARC) at 28°C (http://archemcalc.com/sparc). The above equations are valid providing phenanthrene is not significantly depleted in the methanol and PDMS phases during each dosing step.

2.2.5. Maintenance of D. rerio culture and spawning procedures

An outbred strain of wild type zebrafish genotyped by Charles River Laboratories International, Inc. (Wilmington, MA), obtained from Aquatic Research Organisms (Hampton, NH), were used in this study. Twenty four adult fish of the same age were held at a ratio of 2 males per female and maintained in a 30-liter flow-through customized breeding trap positioned inside a 132-liter aquarium with constant mechanical filtration. Oxygen concentration was held above 80% saturation. Culture conditions were 12:12 light/dark photoperiod with an intensity between 400 – 800 lux and water temperature of 28 ±1°C. The bottom of the breeding trap consisted of 2 mm wire mesh to allow eggs to fall through to prevent cannibalization by adult fish.

Zebrafish spawning and fertilization took place within 30 minutes after the onset of light. After this period eggs were collected and immediately rinsed with water, transferred to a 125x65 mm crystallization dish filled with reconstituted water and examined for viability before use in toxicity testing.

2.2.6. Experimental Test Design

In the first test phase embryos were exposed to three concentrations (nominally 35, 122, 405 µg/L) and a control for 5 days. These concentrations were selected based on initial range finding studies. Embryos were exposed in 22-mL GC vials that had been coated with 108µm of PDMS. Embryos were sequentially added to each vial, capped with teflon coated screw caps and placed into an environmental chamber at 28±1°C. Microscopic observations were performed in 24-hour intervals. Lethal (coagulation, lack of heartbeat) and sub-lethal (pericardial and yolk sac edemas, spinal curvature, hatching time) endpoints included those defined in the OECD FET draft guideline (OECD, 203). The OECD FET draft guideline (OECD, 203) referenced within the manuscript provides justification for using twenty single embryos. Exposing one embryo per vial allowed us the opportunity to observe the same embryo through the first 5 days of development and also prevented the impact of dead embryos and fungal formation on the survival of other embryos.

This design also allowed sufficient numbers of surviving embryos to be transferred to replicate flow-through chambers to monitor further effects on larval survival and growth. The acute test duration was based primarily on previous work suggesting zebrafish develop the ability to feed exogenously on day 5 (Belanger et al., 2010).

In the second test phase (days 5-30), embryos that hatched into larval fish as well as any un-hatched embryos exposed in vials were transferred into 300-mL customized flow-through exposure chambers dosed to nominal concentrations. Depending on treatment related mortality individuals were divided into as many as four different flow-through

chambers each with up to 5 fish per replicate concentration. Four-liter mixing vessels containing 30, 1.0g O-rings for each concentration were prepared. Three, 1.0g O-rings were also added to each customized flow through chamber. Control systems were set up using O-rings dosed with clean methanol. Figure 2.2A provides a conceptual model for the recirculating flow-through test system. While figures 2.2B and C illustrates the recirculating flow through system which provided a 5 mL/min flow rate. Mixing vessels supersaturated with oxygen, were exchanged every seven days ± 1 day with a new set of O-rings to ensure optimal water quality, decrease test material loss due to bio-fouling and limit possible PDMS depletion.

Upon transfer to the flow through chambers, fish were fed Paramecium multimicronucleatumn. Larval fish were fed Artemia nauplii starting at 12 days. Paramecium and brine shrimp were fed ad libitum, twice daily throughout the experiment. During the feeding period, flow was turned off. After approximately 20 minutes of feeding, the flow-through system was re-started and flushed into a drainage basin for 10 minutes. After 10 minutes of flushing with test media all mixing vessels were filled and supersaturated with oxygen before starting flow recirculation. During the second phase, starting on day 6, fish were observed daily for immobility, lack of respiratory movement and lack of reaction to mechanical stimulus (OECD, 210). To evaluate potential larval growth effects, surviving fish were measured (total length) at study termination. The greater volume provided by the flow through system helped dilute the potentially elevated levels of ammonia, nitrite and nitrate as well as overcome oxygen depletion due to addition of paramecium to the test chamber and fecal egestion of larval fish.

2.2.7. Sampling and GC-FID-HS Analysis

During the acute phase of the study 2 mLs was pipetted from 10 vials for a total sample volume of 20 mLs for each exposure concentration and control. During the chronic phase of the experiment 20 mL samples were taken from the mixing vessel or the outflow just prior to the water re-entering the mixing vessel. A 1:1 dilution scheme containing 1 part test water to 1 part moderately hard reconstituted diluent water ($NaHCO_3$, 12 mg/L; $CaSO_4$ $2H_2O$, 60 mg/L; $MgSO_4$, 60 mg/L; KCl, 4 mg/L; water hardness, 80-100 mg $CaCO_3$/L; Alkalinity, 60 mg $CaCO_3$/L; pH 7.4-7.8) was used for analysis. Phenanthrene analysis was performed using automated static headspace gas chromatography with flame ionization detection (HS-GC-FID) using a Perkin Elmer Autosystem XL gas chromatograph with a 30 mm x 0.53 mm id, 1.5 µm film DB-5 (J&W Scientific) analytical column. The transfer line of a Perkin-Elmer TurboMatrix 40 Trap Headspace Sampler was connected directly to the analytical column. Samples and standards were equilibrated for 45 minutes at 90°C. The needle and transfer lines were held at 175°C, the pressurization time was 3 minutes. The injector temperature was 50°C and column pressure was 28 psi. The FID was set at 275°C and the oven temperature was held at 40°C for 1 minute and then ramped up to 275°C at 20°C/minute. Data were acquired and processed using Perkin Elmer TotalChrom Workstation software (version 6.3.1). Analysis of standards resulted in a linear response over the calibration range (9.8 – 245.3 µg/L).

2.2.8. Statistical Methodology

Acute and chronic endpoint effect concentrations were calculated using either probit transformations (Finney et al., 1971) in SAS (SAS V9.2) or a linear model using the Benchmark Dose method (USEPA Benchmark Dose Method V2.1.2.). The T-test with Bonferroni adjustment (Bland, 1995) or Fisher's Exact Test (Finney, 1948; Pearson, 1962) using TOXSTAT (Gulley, 1994) software was used to determine significant differences from the Control.

2.3. Results and Discussion

2.3.1. Method Development

In an attempt to clarify the dosing methodology, water samples were taken from coated vials and 1.0 g O-rings prepared as previously discussed, in 40 mL Volatile Organic Acid (VOA) vials with no headspace at test temperature 28±1°C. This allowed analytical verification of aqueous concentrations as well as comparison of the two passive dosing devices. Measured concentrations demonstrate a linear correlation between predicted and measured values over a 5 day test period with both passive dosing devices (Figure 2.3). There was no significant difference in the dosing devices as concentrations remained constant throughout the exposure duration. For the definitive test, it was hypothesized that while vials may provide a quicker kinetic release of phenanthrene into the aqueous phase due to a decreased PDMS thickness, the O-ring would provide greater mass of test

compound for delivery into the aqueous phase for longer duration, flow-through tests and therefore be less vulnerable to depletion through loss processes. As such, coated vials were used in the first phase with embryos involving static exposures while O-rings were utilized in the second flow-through test phase.

2.3.2. Analytical confirmation of exposure concentrations in toxicity test

During the first test phase, phenanthrene concentrations were analytically confirmed at test start and then again on day 5 prior to the addition of larval fish to the customized flow through test chamber. While fish were in exposure chambers, measurements were taken from each new and corresponding old mixing vessel (every 5-7 days) (Figure 2.4). Test substance analysis in the high exposure level (423 µg/L) was terminated due to complete mortality on day 12. As shown within figure 2.4 data from all three exposure levels are in good agreement with calculated concentrations (Eqn. 2.3) Further the data show no more than 20% loss throughout the entirety of the study in any of the exposure levels.

2.3.3. Acute effects of phenanthrene on zebrafish embryos

Similar to recent studies investigating the acute toxicity of phenanthrene to zebrafish, (Incardona et al., 2004; Incardona et al., 2005; Billiard et al 2008; Wolinska et al., 2011) sub-lethal (pericardial edema, yolk sac edema and spinal curvature) and lethal effects were observed. These data are displayed within table 2.1. Pericardial edema was the most

sensitive sub-lethal effect and was observed in the two highest concentrations after 24 hours. Figure 2.5 shows a phenotypic representation of all sub-lethal side effects observed during the acute phase of the experiment.

Sub-lethal effects were often observed prior to treatment related mortality in both a time and dose response fashion. However, in some instances sub-lethal effects, mainly pericardial and yolk sac edema were observed to decrease over time which suggests potential reversibility in these endpoints. After 120 hours, mortality at the high exposure concentration of 423 µg/L was observed to be significantly different from the control (p=0.05). At the end of the acute phase of the experiment (120 hours) the EC_{10} and EC_{50} values with their 95% confidence intervals were 189 µg/L (49–274) and 386 µg/L (300–536) respectively, based on spinal curvature which was the only sub-lethal effect remaining at 120 hours. A LC_{50} value could not be calculated at this time period (LC_{50} > 423µg/L). However, the TLM predicted a phenanthrene LC50 for zebrafish of 510 µg/L (McGrath & DiToro et al., 2009) which is not inconsistent with our results. These findings are also consistent with a recent study that reported the 96 hour no effect concentration for phenanthrene of 870 µg/L based on nominal exposure concentrations (Wolinska et al., 2011).

2.3.4. Chronic effects of phenanthrene on embryos

It is interesting to note that the 144 hour LC_{50} for survival decreased from the 120 hour LC_{50} of > 423 µg/L to 160 µg/L (Table 2.1 and Figure 2.6). Further effects on survival were observed in the high exposure group with one mortality on day 7 and one mortality

in the low exposure group on day 21. Increased mortality was observed on days 5 and 6 in the high and mid exposure groups. These data suggest that the most sensitive time point based upon lethality for zebrafish ELS development to phenanthrene is within the first 6 days PF. Once fish mature past the elutheroembryo stage of development further toxicity appears limited. Data presented within table 2.2 and figure 2.7A and B show mortality during the chronic phase of the experiment (Days 6-30, based on observations) relative to cumulative mortality observed during the entire 30 day exposure. Furthermore, statistically significant growth differences were observed in the mid exposure group (105 µg/L) at test termination (Figure 2.7C). Due to complete mortality growth measurements were not collected for the high exposure concentration.

2.3.5. Utility of passive dosing design

This study demonstrates the utility of the passive dosing design for achieving and maintaining constant aqueous concentration over the 30 day chronic test period with little loss. The aqueous phase concentrations were in reasonable agreement with predicted values and constant exposure was maintained within 20% throughout the test for the three test concentrations used. A key criterion for successful application of the passive dosing system used in this study was ensuring the mass of phenanthrene dissolved into methanol was much greater than that partitioned into the PDMS O-rings which in turn was much greater than that partitioned into the aqueous phase. This ensured that the test systems

were not depleted and served as a constant dosing source (methanol:PDMS; PDMS:water).

2.3.6. Comparative Toxicity

Fish ELS test data for phenanthrene from the literature were compiled for comparison to the current study. Data from six studies utilizing semi-static renewal and passive dosing methods (Call et al., 1986; Hooftman & Evers de Ruiters, 1992; Passino-Reader, 1995; Roex et al., 2001; Rhodes et al., 2005; Turcotte et al., 2011) are provided in Table 2.3. These studies bracket effect concentrations reported in the present study. The study performed by Hooftman and Eves-de Ruiter (1992) best matched the duration of our test. These authors observed survival and growth to be the most sensitive parameters with a reported 42 day No Observed Effect Concentration (NOEC) of 56 µg/L which is within a factor of two of the reported NOEC in the current study of 32 µg/L. The NOEC in the present study is lower and may reflect the more well defined, constant exposure provided by the passive dosing system as suggested in previous studies (Kiparissis et al., 2003). The most sensitive acute (days 0-5) early life stage sub-lethal endpoint appeared to be pericardial edema (Table 2.1). These data align with cumulative mortality data (Table 2.2). However, this endpoint was shown to exhibit reversibility in that at 120 hours none of the surviving larvae were observed to have pericardial edema. Spinal curvature, did not appear until 72 hours post treatment, but affected 58% of the remaining embryos in the high concentration at 120 hours. Growth and survival were observed to be the most sensitive larval (days 5-30) life stage endpoint in our study.

The NOEC of 32 µg/L determined in this study was also compared to the hazard concentration (HC5) of 10.4 µg/L for phenanthrene reported by McGrath and DiToro (2009). These results indicate the HC5 is protective of ELS toxicity associated with chronic phenanthrene exposure to zebrafish.

2.3.7. Conclusions

Passive dosing provides an innovative method for maintaining aqueous exposure concentrations of poorly water soluble compounds. Using phenanthrene the validity of this approach was demonstrated by keeping aqueous concentrations within 20% of initial values during a 30-day fish ELS test. Most of the observed toxicity from phenanthrene occurred within the first 6 days of zebrafish development. Chronic effect concentrations derived from this study for zebrafish were comparable to earlier literature data on other test species under similar test durations. Further data are needed to determine the relative sensitivity of embryo versus larval chronic endpoints and to support further evaluation of toxicity model predictions for additional hydrocarbons and fish species. Although more experimental data are needed, the results in this exposure suggest the possibility of a shortened ELS test. Furthermore, the HC5 derived PNEC values are more sensitive and provide sufficient protection when compared to the chronic effect concentrations observed in the current study.

Reference for this chapter:
Butler, J.D., Parkerton, T.F., Letinski, D.J., Bragin, G.E., Lampi, M.A., Cooper, K.R., 2013. A Novel Passive Dosing System for Determining the Toxicity of Phenanthrene to Early Life Stages of Zebrafish. *Science of The Total Environment, Volumes 463–464, 1 October 2013, Pages 952-958*

Table 2.1: Summary of phenanthrene effects on zebrafish embryo/larvae.

Lethal Endpoints	Mean Exposure Concentration (µg/L) ± SD	Percent Mortality					
		24 hours (%)	48 hours (%)	72 hours (%)	96 hours (%)	120 hours (%)	144 hours (%) [b,c]
Mortality[a]	0 ± 0	0	0	0	5	5	5
	32 ± 4	0	0	0	0	0	0
	105 ± 17	0	0	5	25	25	60*
	423 ± 41	5	5	10	10	40*	95*
Sub-Lethal Endpoints	Percentage of Remaining Embryos Effected						
Pericardial Edema	0 ± 0	0	0	0	0	0	†
	32 ± 4	0	0	0	0	0	†
	105 ± 17	65*	100*	100*	27*	0	†
	423 ± 41	95*	100*	100*	0	0	†
Yolk Sac Edema	0 ± 0	0	0	0	0	0	†
	32 ± 4	0	0	0	0	0	†
	105 ± 17	0	100*	0	0	0	†
	423 ± 41	89*	100*	0	0	0	†
Tail Curvature	0 ± 0	0	0	0	0	0	0
	32 ± 4	0	0	0	0	0	0
	105 ± 17	5	5	5	7	7	13
	423 ± 41	5	5	22*	56*	58*	100*
Sub-Lethal Endpoints	Percentage of Total Number of Embryos						
Hatching Time	0 ± 0	0	0	40	100	100	100
	32 ± 4	0	0	45	100	100	100
	105 ± 17	0	0	5*	50*	75	75
	423 ± 41	0	0	15	85	85	85

*Statistically Significant from controls ($p \leq 0.05$).

[a]Mortality is defined as coagulation during the first 48 hours of the test, and thereafter by a lack of heartbeat.

[b] Column was not included in effect concentration determination. ComLum was only included for visual clarity of steep increase in mortality between 120 and 144 hours.

[c] Microscopic observations were not performed.

Table 2.2: Summary of phenanthrene effects on zebrafish larvae.

Mean Measured Exposure Concentration (µg/L) ± SD	% Larval Mortality (Days 6-30)	% Cumulative Mortality (Days 0-30)	30 Day Larval Fish Length (cm) ± SD
0 ± 0	5	5	1.19 ± 0.14
32 ± 4	5	5	1.15 ± 0.11
105 ± 17	35*	60*	1.01 ± 0.06*
423 ± 41	60*	100*	N/A

* Statistically different than control at $P \leq 0.05$.

Table 2.3: Comparison of fish ELS toxicity studies with phenanthrene as the exposure compound.

Study	Exposure Method	Species	Results
Call (1986)	Semi-static/renewal	*Oncorhynchus mykiss*	90 d NOEC survival/growth: 5µg/L
Hooftman (1992)	Semi-static/renewal	*Danio rerio*	42 d NOEC survival/growth: 56 µg/L
Passino-Reader (1995)	Semi-static/renewal	*Oncorhynchus mykiss*	60 d NOEC weight: 44 µg/L
Rhodes (2005)	Semi-static/renewal	*Orzias latipes*	17 d NOEC deformation: >200 µg/L
Turcotte (2011)	Passive dosing	*Orzias latipes*	17 d EC10 embryo development: 50 µg/L
Current Study	Passive dosing/flow - through	*Danio rerio*	30 d NOEC survival/growth: 32 µg/L

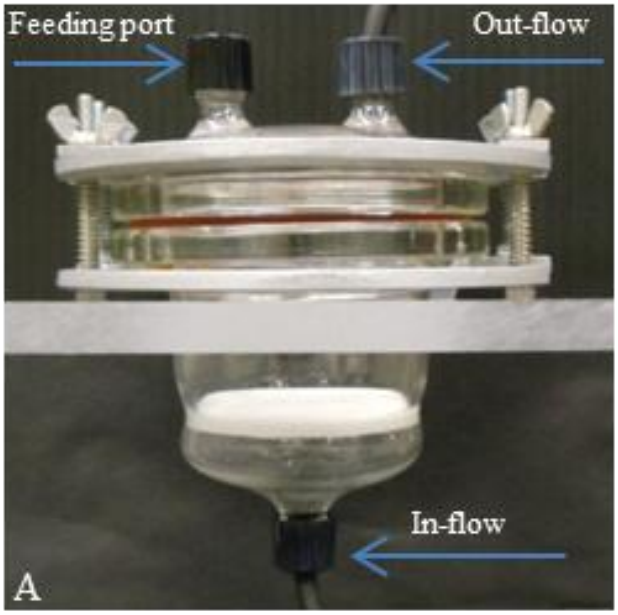


Figure 2.1: 300mL customized flow-through chamber labeled with port functionality.

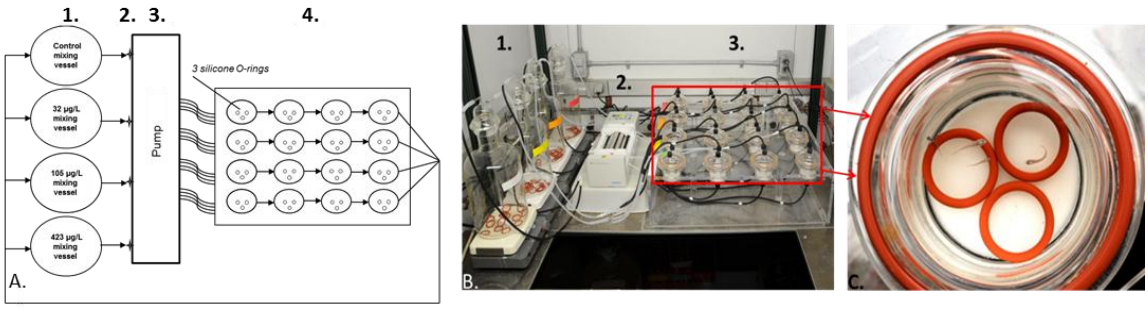


Figure 2.2: Conceptual and experimental design of recirculating flow-through test system used for larval zebrafish exposures. Figure 2A. Conceptual design of recirculating flow-through test system used for larval zebrafish toxicity tests: (1) 4L mixing vessels containing phenanthrene-dosed O-rings serve as the main dosing reservoir; aqueous concentrations obtained from these vessels are split into 4 different channels with a glass manifold (2) and pumped (3) through 4 replicates (300 mL customized, glass flow through exposure chambers) each containing three supplemental passive dosing O-rings (4). After flowing sequentially through each chamber each effluent is re-circulated back to the respective mixing vessel. Arrows indicate flow direction. Figure 2B. Experimental Photo-representation of recirculating flow through system used for larval toxicity tests: (1) 4L mixing vessels containing phenanthrene-dosed O-rings served as the main dosing reservoir; aqueous concentrations obtained from these vessels are split into 4 different channels with a glass manifold and pumped (2) through 4 replicates (300 mL customized, glass flow through exposure chambers) (3) each containing three supplemental passive dosing O-rings (figure 2.C.).

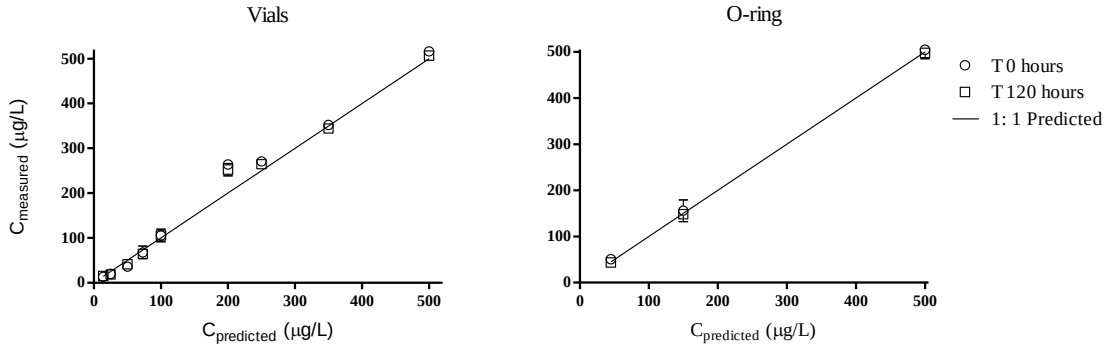


Figure 2.3: Comparison of phenanthrene exposure concentrations in water obtained using passive dosing with PDMS coated vials and O-rings. The 1:1 predicted line shows the agreement between the model predicted (equation 2.3, with partition coefficient derived from SPARC), and observed concentrations. Each point represents the mean concentration and standard deviation of three replicates.

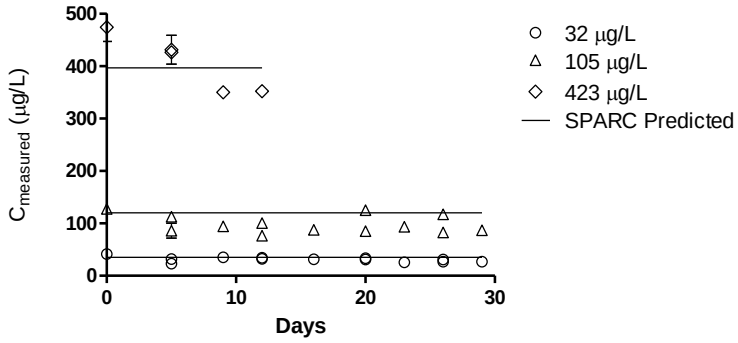


Figure 2.4: Measured aqueous concentrations throughout test duration. Each symbol represents the mean of three replicates plus the standard deviation The nominally predicted concentrations were obtained by dividing the methanol spiking concentration used to load the silicone O-rings by the methanol-water partition coefficient for the test compound that was calculated using SPARC. The concentration reported for each compound in the legend represents the 30 day mean value for each treatment investigated.

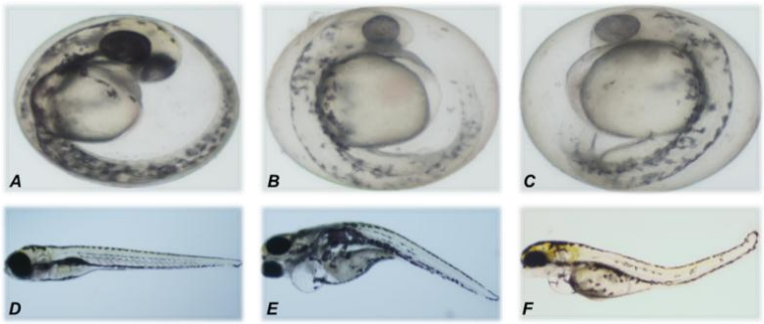


Figure 2.5: Most sensitive sub-lethal effects observed throughout exposures. Row 1, pictures B and C show edema in the pericard and yolk sac region at 48 hours post fertilization (PF), these can be compared to the control (row 1, A). Row 2, pictures E and F show tail curvature and pericardial edema at 120 hours PF, these can be compared to the control at 120 hours pf (row 2, D).

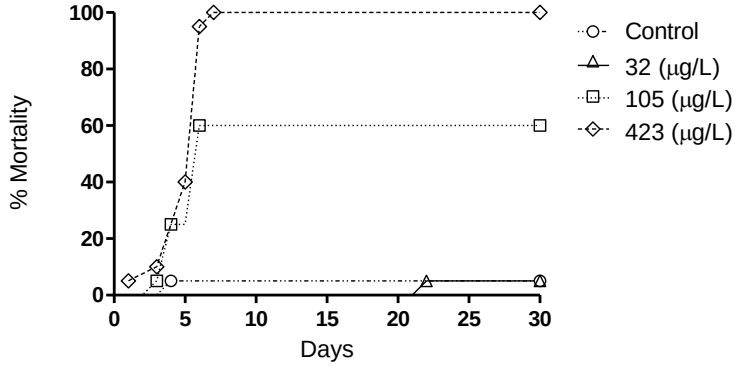


Figure 2.6: Cumulative zebrafish embryo-larval mortality over 30 days. Each data point reflects a change in percent mortality for a specific concentration on the day it was observed.

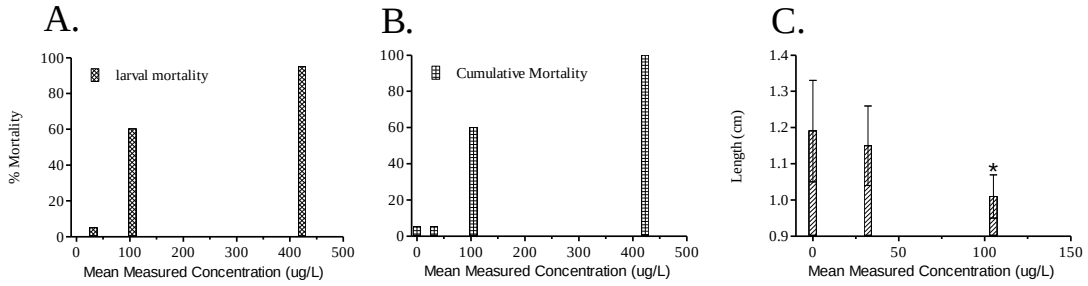


Figure 2.7: Chronic sub-lethal and lethal effects. Figure 2.7A. Mortality to larval life stage of zebrafish exposure (days 6-30). There were no mortalities observed for the control exposure during this time period. B. Cumulative zebrafish mortality throughout 30 days of exposure. C. Mean measured total length (cm) of remaining larval fish measured at study termination. Error bars represent the standard deviation of the mean between all fish measured for that concentration. The mean measured concentration of 105 (µg/L) produced a statistically significant difference from controls (*) at a ($P \geq 0.05$). Complete mortality observed in the high exposure concentration of 423 (µg/L), thus no fish were measured for this concentration.

Chapter 3

Determining the Toxicity of 5 Aromatic Hydrocarbons to Early Life Stages of Zebrafish

Abstract

The target lipid model (TLM) has been used to derive water quality objectives (HC-5's) that are intended to be protective of chronic effects posed by hydrocarbons. However, reliable experimental early life stage chronic toxicity data for fish are limited and further data are needed to confirm model predictions. Single compound exposures were performed using 8 aromatic hydrocarbons with a K_{OW} range of ca. 3 to 6. Zebrafish were exposed to six aromatic hydrocarbons acutely (120 hours) and five aromatic hydrocarbons chronically (30 days). During acute exposures sub-lethal and lethal endpoints were assessed after 120 hours, whereas larval survival was assessed during chronic testing. To deliver controlled exposure concentrations, a passive dosing system consisting of silicone o-rings was employed. In each exposure, results indicated that 10 day LC10 values were similar to 30 day embryo-larval LC10 values. Coagulation, lack of somite development, lack of tail bud detachment, heart-beat and immobilization were lethal endpoints observed. Pericardial edema, yolk sac edema and tail curvature were the most sensitive sub-lethal side effects observed. Furthermore, results confirm that TLM predicted water quality objectives for all aromatic hydrocarbons tested are protective for early life stage effects to zebrafish. Further work is needed to determine the relative

sensitivity of embryo versus larval chronic endpoints for additional hydrocarbons and test species.

3.1. Introduction

Performing risk assessments on the large number of chemicals in current use as well as new chemicals entering commerce is a large task. In reality some chemicals have never gone through a formal risk assessment nor do they have sufficient toxicity data (Garay & Isnard et al., 2000; EC., 2003). Experimental costs as well as extensive regulatory requirements have made the generation of quality data for use in environmental risk assessments challenging. Reliable toxicity data and predictive modeling are needed to help accurately characterize a large number of chemical substances in a timely manner.

The Target Lipid Model (TLM) has been successfully used to predict water quality objectives such as LC50 and Predicted No Effect Concentration (PNEC) of non-polar organic chemicals including cyclic hydrocarbons (DiToro et al., 2000; McGrath et al., 2004; McGrath and DiToro, 2009). Briefly, the TLM is used to characterize the species sensitivity to hydrocarbons using the Critical Target Lipid Body Burden (C_L^*) (DiToro et al., 2000; McGrath and DiToro et al., 2009). The TLM relies on the octanol-water partition coefficient (K_{OW}) to describe lipid to water partitioning (K_{TLW}) of a substance into the lipid of an organsim. This relationship, currently calibrated to 47 organisms, is observed to be linear in the K_{OW} range of ca. 0-6 with a slope of -0.936 and minor variations (ΔC) for certain chemical classes (e.g., mono-aromatic, aromatic) (McGrath et

al., 2009) (eqn., 3.1). The acute median lethal concentration can then be predicted by subtracting the $\log K_{TLW}$ from the *log CTLBB* (eqn., 3.2).

$$logK_{TLW} = 0.936logK_{ow} + \Delta C \qquad (3.1)$$

$$logLC_{50} = \log CTLBB - logK_{TLW} \qquad (3.2)$$

Predictions of chronic effects for a particular chemical to a specific organism can then be performed using empirically derived distributions of acute to chronic ratios (ACRs) (DiToro et al., 2000; McGrath et al., 2004) (eqn., 3.3).

$$ACR = \frac{Acute\ Effect\ Concentration\ (LC_{50})}{Chronic\ Effect\ Concentration\ (EC_{10})} \qquad (3.3)$$

Distributions of CTLBBs and ACRs are used to establish protective concentration levels using statistical extrapolation methods (McGrath and DiToro., 2009).

Recent reviews suggest the selection of a hazard concentration protecting 95% of the organisms exposed to a toxicant also known as the HC-5 appears to provide an appropriate level of protection (Versteeg et al 1999; van de Brink et al., 2002). Within the TLM framework PNEC values (HC5) can also be accurately derived with log K_{OW} values up to 6.4 (McGrath et al., 2004; McGrath and DiToro., 2009). Thus it is possible to extend the TLM to predict HC5 values for use in risk assessments (Parkerton et al., in press).

To test the robustness of the TLM for predicting toxicity of fish early life stages (ELSs) toxicity testing, 8 aromatic hydrocarbons (AHs) (Table 3.1.) ranging in log K_{ow} values

from ca. 3-6 were investigated. Exposures were performed with AH loaded silicone O-rings as a passive dosing source (Smith et al., 2010; Smith et al 2012; Butler et al., 2013). The objectives of this paper were to: 1) Apply the passive dosing approach to a series of AHs in acute (120-hour) and chronic (30-day) fish ELS tests, 2) Assess sensitivity of embryo/larval effect endpoints and use data generated to compare and refine the current model, 3) Compare the acute to chronic ratio (ACR) and TLM derived HC5 to experimental results.

3.2. Methods and Materials

3.2.1. Chemicals and materials

Twenty mL headspace vials were purchased from MicroLiter Analytical Supplies, Inc. (Suwanee, Georgia). Silicone O-rings were obtained from O-rings West (Seattle, WA). Phenanthrene (purity of >97%), was obtained from TCI America (Portland, OR). naphthalene, 1-methylnaphthalene, biphenyl, pyrene and benzo(a)pyrene (purity 99%, 95%, 96%, 98% and 96% respectively) were obtained from Sigma Aldrich (Milwaukee, WI). 1, 2, 3, 4, 5, 6, 7, 8 octahydrophenanthrene and chrysene (purity 99% and 95% respectively) were obtained from City Chemical (West Haven, CT). Stock AH solutions were prepared in methanol (HPLC grade) obtained from J.T. Baker (Phillipsburg, NJ). Paramecium and brine shrimp (*Artemia nauplii*) used for feeding larval zebrafish were

purchased from Z-FIN (Portland, OR) and Argent Foods (Salt Lake City, UT), respectively.

3.2.2. Loading and dosing with PDMS silicone O-rings

Silicone O-rings were loaded with a single AH as a dosing source for embryo/larval exposures. 0.13 g O-rings were used for acute embryonic exposures (up to 120 hours) and 1.0 g O-rings were used during the chronic larval exposure period (days 5-30). Methods were employed to load AHs from a methanol stock solution into O-rings following the procedures reported by Butler et al. (2013) and chapter 2 of this thesis. Nominal concentrations in water were predicted by dividing the test substance concentration in the methanol stock solution by the methanol-water partition coefficient of the substance that was predicted using the SPARC model (Sparc Performs Automated Reasoning in Chemistry (SPARC) (www.ibmLc2.chem.uga.edu) (eqn. 2.3). Depletion of the methanol stock solutions during the methanol:PDMS partitioning phase and the silicone O-rings during the PDMS:water partitioning phase was assumed to be negligible. A key criterion for successful application of the passive dosing system used in this study was ensuring the mass of AH dissolved into methanol was much greater than that partitioned into the PDMS O-rings which in turn was much greater than that partitioned into the aqueous phase. This ensured that the test systems were not depleted and served as a constant dosing source.

3.2.4. Maintenance of D. rerio culture and spawning procedures

An outbred strain of wild type zebrafish genotyped by Charles River Laboratories International, Inc. (Wilmington, MA), obtained from Aquatic Research Organisms (Hampton, NH), were used in this study. Twenty-four adult fish of the same age were held at a ratio of 2 males per female and maintained in a 30-liter flow-through customized breeding trap positioned inside a 132-liter aquarium with constant mechanical filtration. Oxygen concentration was held above 80% saturation. Culture conditions were 12:12 light/dark photoperiod with intensity between 400 – 800 lux (fluorescent lighting) and water temperature of 28 ±1°C. The bottom of the breeding trap consisted of 2 mm wire mesh which allowed eggs to fall through and prevent cannibalization by adult fish. Zebrafish spawning and fertilization took place within 30 minutes after the onset of light. After this period eggs were collected and immediately rinsed with water, transferred to a 125x65 mm crystallization dish filled with reconstituted water and examined for viability before being placed into an environmental chamber at 28 ±1°C.

3.2.5 Experimental Test Design

D. rerio embryo/larvae were exposed to naphthalene, 1-methylnaphthalene, biphenyl, phenanthrene, pyrene, 1, 2, 3, 4, 5, 6, 7, 8 octahydrophenanthrene, in acute tests and to 1-methylnaphthalene, phenanthrene, 1, 2, 3, 4, 5, 6, 7, 8 octahydrophenanthrene, chrysene and BaP in chronic exposures. Acute exposures were performed for 120 hours post-fertilization (PF) while chronic exposures lasted 30 days PF. Including two different exposure periods afforded the opportunity to select exposure concentrations which

targeted different developmental life stages. Further, this design allowed empirical ACRs to be calculated and compared to earlier compilations across hydrocarbons and test species (McGrath and DiToro (2009). Inclusion of chronic tests also allowed direct comparison of chronic endpoints to TLM-derived HC5 predictions to determine if this framework was protective for chronic effects on fish ELSs.

3.2.5.1 Acute tests

Embryos were exposed to 5 concentrations and a control per exposure. Concentrations were selected with the use of literature values as well as TLM predictions. Embryos were exposed in 20 mL headspace vials that had 0.13 g O-rings resting on the bottom. Vials were then capped with teflon coated screw caps and placed into an environmental chamber at 28±1°C. Twenty replicates with one embryo per vial were tested for each treatment. Microscopic observations were performed at 24-hour intervals. Lethal (coagulation, lack of heartbeat) and sub-lethal (pericardial and yolk sac edemas, tail curvature, hatching time) endpoints included those defined in the OECD (2006) FET draft guideline up to 120 hours post fertilization. The test media for the acute phase was prepared using Instant Ocean® sea salt, 60ug/mL stock salts as described in Westerfield., (2001).

3.2.5.2 Chronic tests

Larvae were exposed for 30-PF to 3 AHs (1-methylnaphthalene, phenanthrene, 1, 2, 3, 4, 5, 6, 7, 8, octahydrophenanthrene) in separate exposures with each AH having 3

concentrations and a control. For chrysene and BAP which exhibit a low aqueous solubility (Table 3.1), only a single test concentration and control was investigated at the highest aqueous test concentration that could be achieved in the test system. During the chronic exposures, embryos were initially exposed in vials for 120 hours, similar to the acute experiments discussed above. Upon 120 hours PF, embryos that hatched into larval fish as well as any unhatched embryos exposed in vials were divided and transferred into four different 300 mL flow-through chambers, each with up to 5 fish per replicate chamber (Figure 3.1B, 3 and Figure 3.1C). Four-liter mixing vessels containing 30, 1.0g O-rings for each concentration were prepared (Figure 3.1B, 1). Three, 1.0g O-rings were also added to each customized flow through chamber (Figure 3.1C). This process is shown conceptually in figure 3.1A. The flow through each test chamber was supplied by a peristaltic pump (Figure 3.1B, 2) and was set to a rate of 5 mL/min. Mixing vessels supersaturated with oxygen, were exchanged every 5 days ± 24 hours with a new set of O-rings to ensure optimal water quality, avoid biofouling and limit possible PDMS depletion.

The customized flow through chambers contained three individual ports; two ports were located on top, one which served as an out-flow port and the other strictly for feeding purposes. The third port was located on bottom and served as the in-flow port. This design allowed for continuous flow with optimum mixing. The out-flow port was partially blocked with steel mesh to prevent larvae from swimming out of the exposure chamber (Figure 3.2). Test media for the chronic phase (days 5-30) was prepared according to *Standard Methods for the Examination of Water and Wastewater* 21st Edition method 8010E (2005), moderately hard reconstituted water ($NaHCO_3$, 12 mg/L;

$CaSO_4$ $2H_2O$, 60 mg/L; $MgSO_4$, 60 mg/L; KCl, 4 mg/L; water hardness, 80-100 mg $CaCO_3$/L; Alkalinity, 60 mg $CaCO_3$/L; pH 7.4-7.8).

Upon transfer to the flow through chambers, fish were considered to be exogenously feeding (Belanger et al., 2010) and were fed *Paramecium multimicronucleatumn.* Larval fish were fed *Artemia nauplii* starting at 12 days. Paramecium and brine shrimp were fed *ad libitum*, twice daily throughout the experiment. During the feeding period, flow was turned off. After approximately 20 minutes of feeding, the flow-through system was re-started and flushed into a drainage basin for 10 minutes. After 10 minutes of flushing with test media all mixing vessels were filled and supersaturated with oxygen before starting flow recirculation. Fish were observed daily for immobility, lack of respiratory movement and lack of reaction to mechanical stimulus (OECD 210, 1992). To evaluate potential larval growth effects, surviving fish were measured (total length) at study termination.

3.2.6. Chemical Analysis

Chemical analysis for naphthalene, biphenyl, phenanthrene and 1-methylnaphthalene in water was performed using static headspace gas chromatography with flame ionization detection (HS-GC-FID) using a Perkin Elmer Autosystem XL gas chromatograph. Analysis of 1, 2, 3, 4, 5, 6, 7, 8 octahydrophenanthrene was performed by HS-GC-FID modified with a trap accessory to provide additional sensitivity. Pyrene, B(a)P and chrysene were analyzed by automated direct immersion (DI) solid phase microextraction (SPME) coupled with gas chromatography with mass selective detection (GC-MSD).

The HS-GC-FID was equipped with a 30 mm x 0.53 mm id, 1.5 µm film DB-5 (J&W Scientific) analytical column which was connected directly to the transfer line of a Perkin-Elmer TurboMatrix 40 Trap Headspace Sampler. For 1, 2, 3, 4, 5, 6, 7, 8 - octahydrophenanthrene analysis, a sorbent trap containing fused silica beads and Carbopack C was placed in-line of the headspace sampler. All water samples for headspace analysis and standards spiked into water were equilibrated for 45 minutes at 90°C. The needle and transfer line temperatures were both 175°C, the pressurization time was 3 minutes. The injector temperature was 50°C and column pressure was 28 psi. The FID was 275°C and the oven temperature was held at 40°C for 1 minute and then ramped up to 275°C at 20°C/minute. Data were acquired and processed using Perkin Elmer TotalChrom Workstation software (version 6.3.1). Standards analysis resulted in a linear response over concentration ranges bracketing the respective water concentrations for the test compounds.

The DI-SPME coupled with GC-MSD was equipped with an Agilent 6890N gas chromatograph and Agilent 5975 mass selective detector operated in the selective ion monitoring mode (SIM) was used for analysis. The analytical column was a 25 m x 0.2 mm id, 0.33 µm DB-5MS (J&W Scientific) capillary column. The GC-MSD system was equipped with a Gerstel CIS 4 inlet and interfaced with a Gerstel MPS 2 autosampler operated in the DI-SPME mode using a 7-µm PDMS fiber (Supelco) with a 90-minute extraction time at 30^{o}C. The SPME fiber was automatically desorbed for 5 minutes at 300^{o}C. The GC oven temperature was programmed from 50^{o}C for 5 minutes to 340^{o}C at 12^{o}C/minute. After an initial 25 psi pressure pulse for one minute, the helium carrier gas

pressure was set at 13.8 psi corresponding to a constant flow of approximately 0.8 mL/min for the duration of the run.

3.2.7. Statistical Methodology

Acute and chronic endpoint effect concentrations were calculated using either probit transformations (Finney., 1971) in SAS (V9.2, 2002-2008) or non-linear regressions using the Spearman-Karber method (Hamilton et al., 1977)

3.3 Results and Discussion

3.3.1. Exposure confirmation

Dosing poorly water soluble compounds in aquatic toxicity tests pose many challenges (i.e., chemical losses). Losses can occur as a result of sorption, volatilization, biodegradation and metabolism during exposures. The ability of the passive dosing system to accurately and precisely dose to nominal concentrations was tested and confirmed through varying exposure durations with 8 separate AHs with K_{OW} values ranging three orders of magnitude. During the acute exposure period, samples were taken at test start and then again on day 5. Mean measured test substance losses observed in acute tests were minimal (3-8%) and observed to deviate by no more than 10 % of the mean measured concentrations (table 3.2).

During chronic exposures, measurements were taken at test start and then again on day 5 prior to the addition of larval fish to the customized flow through test chamber. While fish were in exposure chambers, measurements were taken from each new and corresponding old mixing vessel every 5-7 days. Additionally, random concentration checks for some exposures were performed (Figure 3.3, A-D). Analysis of the high treatment level in the phenanthrene and 1-methylnaphthalene studies (Figure 3.3, A and B) was terminated before the full duration of the test due to complete mortality. Figure 3.3 reports data from all experiments and treatments and shows measured concentrations were in excellent agreement with nominal predicted concentrations.

3.3.2. Acute effects of PAH exposures on embryos

Sub-lethal effects during the acute phase of exposures consisted of pericardial edema, yolk sac edema and tail curvature. Pericardial edema was the most sensitive sublethal effect and was observed in each exposure. Tail curvature was also observed in each exposure. Similar to Butler et al., 2013 and previous intra-laboratory studies sub-lethal effects were often observed prior to treatment related mortality. However, in some instances sub-lethal effects, mainly pericardial and yolk sac edema were observed to decrease over time which indicates reversibility in these sublethal endpoints (Figure 3.4). At the end of the acute phase of the experiment (120 hours) LC50 values with their 95% confidence intervals were 6309 (5888-6760), 1013 (1182-1703), 1548 (1148-2290), 334 (184-1493), 96 (87-102) and 52 (39-80) µg/L for naphthalene, 1-methylnaphthalene, biphenyl, phenanthrene, pyrene and 1, 2, 3, 4, 5, 6, 7, 8 octahydrophenanthrene,

respectively (Table 3.3). The majority of the lethality was observed during the latter portion of the test. EC10 and EC50 values based upon sub-lethal effects were not calculated due to the reversible toxicity observed in sub-lethal effects. The 120 hour concentration-response for each test compound is shown in Figure 3.5. The horizontal lines denote the LC10 and LC50 endpoints.

3.3.3. Chronic effects of PAH exposures on embryos

A summary of cumulative mortality of embryos/larvae throughout the test duration (days 0-30) is shown in figure 3.6. All mortality data for compounds tested show a similar trend in life stage specific toxicity. Once fish mature past the elutheroembryo stage of development (days 5-7) further toxicity appears limited. Data suggest that the most sensitive time point of zebrafish ELS development to all compounds tested is within the first 10 days PF.

The results shown in table 3.3 indicate 1, 2, 3, 4, 5, 6, 7, 8 - octahydrophenanthrene was the most toxic followed by phenanthrene and 1-methylnaphthalene with LC10 values of 9, 44, and 141 µg/L respectively. Chrysene and BaP were observed to be non-toxic at the maximum concentration that could be achieved using the passive dosing test system (i.e. mean measured exposure concentrations of 1.4 and 1.7 µg/L, respectively). It is important to note that at the end of each chronic exposure total length of larvae/juvenile zebrafish was measured. However, in all exposures except for phenanthrene survival was consistently observed to be the most sensitive ELS effect.

3.3.4. TLM, ACR and HC5 comparison

Five day LC50s for zebrafish from the current study as well as 4 day values from FhG (1979) and 2 day values from Sloof et al., (1979) are compared to the baseline TLM (McGrath and DiToro et al., 2009) (eqn. 3.4) in Figure 3.7. Data appear to be in agreement with the baseline TLM. The initial log CTLBB (2.099) for zebrafish was taken from McGrath & DiToro (2009). To compare results obtained in this study to the TLM, the appropriate class corrections were applied. Adjusted values are reported in table 3.4 and plotted in Figure 3.7.

$$logLC_{50} = 0.936logK_{ow} + 2.099 \qquad (3.4)$$

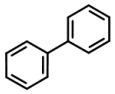
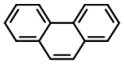
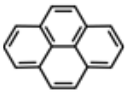
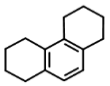
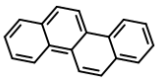
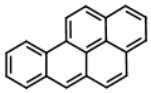
A summary of the 5 day LC50 and 30 day LC10 and resulting ACR values are summarized in Table 3.3. McGrath and DiToro (2009) calculated ACRs for 29 paired data sets with a geometric mean of 3.83 (ranging from 1.2-12.7) using 11 different species with varying endpoints. Of the 29 data sets 17 were Polycyclic Aromatic Hydrocarbons (PAHs), 6 were Mono-Aromatic Hydrocarbons (MAHs) and 6 were aliphatic hydrocarbons. Our geometric mean of 6.8 was calculated using 3 hydrocarbons; two PAHs (1-methylnaphthalene and phenanthrene) and one MAH (1, 2, 3, 4, 5, 6, 7, 8-octahydrophenanthrene). Survival was observed to be our most sensitive endpoint. Although the initial ACR calculation performed by McGrath and DiToro (2009) did not include zebrafish as a test species nor did it include 1-methylnaphthalene or 1, 2, 3, 4, 5, 6, 7, 8 - octahydrophenanthrene, our data were observed to be within the range of their

data (Figure 3.8). HC-5 values derived from the TLM framework were also calculated and compared to observed chronic LC_{10} values (Figure 3.9) (McGrath and DiToro., 2009). In all cases the HC5 was lower than and the observed 30 day LC10 values and thus protective for ELS effects on zebrafish (Table 3.3).

3.3.5 Conclusions

The results of these studies demonstrate the ability of the TLM to accurately characterize the toxicity of a series of AHs during acute (120 hour) toxicity tests. Passive dosing provided an innovative method for maintaining aqueous exposure concentrations of poorly water soluble compounds during both acute and chronic exposures. Acute test results were consistent with TLM derived predictions for zebrafish. Using results of both acute and chronic test endpoints, ACRs derived in this study were consistent with earlier literature that have been compiled on PAHs and other test species. The TLM-derived HC-5 was observed to be protective of exposure concentrations demonstrating chronic effects to early life stages of zebrafish.

Table 3.1: Physical-chemical properties provided by SPARC (unless otherwise noted by*) of 6 AHs used in ELS exposures.

Compound Name	Molecular Structure	Molecular Weight (g/mol)	Water Solubility (mg/L)	logKow
Naphthalene		128.2	58	3.35
1-Methylnaphthalene		142.2	25	3.88
Biphenyl		154.2	7.0	4.16
Phenanthrene		178.2	1.2	4.74
Pyrene		202.3	0.24	5.25
1, 2, 3, 4, 5, 6, 7, 8, -Octahydrophenanthrene		186.3	0.47	5.19
Chrysene		228.3	0.0014*	5.90
Benzo(a)Pyrene		252.3	0.0017*	6.54

* Experimentally derived water solubility's

Compound	Mean measured exposure concentration (ug/L) ± SD	Day 0 mean measured concentration (ug/L) ± SD	Day 5 mean me concentration (ug
1-methylnaphthalene	0 ± 0.0	0 ± 0.0	0 ± 0.0
	118 ± 1.4	112 ± 2.0	125 ± 3.8
	204 ± 0.09	199 ± 2.1	209 ± 2.2
	401 ± 7.2	360 ± 14	437 ± 4.1
	913 ± 0.26	825 ± 6.	980 ± 6.4
	1716 ± 36	1606 ± 8.7	1826 ± 6
naphthalene	0 ± 0.0	0 ± 0.0	0 ± 0.0
	420 ± 52	416 ± 85	424 ± 11
	875 ± 0.0	859 ± 144	891 ± 14
	2183 ± 51	2196 ± 156	2170 ± 8
	5415 ± 38	5030 ± 2.1	5800 ± 5
	19351 ± 9.5	20036 ± 28	18667 ± 4
biphenyl	0 ± 0.0	0 ± 0.0	0 ± 0.0
	233 ± 15	246 ± 39	220 ± 59
	339 ± 14	339 ± 20	348 ± 0.0
	958 ± 88	958 ± 129	939 ± 5.6
	1670 ± 29	1670 ± 35	1751 ± 7
	3887 ± 145	3887 ± 39	3787 ± 24
phenanthrene	0 ± 0.0	0 ± 0.0	0 ± 0.0
	36 ± 0.13	35 ± 0.28	37 ± 0.10
	70 ± 1.0	72 ± 1.8	69 ± 3.2
	159 ± 1.0	168 ± 3.1	149 ± 4.5
	415 ± 1.9	430 ± 3.1	385 ± 0.3
	948 ± 9.8	1010 ± 0.0	886 ± 14
1, 2, 3, 4, 5, 6, 7, 8 - octahydrophenanthrene	0 ± 0.0	0 ± 0.0	0 ± 0.0
	9 ± 0.0	9 ± 0.25	8 ± 0.24
	29 ± 2.7	31 ± 4.0	27 ± 0.18
	79 ± 7.0	86 ± 18	73 ± 8.5
	221 ± 0.65	216 ± 0.37	226 ± 1.3
	714 ± 3.5	630 ± 14	784 ± 8.9
pyrene	0 ± 0.0	0 ± 0.0	0 ± 0.0
	8 ± 0.0	8 ± 0.06	7 ± 0.07
	17 ± 0.05	17 ± 0.0	15 ± 0.0
	30 ± 0.85	30 ± 1.3	28 ± 0.14

Table 3.2: Analytical summary of 6 acute zebrafish exposures. Concentrations deviated from the mean by no more than 10 percent.

Compound	Mean measured exposure concentration (µg/L) ± SD	Day 0 mean measured concentration (µg/L) ± SD	Day 5 mean measured concentration (µg/L) ± SD
1-Methylnaphthalene	0 ± 0.0 118 ± 1.4 204 ± 0.09 401 ± 7.2 913 ± 0.26 1716 ± 36	0 ± 0.0 112 ± 2.0 199 ± 2.1 360 ± 14 825 ± 6. 1606 ± 8.7	0 ± 0.0 125 ± 3.8 209 ± 2.2 437 ± 4.1 980 ± 6.4 1826 ± 60
Naphthalene	0 ± 0.0 420 ± 52 875 ± 0.0 2183 ± 51 5415 ± 38 19351 ± 9.5	0 ± 0.0 416 ± 85 859 ± 144 2196 ± 156 5030 ± 2.1 20036 ± 28	0 ± 0.0 424 ± 11 891 ± 144 2170 ± 84 5800 ± 56 18667 ± 42
Biphenyl	0 ± 0.0 233 ± 15 339 ± 14 958 ± 88 1670 ± 29 3887 ± 145	0 ± 0.0 246 ± 39 339 ± 20 958 ± 129 1670 ± 35 3887 ± 39	0 ± 0.0 220 ± 59 348 ± 0.0 939 ± 5.6 1751 ± 75 3787 ± 243
Phenanthrene	0 ± 0.0 36 ± 0.13 70 ± 1.0 159 ± 1.0 415 ± 1.9 948 ± 9.8	0 ± 0.0 35 ± 0.28 72 ± 1.8 168 ± 3.1 430 ± 3.1 1010 ± 0.0	0 ± 0.0 37 ± 0.10 69 ± 3.2 149 ± 4.5 385 ± 0.38 886 ± 14
1, 2, 3, 4, 5, 6, 7, 8 - Octahydrophenanthrene	0 ± 0.0 9 ± 0.0 29 ± 2.7 79 ± 7.0 221 ± 0.65 714 ± 3.5	0 ± 0.0 9 ± 0.25 31 ± 4.0 86 ± 18 216 ± 0.37 630 ± 14	0 ± 0.0 8 ± 0.24 27 ± 0.18 73 ± 8.5 226 ± 1.3 784 ± 8.9
Pyrene	0 ± 0.0 8 ± 0.0 17 ± 0.05 30 ± 0.85 63 ± 0.60 107 ± 1.0	0 ± 0.0 8 ± 0.06 17 ± 0.0 30 ± 1.3 63 ± 0.85 107 ± 0.71	0 ± 0.0 7 ± 0.07 15 ± 0.07 28 ± 0.14 62 ± 1.7 97 ± 2.1

Table 3.3: Summary of embryo-larval effects on zebrafish exposed to 5 separate AH compounds.

Compound Name	30-day LC10 Embryo/Larval Survival (µg/L)	ACR	Chronic HC-5 (µg/L)
1-Methylnaphthalene	141	7.2	38
Phenanthrene	44	7.6	10
1,2,3,4,5,6,7,8 Octahydrophe	9	5.8	5
Chrysene	>1.4	*	0.77
BaP	>1.7	*	0.21

*Could not calculate, compounds not toxic at water solubility.

Table 3.4: Summary of observed LC50 concentrations compared to TLM adjusted LC50 concentrations.

Compound	5-day LC50 (ug/L) with (95% confidence intervals)	Mol. Wt. (g/mol)	log LC50 (mmol/L)	Class correction factor (ΔC)	Adjusted LC50 value (mmol/L)
Naphthalene	6309 (5888-6760)	128.2	-1.31	-0.352	-0.96
1-methylnaphthalene	1013 (1182-1703)	142.2	-2.15	-0.352	-1.80
Biphenyl	1548 (1148-2290)	154.2	-2.00	-0.352	-1.65
Phenanthrene	334 (184-1493)	178.2	-2.73	-0.352	-2.38
1,2,3,4,5,6,7,8 - Octahydrophenanthrene	52 (39-80)	186.3	-3.55	-0.109	-3.45
Pyrene	96 (87-102)	202.3	-3.32	-0.352	-2.97

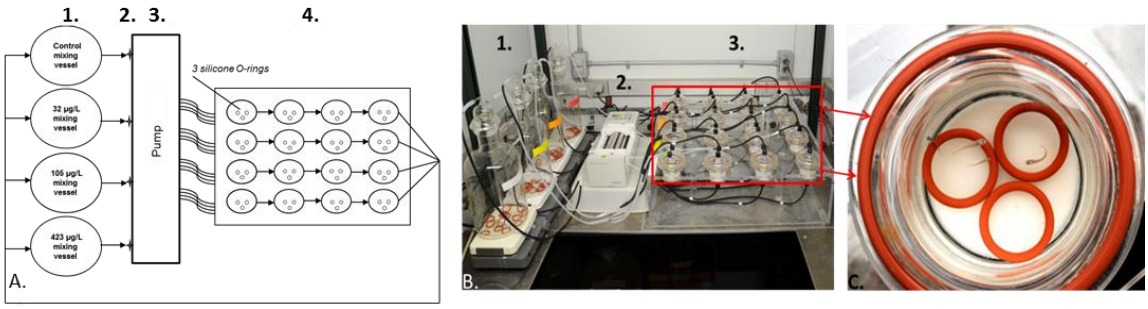


Figure 3.1: Conceptual and experimental design of recirculating flow-through test system used for larval zebrafish toxicity tests. Figure 3.1A (1) 4L mixing vessels containing phenanthrene-dosed O-rings serve as the main dosing reservoir; aqueous concentrations obtained from these vessels are split into 4 different channels with a glass manifold (2) and pumped (3) through 4 replicates (300 mL customized, glass flow through exposure chambers) each containing three supplemental passive dosing O-rings (4). After flowing sequentially through each chamber each effluent is re-circulated back to the respective mixing vessel. Arrows indicate flow direction. Figure 3.1B. Experimental Photo-representation of recirculating flow through system used for larval toxicity tests: (1) 4L mixing vessels containing phenanthrene-dosed O-rings serve as the main dosing reservoir; aqueous concentrations obtained from these vessels are split into 4 different channels with a glass manifold and pumped (2) through 4 replicates (300 mL customized, glass flow through exposure chambers) (3) each containing three supplemental passive dosing O-rings (figure 3.1C.).

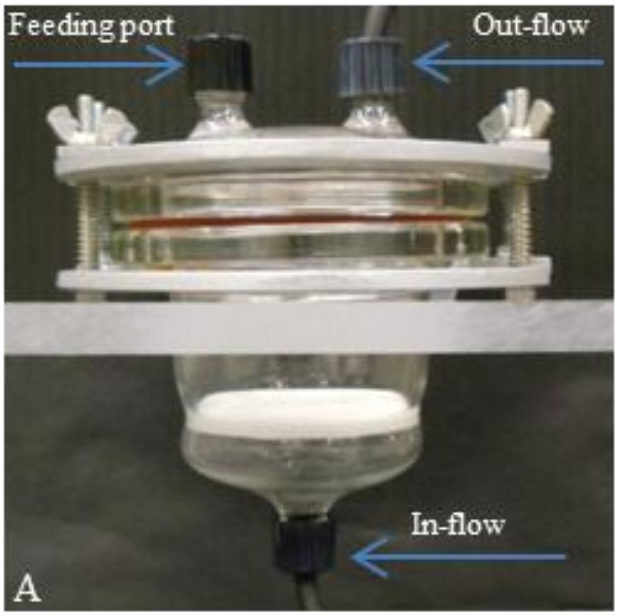


Figure 3.2: 300mL customized flow-through chamber labeled with port functionality.

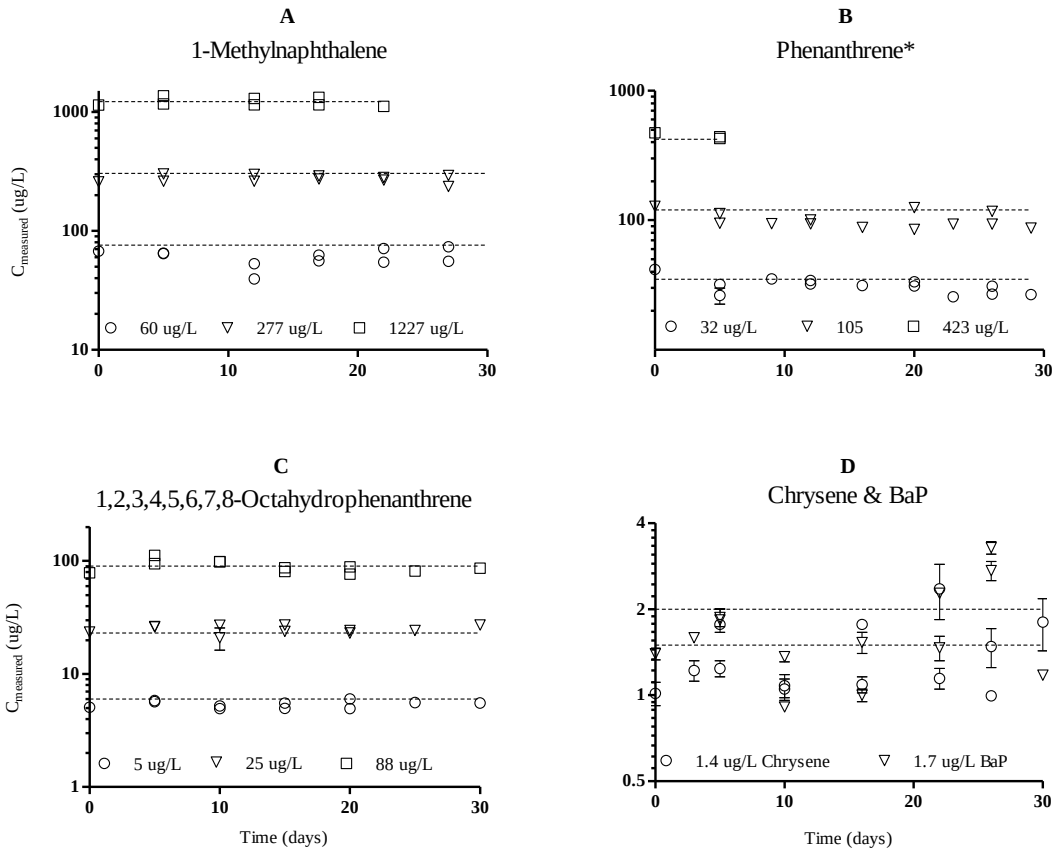


Figure 3.3: Measured aqueous concentrations in chronic tests. Each symbol represents the mean of two replicates plus the standard deviation The nominally predicted concentrations (---------) were obtained by dividing the methanol spiking concentration used to load the silicone O-rings by the methanol-water partition coefficient for the test compound that was calculated using SPARC. The concentration reported for each compound at the bottom of the plot represents the 30 day mean value for each treatment investigated. * Chronic exposure data for phenanthrene was taken from chapter 2.

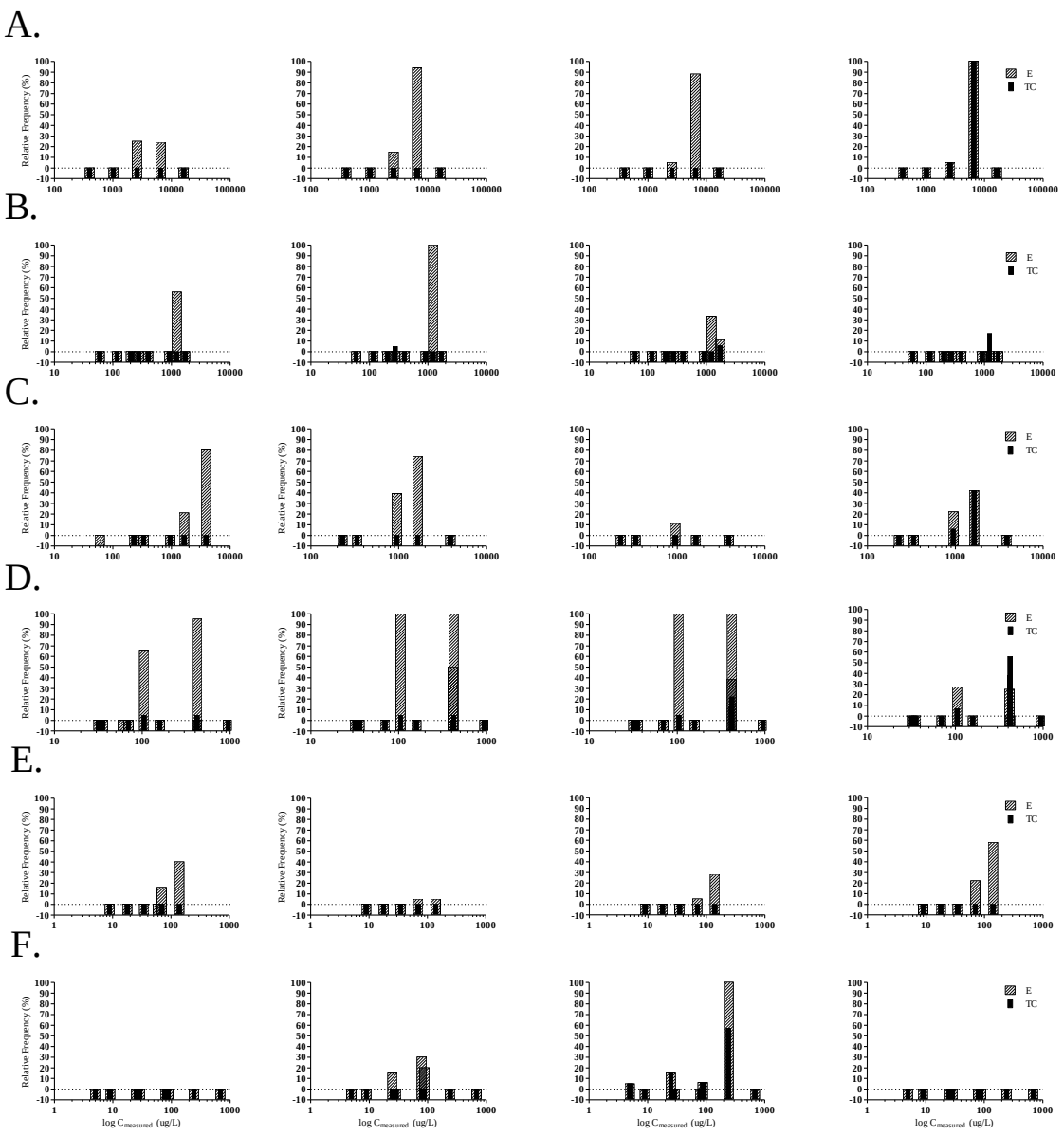


Figure 3.4: Relative frequency of sub-lethal side effects. Edema (E) and Tail Curvature (TC) for naphthalene (row A) 1-methylnaphthalene (row B), biphenyl (row C), phenanthrene (row D), pyrene (Row E) and 1, 2, 3, 4, 5, 6, 7, 8 -octahydrophenanthrene (row F) during the acute exposure period of the acute and chronic experiments. BaP and chrysene were not included because there were no sublethal effects observed. The X-axis is offset from the Y by 10% for visual clarity of each concentration tested.

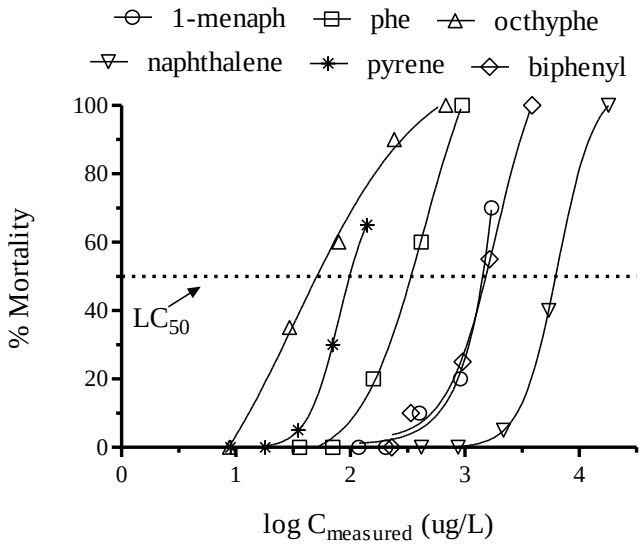


Figure 3.5: Acute (120 hour) concentration response relationships of the AHs tested.

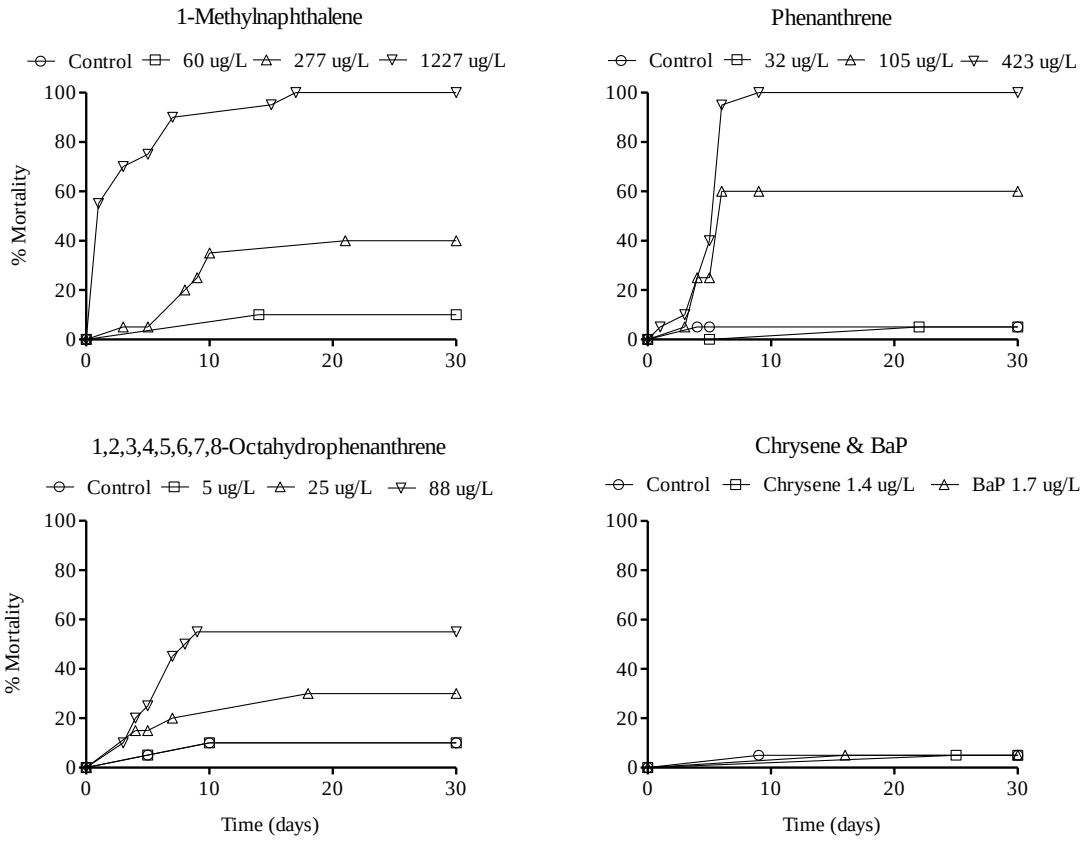


Figure 3.6: Cumulative embryo-larval mortality in 30 day chronic tests.

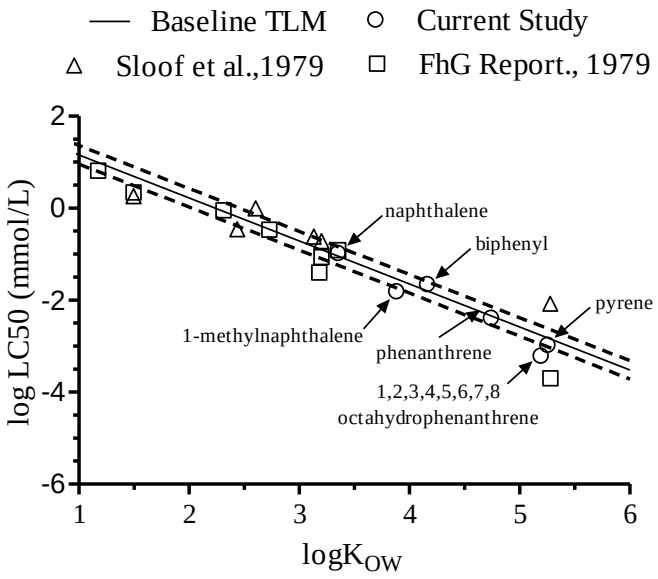


Figure 3.7: Comparison of the baseline TLM slope (—) (McGrath et al., 2009) and experimentally derived 120 hour LC50 values from the current study (○) as well as 4-day values (□) (FhG., 1979) and 2-day values (Δ) (Sloof et al., 1979) are in good agreement with model predicted regression. Dotted lines (------) are the upper and lower bound of baseline TLM.

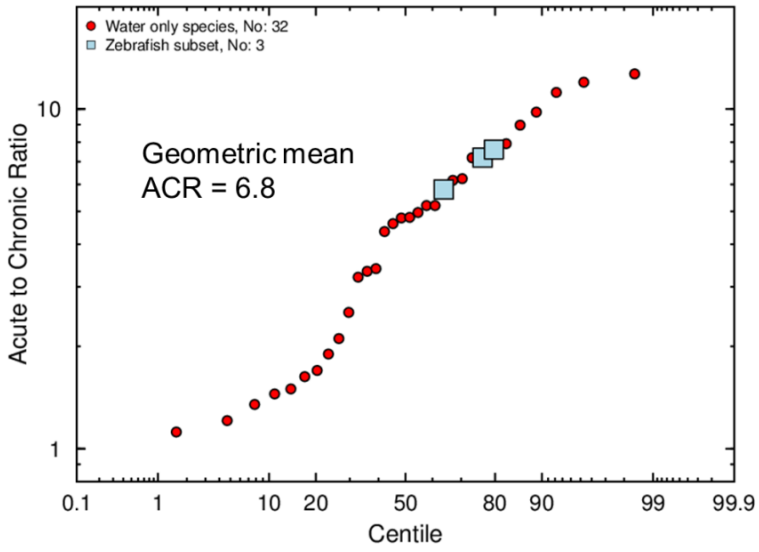


Figure 3.8: Initial ACR calculation performed by McGrath and DiToro (2009) (red circles) compared to our three exposures for which we could calculate ACR (blue squares).

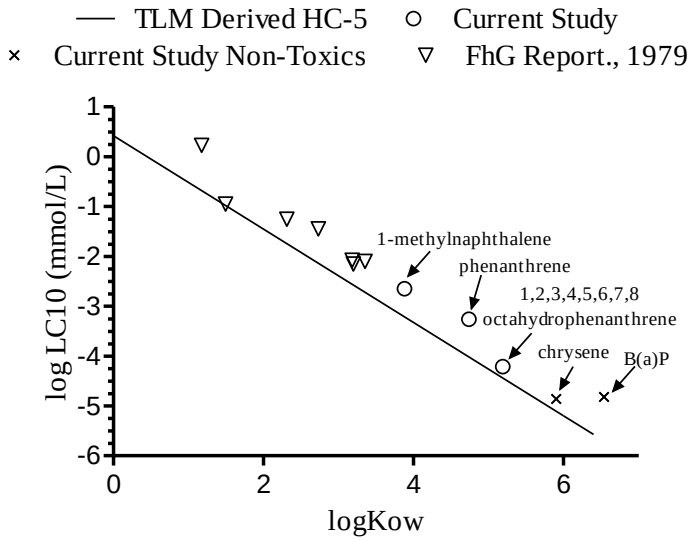


Figure 3.9: Experimentally derived 30-day LC10 values (○) compared to the HC-5 statistical extrapolation regression (—) (McGrath et al., 2009). Data are in good agreement with model predicted regressions.

Chapter 4

Applying the Toxic Unit Approach to Assess Toxicity of a Simple Hydrocarbon Mixture to Early Life Stages of Zebrafish

Abstract

The target lipid model (TLM) has previously been used to provide appropriate treatment level predictions for single compound exposures. Here we investigate its use coupled with the toxic unit (TU - the ratio of concentration in solution to response threshold concentration for the individual chemical) approach to a simple hydrocarbon mixture. Reliable experimental early life stage toxicity data for fish exposed to hydrocarbon mixtures are limited and further data are needed to confirm model predictions. Zebrafish embryo-larval sublethal and lethal developmental endpoints after a 5 day exposure as well as larval survival endpoints during a subsequent 25 day test period using a defined mixture of ten aromatic and naphthenic hydrocarbons with a log K_{ow} range of ca. 4 - 7.5 are described. Three treatment levels corresponding to a toxic unit range above and below unity (assuming concentration addition) were tested. To deliver well-controlled exposure concentrations, a passive dosing system consisting of silicone O-rings was employed. Partition coefficients between a methanol/hydrocarbon mixture and water were measured and calculated. These values were in good agreement with Sparc Performs Automated Reasoning in Chemistry (SPARC) predicted values. Acute and chronic effects were consistent with the assumption of additive toxicity. Furthermore,

Results indicated that effects of this mixture on 30 day embryo-larval survival endpoints were similar to effects on survival after 10 day.

4.1. Introduction

Environmental risk assessments of petroleum hydrocarbons typically focus on organism effects attributed to single compound exposures. However, it is uncommon for single compound exposures to occur in the aquatic compartment. Rather, aquatic organisms are usually exposed to a mixture of compounds. There is strong evidence that suggests chemicals with common specific or diverse modes of action work together to produce combination effects leading to elevated levels of toxicity (Kortenkamp et al., 2009; Dyer et al., 2011; McCarty et al., 2011). Mixture toxicity is usually assessed by treating the mixture as if it were a single substance or by tests of the mixture to establish individual contributions of multiple components. Here, we assess mixture toxicity as multiple components and assume additivity which has been found to be a conservative model for addressing toxicant interaction (Kortenkamp et al 2009; Landrum et al. 2012). Because of the seemingly infinite number of mixtures that could be formed in the environment, it is important to develop models that can accurately predict mixture toxicity for efficient use in environmental risk assessments.

The Target Lipid Model (TLM) is based on the hypothesis that the aqueous concentration corresponding to a toxic endpoint (i.e. LC50) can be predicted from the Critical Target Lipid Body Burden (CTLBB). The CTLBB (C_L^*) is the critical concentration in the target lipid of an organism where a toxic endpoint occurs (i.e. LC50) and can be calculated from the target lipid-water partition coefficient (K_{TLW}) (McGrath and DiToro 2009). It is important to note that the CTLBB is species and endpoint specific and that the K_{TLW} which is a chemical specific parameter and has been calibrated to 47 aquatic organisms is

assumed to be constant. The TLM relies on the octanol-water partition coefficient (K_{OW}) to describe lipid to water partitioning (K_{TLW}) of a substance into the lipid of an organism. This relationship, currently calibrated to 47 organisms, is observed to be linear in the K_{OW} range of ca. 0-6 with a slope of -0.936 and minor variations (ΔC) for certain chemical classes (e.g., mono-aromatic, aromatic) (McGrath et al., 2009) (eqn. 4.1).

$$logK_{TLW} = 0.936logK_{ow} + \Delta C \qquad (4.1)$$

The acute median lethal concentration can then be predicted by subtracting the $logK_{TLW}$ from the $logC_L^*$ (eqn. 4.2).

$$logLC_{50} = logC_L^* - logK_{TLW} \qquad (4.2)$$

Substituting Eqn. (4.1) into (4.2) yields:

$$logLC_{50} = logC_L^* + \Delta C - 0.936logK_{OW} \qquad (4.3)$$

Or as previously described by McGrath and DiToro (2009);

$$logLC_{50} = mlogK_{OW} + b \qquad (4.4)$$

where, m and b are the slope and intercept of the regressing line (LC50 as a function of log K_{OW}). The slope, m = -0.936 previously mentioned is the linear free energy relationship between the $logK_{TLW}$ and $logK_{OW}$. The intercept, b= $logC_L^* + \Delta C$.

Predictions of chronic effects for a particular chemical to a specific organism can then be performed using empirically derived distributions of acute to chronic ratios (ACRs) (DiToro et al., 2000; McGrath et al., 2004) (eqn.,4.5). Distributions of CTLBBs and ACRs are used to establish protective concentration levels using statistical extrapolation methods (McGrath and DiToro., 2009).

$$ACR = \frac{Acute\ Effect\ Concentration\ (LC_{50})}{Chronic\ Effect\ Concentration\ (EC_{10})} \qquad (4.5)$$

When assessing the toxicity of hydrocarbon mixtures, the TLM adopts the Toxic Unit (TU, the ratio of concentration in solution to a response threshold concentration for an

individual chemical) approach (eqn. 4.6) which has been validated by McGrath and DiToro (2009).

$$TU = \frac{C_{W,i}}{C_L^*} \quad (4.6)$$

Where $C_{W,i}$ is the concentration of a single compound (i) in water (W) and C_L^* is the predicted concentrations that elicits an endpoint effect (i.e. LC50). The individual TUs are summed to compute the toxicity of the mixture (eqn. 4.7). If the sum of all TUs in the mixture is greater than or equal to 1 the mixture is predicted to be toxic.

$$TU = \sum_i TU_{W,i} \quad (4.7)$$

It has been previously reported that PAHs may cause toxicity to fish via a number of different modes of action targeting specific organ systems and biochemical pathways that are unrelated to a direct effect on membrane effects (narcosis) (Barron et al., 2004; Incardona et al., 2004; Incardona et al., 2006). Regardless of the mode of action data are needed to determine whether TLM predictions are protective of chronic effects.

Over the last several years, short-term novel passive dosing techniques are increasingly being applied with hydrophobic organic compounds (HOCs) to deliver and maintain concentrations in aquatic tests (Brown et al., 2001; Kiparissis et al., 2003; Mayer & Holmstrup, 2008; Smith et al., 2010; Turcotte et al., 2011; Engraff et al., 2011; Butler et al., 2013). Chemical partitioning from a solvent solution into a Polydimethylsiloxane

(PDMS) reservoir has been conveniently used to “load” the test system. Subsequent partitioning from the loaded PDMS phase into the aqueous test media enables test organisms to be constantly exposed despite potential loss processes (e,g, sorption, biodegradation, biotransformation) that occur in the test system. In addition to improving control of test chemical exposure, this technique has the ability to decrease chemical use and costs associated with test design.

In this study, the toxicity of a hydrocarbon mixture to zebrafish (*Danio rerio*) early life stages (ELS) was investigated for 30 days Post Fertilization (PF). The objectives of this study were to: 1) apply the passive dosing method to a simple to moderately complex mixture of hydrocarbons using the TLM coupled with the TU approach in a chronic (30 day) fish ELS toxicity test (OECD 210), 2) Test the hypothesis of additivity associated with hydrocarbon mixtures.

4.2 Methods and Materials

4.2.1. Chemicals and materials

Twenty mL headspace vials were purchased from MicroLiter Analytical Supplies, Inc. (Suwanee, Georgia). Silicone O-rings were obtained from O-rings West (Seattle, WA). Bicyclohexyl, 1-methylnaphthalene, biphenyl, perhydropyrene, and pyrene (purity of 99, 95, 98, 95 and 98% respectively), were obtained from Sigma Aldrich (Milwaukee, WI). 1, 2, 3, 4, 5, 6, 7, 8, - octahydrophenanthrene, phenanthrene and hexahydropyrene (purity

of >95, 95, >98% respectively) were obtained from TCI America (Portland, OR). 1-methylphenanthrene (purity 99.5%) was obtained from Chem Service (West Chester, PA). Finally perhydrophenanthrene was synthesized by ExxonMobil Chemical Company (Clinton, NJ). Stock AH solutions were prepared in methanol (HPLC grade) obtained from J.T. Baker (Phillipsburg, NJ). Paramecium and brine shrimp (*Artemia nauplii*) used for feeding larval zebrafish were purchased from Z-FIN (Portland, OR) and Argent Foods (Salt Lake City, UT), respectively.

4.2.2. Loading and dosing with PDMS silicone O-rings

Silicone O-rings served as the dosing source for embryo/larval exposures. Methods similar to Butler et al (2013) and chapter 2 of this thesis were employed to load the hydrocarbon mixture from the methanol/hydrocarbon mixture solution into O-rings and subsequently dose the aqueous phase. Nominal aqueous concentrations of each component present in the mixture were predicted by dividing methanol concentrations by the component specific methanol-water partition coefficient. Experimentally derived partition coefficients were determined by loading three separate 20 mL glass vials each with four O-rings with 10 mL of the high treatment level in methanol. Equilibrium between methanol:PDMS was assumed after 72 hours and subsequently after 24 hours in the aqueous phase. These assumptions were based on previous intra-laboratory experiments and work reported by Smith et al., (2010). After a 72 hour equilibration on a vial roller at 28^{o}C, the methanol solution was poured off and an aliquot was diluted 1:20 with acetone and analyzed. Two O-rings from each vial were extracted four times each

with 10 mL of acetone on a wrist-action shaker for 1 hour. An aliquot of each extract was analyzed. This extraction was quantitative; the fourth extraction yielded less than 1.2% of masses in the first extraction. The other two O-rings were rinsed with glass distilled water (per chapter 2 rinsing regime) and wiped dry with lint-free tissue. They were added to a 20 mL glass vial containing embryo water. After agitation on an orbital shaker at 400 rpm for 24 hours at 28^{o}C, an aliquot of the water sample from each vial was analyzed. Partition coefficients between methanol and PDMS ($K_{MeOH:PDMS}$) and between PDMS and water ($K_{PDMS:water}$) were calculated following equation 4.8 and 4.9:

$$K_{methanol:PDMS} = \frac{C_{methanol}}{C_{PDMS}} \quad (4.8)$$

$$K_{PDMS:water} = \frac{C_{PDMS}}{C_{water}} \quad (4.9)$$

Where, C_{MeOH} is the methanol concentration (mg/L) (based on nominal loading), C_{PDMS} is the concentration in O-rings calculated from concentrations measured from acetone extracts (mg/L), and C_{water} is the water concentration (µg/L). The distribution between methanol and water can then be calculated my multiplying the $K_{methanol:PDMS}$ and $K_{PDMS:water}$ (eqn., 4.10).

$$K_{methanol:water} = K_{methanol:PDMS} \times K_{PDMS:water} \quad (4.10)$$

Experimentally measured partition coefficients were cross-referenced with those provided by SPARC (Sparc Performs Automated Reasoning in Chemistry) (www.ibmLc2.chem.uga.edu) for comparison.

Depletion of the methanol stock solutions during the methanol:PDMS partitioning phase and the silicone O-rings during the PDMS:water partitioning phase was assumed to be negligible. A key criterion for successful application of the passive dosing system used in this study was ensuring the mass of hydrocarbon components dissolved into methanol was much greater than that partitioned into the PDMS O-rings which in turn was much greater than that partitioned into the aqueous phase. This ensured that the test systems were not depleted and served as a constant dosing source.

To predict acute endpoints of mixture components (Figure 4.1) based on predicted water concentrations the TLM was fit to six previously tested single compounds (naphthalene, 1-methylnaphthalene, biphenyl, phenanthrene, pyrene, 1, 2, 3, 4, 5, 6, 7, 8,-octahydrophenanthrene) (Chapter 3) for which acute LC50 data was available.

To establish treatments for chronic testing, nominal exposure concentrations for each component were selected so that each compound contributed equally to the total acute or chronic toxic units. Acute toxic units were computed by dividing the predicted nominal concentration in water delivered via passive dosing by the substances' LC50 while chronic toxic units were obtained by instead using the estimated EC10 value. Three treatment levels and a control were investigated that provided total acute and chronic toxic units bracketing the theoretical value of unity where effects are predicted assuming additivity. The total acute TUs for the three treatment levels based on nominal exposure concentrations were 0.2, 0.8 and 3 whereas total chronic toxic units were 0.6, 5, and 20.

4.2.3. Maintenance of D. rerio culture and spawning procedures

An outbred strain of wild type zebrafish genotyped by Charles River Laboratories International, Inc. (Wilmington, MA), obtained from Aquatic Research Organisms (Hampton, NH), were used in this study. Twenty-four adult fish of the same age were held at a ratio of 2 males per female and maintained in a 30-liter flow-through customized breeding trap positioned inside a 132-liter aquarium with constant mechanical filtration. Oxygen concentration was held above 80% saturation. Culture conditions were 12:12 light/dark photoperiod with intensity between 400 – 800 lux (fluorescent lighting) and water temperature of 28 ±1°C. The bottom of the breeding trap consisted of 2 mm wire mesh which allowed eggs to fall through and prevent cannibalization by adult fish. Zebrafish spawning and fertilization took place within 30 minutes after the onset of light. After this period eggs were collected and immediately rinsed with water, transferred to a 125x65 mm crystallization dish filled with reconstituted water and examined for viability before being placed into an environmental chamber at 28 ±1°C.

4.2.4. Experimental Test Design

Embryos were exposed in 20 mL headspace vials that had 0.13 g O-rings resting on the bottom. Embryos were sequentially added to each vial, capped with teflon coated screw caps and placed into an environmental chamber at 28±1°C. Microscopic observations were performed in 24-hour intervals. Sub-lethal (pericardial edemas and spinal curvature)

and lethal (coagulation, lack of heartbeat) endpoints included those defined in the OECD FET draft guideline (OECD, 203). Twenty replicates with one embryo per vial were tested for each treatment. Exposing one replicate per vial allowed observations of a single embryo through the first 5 days of development. The acute test duration was based primarily on the assumption that zebrafish develop the ability to feed exogenously on day 5 (Belanger et al 2010).

In the second test phase (days 5-30), embryos that hatched into larval fish as well as any un-hatched embryos exposed in vials were transferred into 300-mL customized flow-through exposure chambers dosed to nominal concentrations. Depending on treatment related mortality individuals were divided into as many as four different flow-through chambers each with up to 5 fish per replicate chamber (Figure 4.2B, 3 and Figure 4.2C). Four-liter mixing vessels containing 30, 1.0g O-rings for each concentration were prepared (Figure 4.2B, 1). Three, 1.0g O-rings were also added to each customized flow through chamber (Figure 4.2C). This process is shown conceptually in figure 4.2A. The flow through each test chamber was supplied by a peristaltic pump (Figure 4.2B, 2) and was set to a rate of 5 mL/min. Mixing vessels supersaturated with oxygen, were exchanged every 5 days ± 24 hours with a new set of O-rings to ensure optimal water quality, avoid biofouling and limit possible PDMS depletion.

The customized flow through chambers contained three individual ports; two ports were located on top, one which served as an out-flow port and the other strictly for feeding purposes. The third port was located on bottom and served as the in-flow port. This design allowed for continuous flow with optimum mixing. The out-flow port was partially blocked with steel mesh to prevent larvae from swimming out of the exposure

chamber (Figure 4.3). Test media for the chronic phase (days 5-30) was prepared according to *Standard Methods for the Examination of Water and Wastewater* 21st Edition method 8010E (2005), moderately hard reconstituted water ($NaHCO_3$, 12 mg/L; $CaSO_4$ $2H_2O$, 60 mg/L; $MgSO_4$, 60 mg/L; KCl, 4 mg/L; water hardness, 80-100 mg $CaCO_3$/L; Alkalinity, 60 mg $CaCO_3$/L; pH 7.4-7.8).

Upon transfer to the flow through chambers, fish were fed *Paramecium multimicronucleatumn.* Larval fish were fed *Artemia nauplii* starting at 12 days. Paramecium and brine shrimp were fed *ad libitum*, twice daily throughout the experiment. During the feeding period, flow was turned off. After approximately 20 minutes of feeding, the flow-through system was re-started and flushed into a drainage basin for 10 minutes. After 10 minutes of flushing with test media all mixing vessels were filled and supersaturated with oxygen before starting flow recirculation. During the second phase fish were observed daily for immobility, lack of respiratory movement and lack of reaction to mechanical stimulus (OECD, 210). To evaluate potential larval growth effects, surviving fish were measured (total length) at study termination. The greater volume provided by the flow through system helped dilute the elevated levels of ammonia, nitrite and nitrate as well as overcome decreased levels of Dissolved Oxygen (DO) caused by DO consumption and biochemical oxygen demand as a result of the addition of paramecium to the test chamber and fecal discharge of larval fish.

4.2.5. Chemical Analysis

During the acute phase of the study 2 mL was pipetted from 10 vials for a total sample volume of 20 mL for each exposure concentration and control. During the chronic phase of the experiment 20 mL samples were taken from the mixing vessel or the outflow just prior to the water re-entering the mixing vessel. A 1:1 dilution scheme containing 1 part test water to 1 part moderately hard reconstituted diluent water ($NaHCO_3$, 12 mg/L; $CaSO_4$ $2H_2O$, 60 mg/L; $MgSO_4$, 60 mg/L; KCl, 4 mg/L; water hardness, 80-100 mg $CaCO_3$/L; Alkalinity, 60 mg $CaCO_3$/L; pH 7.4-7.8) was used. Water standards or hydrocarbon mixture analysis was performed using automated direct immersion (DI) solid phase microextraction (SPME) coupled with gas chromatography with mass selective detection (GC-MSD). The DI-SPME coupled with GC-MSD was equipped with an Agilent 6890N gas chromatograph and Agilent 5975 mass selective detector operated in the selective ion monitoring (SIM) mode. The analytical column was a 25 m x 0.2 mm id, 0.33 µm DB-5MS (J&W Scientific) capillary column. The GC-MSD system was equipped with a Gerstel CIS 4 inlet and interfaced with a Gerstel MPS 2 autosampler operated in the DI-SPME mode using a 7-µm PDMS fiber (Supelco) with a 90-minute extraction time at 30^{o}C. The SPME fiber was automatically desorbed for 5 minutes at 300^{o}C. The GC oven temperature was programmed from 50^{o}C for 5 minutes to 340^{o}C at 12^{o}C/minute. After an initial 25 psi pressure pulse for one minute, the helium carrier gas pressure was set at 13.8 psi corresponding to a constant flow of approximately 0.8 mL/min for the duration of the run.

Acetone samples were analyzed by the same GC-MSD method except for using a liquid injection of 1 µL. Analytical standards were prepared in acetone at average concentrations of 3.2, 9.6, and 64 µg/mL with an approximate concentration of 4 µg/mL of the internal standard. Methanol samples were diluted 1:20 with acetone. A 1 mL aliquot was spiked with 2 µL of 2000 µg/mL internal standard solution prior to analysis. A 1 mL aliquot of the acetone extract from O-rings was analyzed in the same way spiked with an approximate concentration of 4 µg/mL of the internal standard.

4.2.6. Statistical Methodology

Acute and chronic endpoint effect concentrations were calculated using either probit transformations (Finney et al., 1971) in SAS (SAS V9.2) or a linear model using the Benchmark Dose method (USEPA Benchmark Dose Method V2.1.2.). The T-test with Bonferroni adjustment (Bland, 1995) or Fisher's Exact Test (Finney, 1948; Pearson, 1962) using TOXSTAT (Gulley, 1994) software was used to determine significant differences from the Control.

4.3. Results and Discussion

4.3.1. Analytical confirmation of exposure concentrations in toxicity test

Experimentally measured partition coefficients were compared to SPARC predicted coefficients at 28°C (Table 4.1). Excluding hexahydropyrene partition coefficients were within a factor of 3.5 of SPARC predicted values.

Understanding the partitioning relationship of each mixture component allowed exposure concentrations to be predicted. Following equations 4.8 and 4.9, data from the partitioning experiments previously described were used to calculate $K_{MeOH:\ PDMS}$ and $K_{PDMS:water}$. Following equation 4.10, $K_{MeOH:water}$ was calculated and observed to increased linearly with component hydrophobicity (K_{OW}) ($logK_{MeOH:water} = 0.825logK_{OW} + 0.390;\ r^2 = 0.960$) (Figure 4.4). This can be compared to Smith et al., (2010) who reported a similar linear relationship between $K_{MeOH:water}$ and K_{OW} ($logK_{MeOH:water} = 0.939logK_{OW} + 0.039;\ r^2 = 0.978$).

Dissimilar to recent work (chapters 2 and 3) measured $K_{MeOH:water}$ were used for aqueous concentration prediction instead of SPARC predicted partition coefficients. Measured $K_{MeOH:water}$ correlated with component hydrophobicity ($logK_{OW}$) over 4 orders of magnitude. This is useful for calculating freely dissolved concentrations of Hydrophobic Organic Compounds in test water from known concentrations in methanol loading solutions.

During the first test phase, mixture concentrations for each compound were analytically confirmed at test start and then again on day 5 prior to the addition of larval fish to the customized flow through test chamber. While fish were in exposure chambers, measurements were taken from each new and corresponding old mixing vessel at least every 5-7 days with random concentration checks throughout the test duration (Figure 4.5). As shown within figure 4.5, mean measured concentrations decreased by no more than 22% throughout the test. Furthermore, excluding the low treatment level for 1, 2, 3, 6, 7, 8, - hexahydropyrene, perhydropyrene and hexadecahydropyrene, mean measured

water concentrations for all treatment levels and corresponding mixture component concentrations were in agreement with their predicted values.

4.3.2 Measured Toxic Units

To accurately compute TUs from measured water concentrations a C_L^* was calculated by regressing LC50 data from six previously tested acute single compound exposures (naphthalene, 1-methylnaphthalene, biphenyl, phenanthrene, pyrene, 1, 2, 3, 4, 5, 6, 7, 8, - octahydrophenanthrene) (Chapter 3) as a function of $\log K_{OW}$ (Figure 4.6). Following equation 4.3 the Y-intercept or calibrated C_L^* (2.12 µmol/g lipid) was fitted in the TLM (eqn. 4.11) to predict acute LC50s for all components in the mixture (Table 4.2).

$$logLC_{50} = 2.12 + \Delta C - 0.936 \times logK_{OW} \qquad (4.11)$$

Following equation 4.5, chronic endpoint concentrations for each component were predicted by dividing the acute LC50 by the geometric mean of the ACRs reported in Table (3.3) for 1-methylnaphthalene, phenanthrene and 1, 2, 3, 4, 5, 6, 7, 8,- octahydrophenanthrene of 6.8. Following equations 4.6 and 4.7 the measured water concentrations were divided by their respective endpoint concentration. These individual component TUs were added together to provide a summed TU for each treatment level in the mixture. These TUs corresponded to acute TUs of 0.10, 0.61, 1.4 and chronic TUs of

0.54, 4.8, and 13 (Table 3.3). Measured acute and chronic TUs were within a factor of 1.5 of predicted TUs.

4.3.3. Acute effects of hydrocarbon mixture on zebrafish embryos

Sub-lethal (pericardial edema and spinal curvature) and lethal effects were observed (Table 4.3). There were no discernible differences in endpoint sensitivity. Sub-lethal as well as lethal endpoints were observed to be equally sensitive in both a time and dose response fashion. Similar to previous experiments (chapter 3) sub-lethal effects, mainly pericardial edema were observed to slightly decrease over time which suggests potential reversibility in these endpoints. After 96 hours, mortality at the high exposure level was observed to be significantly different from the control ($p \leq 0.05$). Similarly pericardial edema in the mid and high treatment levels was also observed to be significantly different from controls at 96 and 120 hours ($p \leq 0.05$) (Table 4.3). An LC50 value could not be calculated at this time period (LC50 > 1.4 TUs).

Figure 4.7 shows a phenotypic representation of all sub-lethal side effects observed during the acute phase of the experiment. It is important to note that side effect variability in terms of severity was not observed and that the photo-representations of each sub-lethal effect provided are photo-pneumonic of side effects observed.

4.3.4. Chronic effects of hydrocarbon mixture on embryos

Lethality was observed to be the most sensitive endpoint during the larval life stage. Although observations for other endpoints such as reaction to mechanical stimulus, lack of respiratory movement and growth at test termination were performed, they were not observed in this study. Most of the mortality was observed to occur by day 10, with minimal mortality observed thereafter (Figure 4.8). The thirty day LC10 based on chronic TUs was 1.46.

4.3.5. Acute and chronic dose response

Consistent with the TU approach which relied on TLM predicted LC50 concentrations for each component in the mixture, there was 0, 5 and 30% mortality observed at 0.10, 0.61 and 1.4 TU respectively (Figure 4.9A). Extrapolating the observed acute dose response suggests that a 50% response would occur at ca. 2 TUs. This is higher than the theoretical value of one assuming strict additivity but within the uncertainty bounds of the TLM based LC50 estimates which exhibits an approximate 95% confidence interval that varies by a factor of ca. 2.5 (i.e. $10^{((2.099+2*0.101)-(2.099-2*0.101))}$). Similar to the acute TU dose response chronic TU were observed to be in excellent agreement with observed effects (Figure 4.9B). As the dose response approaches 10% mortality a TU of one is expected. The calculated LC10 based on measured chronic TUs was 1.46. This is well within the calculated confidence interval which is greater than acute TUs since additional uncertainty is introduced by applying the acute to chronic ratio.

4.3.6. Conclusions

This study demonstrates the utility of the passive dosing design for achieving and maintaining constant aqueous concentrations of all mixture components over the 30 day chronic test period with little loss. There was no discernible difference between lethal and sub-lethal endpoints for acute exposure. Lethality was observed to be the most sensitive endpoint during larval life stages. Although observations for reaction to mechanical stimulus as well as respiratory movement were performed, they were not observed. Acute and chronic endpoints were consistent with the TU approach with assumed additivity. Depending on severity of the sub-lethal side effects observed during the first 120 hours of exposure, the mid and high exposure concentrations showed slight reversibility in observed sub-lethal side effects. This is consistent with previously generated data using hydrocarbons as test substances.

Table 4.1: Comparison of predicted and measured logKMeOH:water for the test components via O-rings at 28°C.

CHEMICAL	Predicted	Measured		
	$logK_{MeOH:water}$	$logK_{MeOH:PDMS}$	$logK_{PDMS:water}$	$logK_{MeOH:water}$
1-Methylnaphthalene	3.48	0.70	2.98	3.68
Biphenyl	3.76	0.67	3.26	3.92
Phenanthrene	4.34	0.43	3.84	4.27
1-Methylphenanthrene	4.76	0.49	4.26	4.75
1, 2, 3, 4, 5, 6, 7, 8, -Octahydrophenanthrene	4.79	0.10	4.29	4.39
Pyrene	4.85	0.4	4.35	4.74
1, 2, 3, 6, 7, 8, -Hexahydropyrene	5.30	-0.07	4.83	4.76
Bicyclohexyl	5.6	0.56	5.10	5.66
Perhydrophenanthrene	5.63	1.05	5.13	6.17
Hexadecahydropyrene	5.66	1.41	5.16	6.56

Table 4.2: Predicted 5 day and 30 day zebrafish toxicity values and measured exposure concentration for each component and corresponding TU.

Mixture Compound	Predicted Toxicity (µg/L)		Mean Measured Exposure Concentrations (µg/L)					
			Level 1		Level 2		Level 3	
	5 d LC_{50}	30 d LC_{10}	5 d	30 d	5 d	30 d	5 d	30 d
1-methylnaphthalene	1946	282	3.5	3.9	22	26	112	158
biphenyl	1154	167	3.3	4.7	18	25	93	126
phenanthrene	382	55	0.78	0.90	3.2	4.3	15	19
1-methylphenanthrene	167	24	0.26	0.28	0.69	0.86	3.2	3.4
1,2,3,4,5,6,7,8-octahydrophenanthrene	265	38	0.28	0.24	1.1	0.83	4.8	4.1
pyrene	145	21	0.39	0.57	1.4	2.1	7.5	8.8
hexahydropyrene	53	7.7	0.23	0.26	0.48	0.53	2.1	1.8
bicyclohexyl	18	2.6	0.60	0.11	0.57	0.55	0.39	2.7
perhydropyrene	1.1	0.16	0.04	0.04	0.48	0.51	0.70	0.6
perhydrophenanthrene	5.3	0.77	0.07	0.09	0.47	0.73	2.3	4.4
∑ TUs			0.10	0.54	0.61	4.8	1.4	13

Table 4.3: Summary of sub-lethal effects on zebrafish embryo/larvae.Ninitial=20.

Compound	Sub-Lethal Side Effect	TUs	Percent of Remaining Embryo Sub-Lethal Effects				
			24 hours (%)	48 hours (%)	72 hours (%)	96 hours (%)	120 hours (%)
Simple Hydrocarbon Mixture	Mortality	0.00	0	0	0	0	0
		0.10	0	0	0	0	0
		0.61	0	0	0	5	5
		1.4	0	10	20	30*	30*
	Pericardial Edema	0.00	0	0	0	0	0
		0.10	0	0	0	0	0
		0.61	0	0	5	26*	21*
		1.4	10	11	19	35*	29*
	Tail Curvature	0.00	0	0	0	0	0
		0.10	0	0	5	26*	21*
		0.61	10	11	19	35*	29*
		1.4	0	0	6	7	7

* Statistically Significant from controls at a p value of 0.05

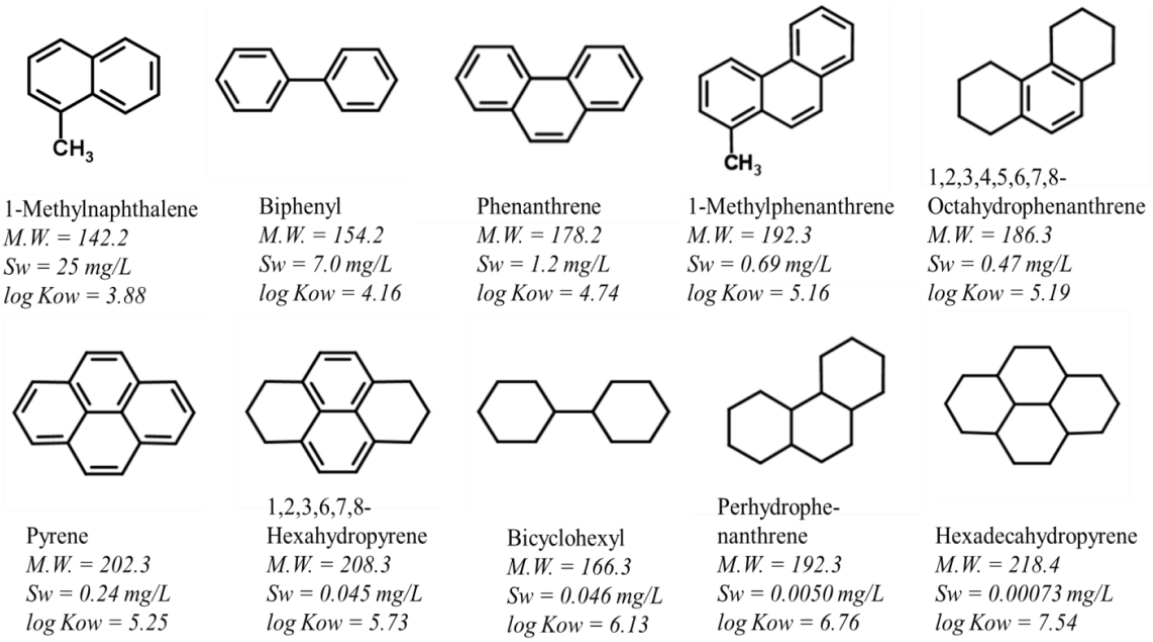


Figure 4.1: Physicochemical properties of aromatic hydrocarbons used in this experiment.

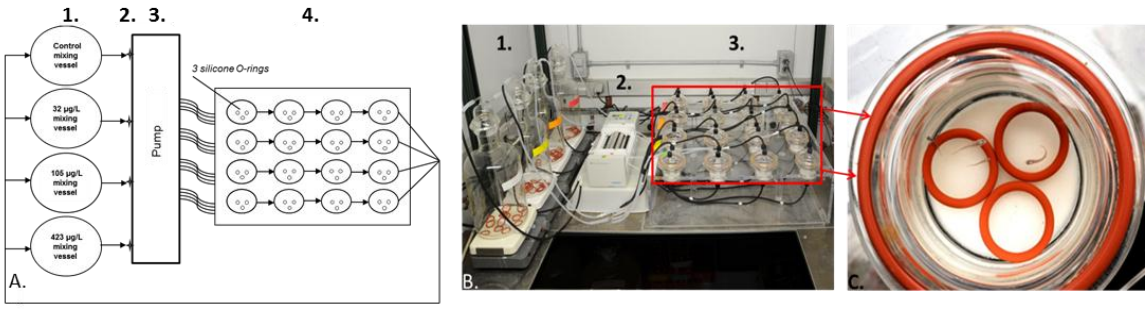


Figure 4.2: Conceptual and experimental design of recirculating flow-through test system used for larval zebrafish toxicity tests: Figure 2A, (1) 4L mixing vessels containing phenanthrene-dosed O-rings serve as the main dosing reservoir; aqueous concentrations obtained from these vessels are split into 4 different channels with a glass manifold (2) and pumped (3) through 4 replicates (300 mL customized, glass flow through exposure chambers) each containing three supplemental passive dosing O-rings (4). After flowing sequentially through each chamber each effluent is re-circulated back to the respective mixing vessel. Arrows indicate flow direction. Figure 2B. Experimental Photo-representation of recirculating flow through system used for larval toxicity tests: (1) 4L mixing vessels containing phenanthrene-dosed O-rings serve as the main dosing reservoir; aqueous concentrations obtained from these vessels are split into 4 different channels with a glass manifold and pumped (2) through 4 replicates (300 mL customized, glass flow through exposure chambers) (3) each containing three supplemental passive dosing O-rings (figure 2.C.).

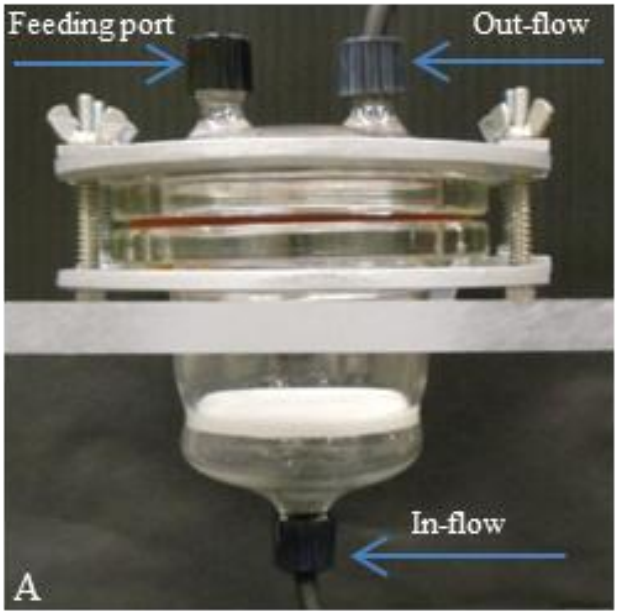


Figure 4.3: 300mL customized flow-through chamber labeled with port functionality.

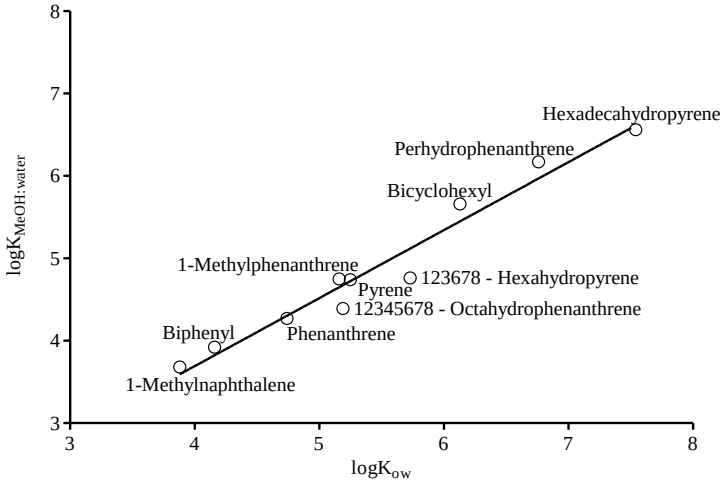


Figure 4.4: Measured Kmethanol:water as a function of Kow.

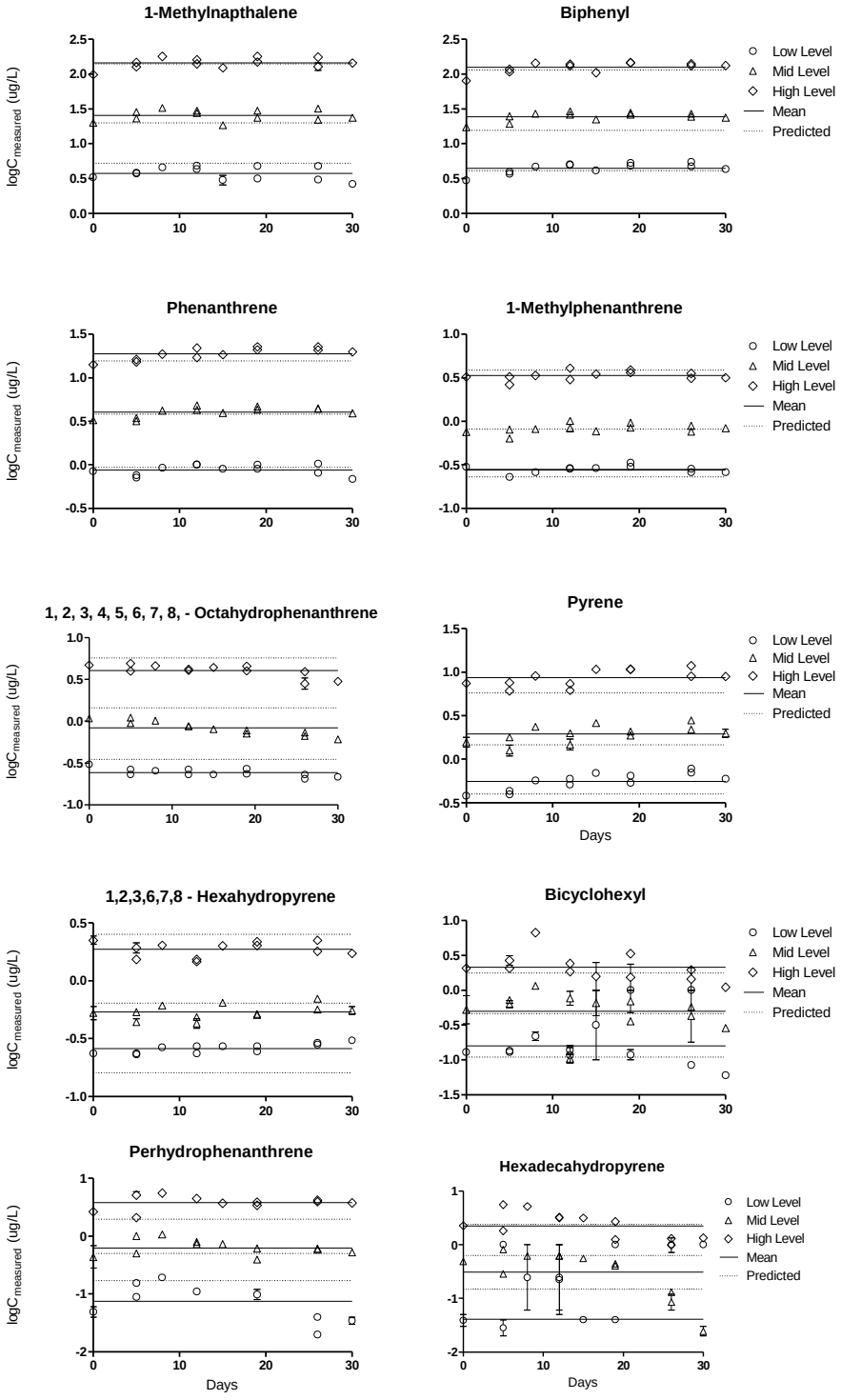


Figure 4.5: Comparison of observed and predicted (lines) exposure concentrations. Each point represents the mean and standard deviation of two replicates.

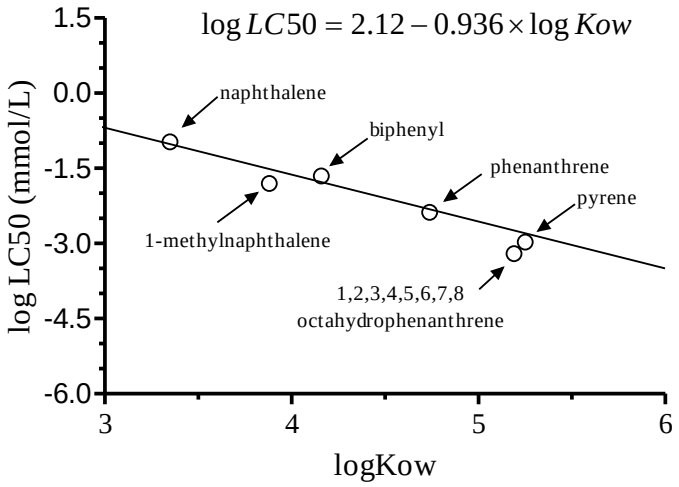


Figure 4.6: Comparison of the baseline TLM slope (—) (McGrath et al., 2009) and experimentally derived 120 hour LC50 values from previous intra-laboratory testing (○). The equation provided within the graph window shows the y-intercept (C_L^*).

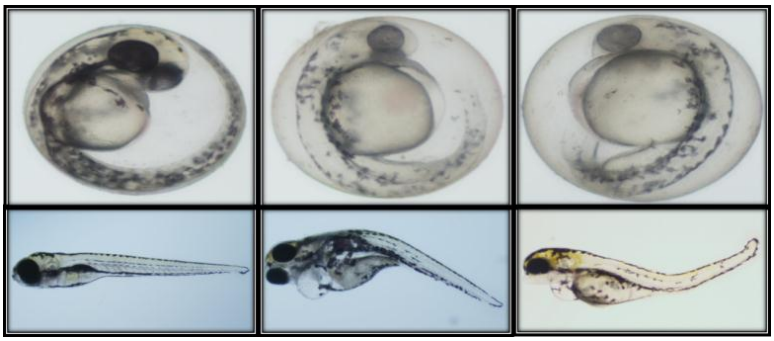


Figure 4.7: Sublethal effects observed throughout exposures. Row 1, pictures B and C show edema in the pericard and yolk sac region at 48 hours post fertilization (hpf), these can be compared to the control (row 1A). Row 2, pictures E and F show tail curvature and edema at 120 hpf, these can be compared to the control at 120 hpf (row 2D).

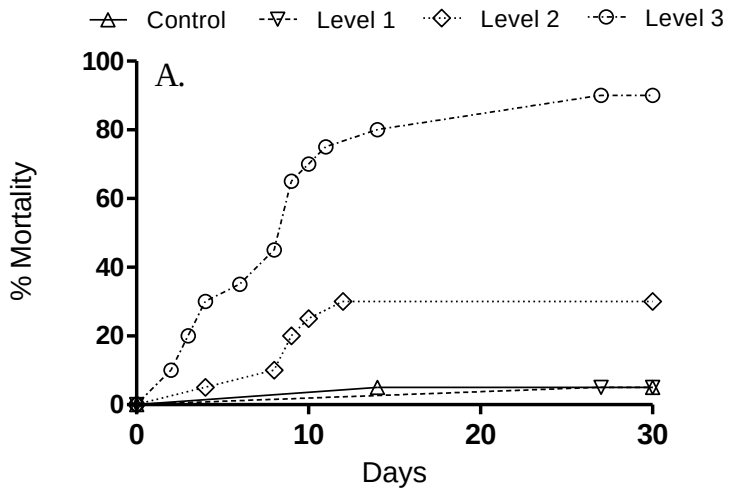


Figure 4.8: Cumulative embryo-larval mortality during the test.

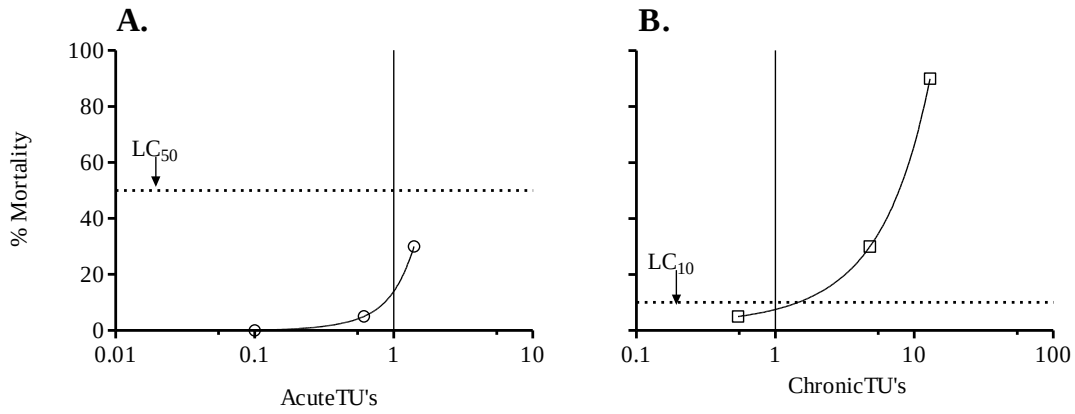


Figure 4.9: Acute and chronic embryo mortality as a function of their respective Toxic Units.

Chapter 5

Summary of chapters

5.1. Summary of Chapters

The experiments performed in this research support the hypothesis that the Target Lipid Model (TLM) can be used to successfully predict aqueous effect concentrations as well as provide a framework to provide chronic protection (HC5 values) to fish Early Life Stages (ELSs). The ability of a passive dosing approach to accurately and precisely dose to nominal concentration was tested and confirmed with an initial sentinel toxicant (phenanthrene) exposure lasting 30 days. Consequently, the approach was then applied to a myriad of acute and chronic exposures using aromatic hydrocarbons (naphthalene, 1-methylnaphthalene, biphenyl, phenanthrene, 1, 2, 3, 4, 5, 6, 7, 8, -octahydrophenanthrene, pyrene) ranging in $\log K_{OW}$ values of over two orders of magnitude (ca. 3 – 5) and water solubilities of ca. 0.24 - 25 mg/L (SPARC) ((http://archemcalc.com/sparc). Additionally, the application of the TLM coupled with the Toxic Unit (TU) approach to predict appropriate component concentrations in a simple mixture experiment including 10 relevant hydrocarbons was verified. Data generated were used to derive species and endpoint specific CTLBBs for ELSs of zebrafish - building robustness in the TLM for predicting endpoint concentrations as well as validating it performance for use in environmental risk assessments.

5.2. Sentinel reference toxicant study using a passive dosing technique

The TLM has been used to derive water quality objectives (LC10, LC50, HC5) that are intended to be protective of chronic effects posed by hydrocarbons (DiToro, 2000). However, reliable experimental ELS chronic toxicity data for fish were limited and further data were needed to confirm model predictions. Efforts have been underway to develop a zebrafish embryo toxicity test guideline to reduce, refine and replace the use of vertebrates in animal testing (Belanger et al., 2010; Strahle et al., 2011). An adaptation of this method which includes embryo lethal and sub-lethal developmental endpoints after a 5 day exposure as well as larval survival and growth endpoints during a subsequent 25 day test period is described within chapter 2 using phenanthrene as a sentinel reference toxicant. To deliver well controlled aqueous exposure concentrations, a passive dosing system consisting of silicone coated vials and silicone O-rings coupled with a flow-through test design was employed (Figure 2.2).

Acute results (0-120 hours) indicated that edema and spinal curvature were the most sensitive sublethal effects observed and in many cases preceded observed mortality. Edema was observed first in all exposure levels followed by spinal curvature during the later stages of physiological development within the acute exposure period. At the end of the acute phase of the experiment the EC10 and EC50 values with their 95% confidence intervals were 189 µg/L (49–274) and 386 µg/L (300–536) respectively. Because edema was observed to be reversible (Table 2.1) during the acute exposure these EC10/50 values were based on spinal curvature. Fifty percent mortality was not observed at 120 hours post fertilization (Table 2.1; Figure 2.6) therefore, a LC50 value could not be calculated

at this time period (LC50 > 423µg/L). However, the TLM predicted phenanthrene LC50 for zebrafish is 510 µg/L (McGrath & DiToro et al., 2009) which is not inconsistent with our results. These findings are also consistent with a recent study that reported a 96 hour no effect concentration for phenanthrene of 870 µg/L based on nominal exposure concentrations (Wolinkska et al. 2011).

The 30 day LC/EC10 values for larval survival and growth were 40 and 67 µg/L respectively. Concentrations shown to cause adverse effects in this study are in the range of previous studies that investigated the chronic effects of phenanthrene on fish (Table 2.3) (Call et al., 1986; Hooftman & Evers de Ruiters, 1992; Passino-Reader, 1995; Roex et al., 2001; Rhodes et al., 2005; Turcotte et al., 2011). These studies bracket effect concentrations reported in the present study. The study performed by Hooftman and Eves-de Ruiter (1992) best matched the duration of our test. These authors observed survival and growth to be the most sensitive parameters with a reported 42 day No Observed Effect Concentration (NOEC) of 56 µg/L which is within a factor of two of the reported NOEC in the current study of 32 µg/L. The NOEC in this study is slightly lower and may reflect the more well defined, constant exposure provided by the passive dosing system as suggested in previous studies (Kiparissis et al., 2003).

Further, results confirm that TLM predicted water quality objectives for phenanthrene were protective of ELS effects on zebrafish. Further work is needed to determine the relative sensitivity of embryo versus larval chronic endpoints for additional hydrocarbons and fish species.

5.3. Single compound exposures to test and calibrate TLM

The target lipid model (TLM) has been used to derive water quality objectives (HC5s) that are intended to be protective of chronic effects posed by hydrocarbons (McGrath & DiToro et al., 2009). However, reliable experimental ELS chronic toxicity data for fish were limited and further data were needed to confirm model predictions. Single compound exposures were performed using 8 aromatic hydrocarbons with a K_{OW} range of ca. 3 to 6 (Figure 3.1). Six aromatic hydrocarbons were exposed acutely (120 hours) (naphthalene, 1-methylnaphthalene, biphenyl, phenanthrene, 1, 2, 3, 4, 5, 6, 7, 8, -octahydrophenanthrene) while five aromatic hydrocarbons were exposed acutely and chronically (30 days) (1-methylnaphthalene, phenanthrene, phenanthrene, chrysene and BaP). Including two different exposure periods afforded the opportunity to select exposure concentrations which targeted different developmental life stages. Further, this design allowed empirical ACRs to be calculated (Table 3.3) and compared to earlier compilations across hydrocarbons and test species (McGrath and DiToro, 2009). Inclusion of chronic tests also allowed direct comparison of chronic endpoints to TLM-derived HC5 predictions to determine if this framework was protective for chronic effects on fish ELSs (Table 3.3).

During acute exposures sublethal and lethal endpoints were assessed after 120 hours (Figure 3.4; Figure 3.5), whereas only larval survival was assessed during chronic testing. To deliver well controlled exposure concentrations, a passive dosing system consisting of silicone O-rings coupled with a flow-through test design was employed (Figure 3.1; Figure 3.2). Following OECD (203) (acute phase) and OECD (210) (chronic phase) observations for coagulation, abnormal somite development, lack of tail bud detachment,

lack of heart-beat and immobilization were performed. However, coagulation, lack of heartbeat and immobilization were the only lethal endpoints observed. Pericardial edema, yolk sac edema and tail curvature were the most sensitive sub-lethal side effects observed. Using LC50 values generated during the acute experiments in chapter 3 (6 total) allowed the opportunity to regress calculated LC50 values as a function of $\log K_{OW}$. The Y-intercept of this regression corresponds to the Critical Target Lipid Body Burdon (CTLBB) in the TLM equation 4.11. The Y-intercept of 2.12 mmol/L could then be compared to the CTLBB derived by McGrath & Ditoro et al., (2009) of 2.10 mmol/L. CTLBBs were in excellent agreement with each other.

Using results of both acute and chronic test endpoints, ACRs derived in this study were consistent with earlier literature that have been compiled on PAHs and other test species. In each exposure, results indicated that 10 day LC10 values were similar to 30 day embryo-larval LC10 values (Figure 3.6). Furthermore, results confirm that TLM predicted water quality objectives for all aromatic hydrocarbons tested are protective for ELS effects to zebrafish (Table 3.3).

5.4. Hydrocarbon mixture experiment

The TLM has previously been used to provide appropriate treatment level predictions for single compound exposures. Within chapter 4 we investigated its use coupled with the TU approach to a simple to moderately complex hydrocarbon mixture. Reliable experimental ELS toxicity data for fish exposed to hydrocarbon mixtures were limited therefore further data were needed to confirm model predictions. Zebrafish embryo-larval sub-lethal and lethal developmental endpoints after a 5 day exposure as well as larval

survival endpoints during a subsequent 25 day test period using a defined mixture of ten aromatic and naphthenic hydrocarbons with a log K_{OW} range of ca. 4 - 7.5 was described. Three treatment levels corresponding to a TU range above and below unity (assuming concentration addition) were tested. To predict acute endpoints of mixture components the TLM was fit to six previously tested single compounds (naphthalene, 1-methylnaphthalene, biphenyl, phenanthrene, pyrene, 1, 2, 3, 4, 5, 6, 7, 8,-octahydrophenanthrene) (Chapter 3) for which acute LC50 data was available. Chronic LC10 concentrations were then provided by applying the ACR derived from previous experiments for which acute LC50 and chronic EC10 data were available (Table 3.3). To determine the normalized amount of each component added to the solution each EC10 concentrations was multiplied by a TU which corresponded to a total summed chronic TU for all compounds of 0.6, 5 and 20 (treatment level 1, 2, and 3 respectively). Because the test design was focused primarily on chronic endpoints initial acute TUs were not calculated. To deliver well-controlled exposure concentrations (Figure 4.5), a passive dosing system consisting of silicone O-rings was employed (Figure 4.2;4.3). Dissimilar to previous experiments partition coefficients between a methanol/hydrocarbon mixture and water were measured and calculated (Table 4.1). These values were in good agreement with previously used Sparc Performs Automated Reasoning in Chemistry (SPARC) predicted values. Following equations 4.6 and 4.7 individual component TUs for each treatment level were calculated and added together to provide a summed TU for each treatment level in the mixture. These TUs corresponded to acute TUs of 0.10, 0.61, 1.4 and chronic TUs of 0.54, 4.8, and 13 (Table 4.2). Measured chronic TUs were within a factor of 1.5 of predicted TUs. Acute and chronic effects were consistent with the

assumption of additive toxicity (Figure 4.9). TUs from individual mixture components were calculated (Table 5.1). Individual component TU contributions were calculated and were observed to be relatively similar to eachother with the exception with the larger K_{OW} compounds throughout all treatment levels. Furthermore, similar to previous experiment performed within chapter 2 and 3 results indicated that the 10 day LC10 value of 2.23 was similar to 30 day LC10 value of 1.46 based on TUs.

5.5. Conclusions and future work

Passive dosing provided an innovative method for maintaining aqueous exposure concentrations of poorly water soluble compounds. The validity of this approach was demonstrated by keeping aqueous concentrations constant during acute and chronic exposures. Endpoint values were observed to be slightly more sensitive when compared to previous exposures of similar duration reflecting the better defined, constant exposure provided by the passive dosing system. This research demonstrated the ability of the TLM to accurately characterize acute and chronic toxicity of a series of aromatic hydrocarbons. Using results of both acute and chronic endpoints, acute to chronic ratios derived in this study were consistent with earlier literature that has been compiled on hydrocarbons and other test species. The TLM derived HC-5 was observed to be protective of acute and chronic exposure concentrations demonstrating effects to ELS s of zebrafish. Additionally, the TLM was employed to successfully predict individual component concentration for a chronic simple hydrocarbon mixture experiment. The predicted individual component concentrations were used to derive appropriate treatment level predictions for the ELS exposure. Throughout all chronic single compound (1-

methylnaphthalene, phenanthrene, 1, 2, 3, 4, 5, 6, 7, 8, - octahydrophenanthrene) and mixture exposures, 10 day LC10 values were similar to 30 day LC10 values. Although more experimental data are needed, the results in all chronic exposures including the hydrocarbon mixture experiment suggest the possibility of a shortened ELS test.

The success of the passive dosing methodology suggests that it could be successfully used in other scenarios. These would include; toxicity testing of rapidly photo-degrading and readily photo-activated compounds, performing time variable toxicity studies with similar compound classes, investigating developmental and genetic effects of similar compounds to different species for deriving endpoint specific CTLBBs.

Utilizing passive dosing with PAHs coupled with the confounding effects posed by increase rates of photolysis and consequent decreased half-lives as well as photo-transformation reported in Salihoglu et al., (2012) should be investigated. Many PAHs are considered to be teratogenic, mutagenic and tumorigenic. Although the mechanisms by which PAHs elicit toxicity have been well studied, the environmental fate of PAHs and the photo-toxicity exerted by PAHs, as well as their photoreaction products formed in the environment, have received much less attention. Upon UV exposure, PAHs and all their photoreaction products have the ability to absorb light energy which leads to photo-excited states. The photo excited compound can then react with molecular oxygen, to produce reactive oxygen species (ROS) and other reactive intermediates, such as oxygenated PAHs and free radicals. These intermediates, induce DNA damage and cause lipid peroxidation (PP. Fu et al., 2012). Ultraviolet (UV) radiation in some cases degrades and in other cases alters the structure of organic contaminants such as hydrocarbons in the aqueous phase (Bertilsson & Widenfalk 2002). Maintaining constant aqueous

exposure despite the rapid onset of photolysis and consequent degradation of the compound has traditionally been challenging. The passive dosing methodology developed in this thesis may provide a means for constant exposure for even the most photo-degraded compounds. Furthermore, The TLM could then be calibrated to endpoints specifically calibrated to photomodified or photosensitized exposures. Relationships between dose and exposure leading to toxic effects have been well recognized, although data for this type of toxicity testing is limited. Performing toxicity experiments while varying the time of exposure gives the researcher new insights into time dependent effect concentrations. This is particularly useful when assessing toxicity to transient exposures (i.e. oil spills). Generating time-variable data would allow the ability to calculate an LC50 at different time points.

Currently the TLM is calibrated to LC50s provided by acute exposures. These LC50 values can then be extrapolated by ACRs to predict chronic effect endpoints. Time-variable exposure would build robustness into the model and therefore make it more appealing to other researchers and increase acceptance into the scientific community. Although this research has built credible robustness into the TLM for ELS testing of zebrafish, data for other species and are still considered limited (McGrath & DiToro, 2009). Additional research is needed in the form of single compound and mixture exposures with varying exposure periods to petroleum products regarding species for which CTLBBs are available. These data would further validate the TLM and TU concepts.

From rapid development and organogenesis to clear chorion and transparent larvae using zebrafish as a model organism in this research had many advantages. Worldwide,

zebrafish have become a popular model for biomedical research and aquatic toxicology (Strahle et al., 2011). They allow scientist to assess simple developmental abnormalities, but can also be used to determine more complex genetic abnormalities. Future experiments training a CTLBB to genetic alterations within an animal could be helpful in using the TLM to predict acute and chronic reproductive, growth and mortality effects.

Table 5.1: Predicted 5 day and 30 day endpoint values as well as measured exposure concentration and corresponding TU contribution for each component.

Mixture Compound	Predicted Toxicity (µg/L)		Level 1				Level 2				Level 3			
	5 day LC_{50}	30 day LC_{10}	5 day[a] (µg/L)	TU[b]	30 day[a] (µg/L)	TU[b]	5 day[a] (µg/L)	TU[b]	30 day[a] (µg/L)	TU[b]	5 day[a] (µg/L)	TU[b]	30 day[a] (µg/L)	TU[b]
1-Methylnaph	1946	282	3.5	0.002	3.9	0.014	22	0.011	26	0.093	112	0.057	158	0.561
Biphenyl	1154	167	3.3	0.003	4.7	0.028	18	0.016	25	0.150	93	0.081	126	0.752
Phe	382	55	0.78	0.002	0.90	0.016	3.2	0.008	4.3	0.078	15	0.040	19	0.352
1-Methylphe	167	24	0.26	0.002	0.28	0.012	0.69	0.004	0.86	0.036	3.2	0.019	3.4	0.141
1,2,3,4,5,6,7,8-Octahydrophe	265	38	0.28	0.001	0.24	0.006	1.1	0.004	0.83	0.022	4.8	0.018	4.1	0.106
Pyrene	145	21	0.39	0.003	0.57	0.027	1.4	0.010	2.1	0.098	7.5	0.052	8.8	0.418
Hexahydropyrene	53	7.7	0.23	0.004	0.26	0.034	0.48	0.009	0.53	0.069	2.1	0.039	1.8	0.233
Bicyclohexyl	18	2.6	0.60	0.034	0.11	0.042	0.57	0.032	0.55	0.212	0.39	0.022	2.7	1.03
Perhydropyrene	1.1	0.16	0.04	0.034	0.04	0.245	0.48	0.430	0.51	3.123	0.70	0.621	0.6	3.67
Perhydrophe	5.3	0.77	0.07	0.012	0.09	0.117	0.47	0.088	0.73	0.945	2.3	0.440	4.4	5.76
∑ TUs			0.10	0.10	0.54	0.54	0.61	0.61	4.8	4.8	1.4	1.4	13	13

[a] Mean measured concentration.
[b] Calculated individual component TUs.

REFERENCES CHAPTER 1

American Public Health Association, American Water Works Association and Water Environment Federation. 2005. Method 8010E (Table 8010-I). Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health Association, Washington, D.C. Agency for Toxic Substances amd Disease Registry(ATSDR). 1999. Toxicological profile for total petroleum hydrocarbons (TPH) . Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

Antkiewicz DS, Burns CG, Carney SA, Peterson RE, and Heideman W (2005) Heart malformation is an early response to TCDD in embryonic zebrafish. Toxicol Sci 84**:** 368-377.

Arnot, J. A., & Gobas, F. A. (2006). A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. *Environmental Reviews*, *14*(4), 257-297.

ATSDR. 1995. Toxicological profile for polycyclic aromatic hydrocarbons. Atlanta, GA: U.S. Department of Health and Human Services, Agency for Toxic Substances and Disease Registry.

Augustine-Rauch, K., Zhang, C. X., & Panzica-Kelly, J. M. (2010). In vitro developmental toxicology assays: a review of the state of the science of rodent and zebrafish whole embryo culture and embryonic stem cell assays. *Birth Defects Research Part C: Embryo Today: Reviews*, *90*(2), 87-98.

Azimi, S., Rocher, V., Muller, M., Moilleron, R., & Thevenot, D. R. (2005). Sources, distribution and variability of hydrocarbons and metals in atmospheric deposition in an urban area (Paris, France). *Science of the total environment*, *337*(1), 223-239.

Belanger, S.E., Balon, E.K., Rawlings, J.M., 2010. Saltatory ontogeny of fishes and sensitive early life stages for ecotoxicology tests. Aquat. Toxicol. 97, 88–95.

Billiard, S.; Querbach, K.; Hodson, P. *Environmental Toxicology and Chemistry* **1999**, *18*, 2070-2077.

Billiard, S. M., Meyer, J. N., Wassenberg, D. M., Hodson, P. V., & Di Giulio, R. T. (2008). Nonadditive effects of PAHs on Early Vertebrate Development: mechanisms and implications for risk assessment. Toxicological sciences, 105(1), 5-23.

Boelsterli, U. A. (2003). Idiosyncratic drug hepatotoxicity revisited: New insights from mechanistic toxicology. *Toxicology Mechanisms and Methods*, *13*(1), 3-20.

Butler, J.D., Parkerton, T.F., Letinski, D.J., Bragin, G.E., Lampi, M.A., Cooper, K.R., 2013. A Novel Passive Dosing System for Determining the Toxicity of Phenanthrene to Early Life Stages of Zebrafish. Science of the Total Environment.

Carls, M. G., Rice, S. D., & Hose, J. E. (1999). Sensitivity of fish embryos to weathered crude oil: Part I. Low-level exposure during incubation causes malformations, genetic damage, and mortality in larval pacific herring (Clupea pallasi). Environmental Toxicology and Chemistry, 18(3), 481-493.

Carls, M. G., Holland, L., Larsen, M., Collier, T. K., Scholz, N. L., & Incardona, J. P. (2008). Fish embryos are damaged by dissolved PAHs, not oil particles. *Aquatic toxicology*, *88*(2), 121-127.

Chen, Y., Tang, Y., Guo, C., Wang, J., Boral, D., & Nie, D. (2012). Nuclear receptors in the multidrug resistance through the regulation of drug-metabolizing enzymes and drug transporters. *Biochemical Pharmacology*, *83*(8), 1112-1126.

Di Toro, D. M., McGrath, J. A., and Hansen, D. J. (2000). Technical basis for narcotic chemicals and polycyclic aromatic hydrocarbon criteria: I. Water and tissue. *Environ. Toxicol. Chem.* 19, 1951–1970.

Elskus, A. A., Collier, T. K., & Monosson, E. (2005). Interactions between lipids and persistent organic pollutants in fish. *Biochemistry and Molecular Biology of Fishes*, *6*, 119-152.

Eisler, R. 1987. Polycyclic Aromatic Hydrocarbon Hazards to Fish, Wildlife, and Invertebrates: A Synoptic Review. U.S. Fish Wildl. Serv. Biol. Rep. 85(1.11).

Elonen, G. E., Spehar, R. L., Holcombe, G. W., Johnson, R. D., Fernandez, J. D., Erickson, R. J., ... & Cook, P. M. (1998). Comparative toxicity of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin to seven freshwater fish species during early life-stage development. Environmental toxicology and chemistry, 17(3), 472-483.

Ferreira, M., Moradas-Ferreira, P., & Reis-Henriques, M. A. (2006). The effect of long-term depuration on phase I and phase II biotransformation in mullets (< i> Mugil

cephalus</i>) chronically exposed to pollutants in River Douro Estuary, Portugal. Marine environmental research, 61(3), 326-338.

Foekema, E. M., Fischer, A., Parron, M. L., Kwadijk, C., de Vries, P., & Murk, A. J. (2012). Toxic concentrations in fish early life stages peak at a critical moment. Environmental Toxicology and Chemistry, 31(6), 1381-1390.

Gary, J. & Handwerk, G. (2001), Petroleum Refining: Technology and Economics, 4th Edition, CRC Press, ISBN 0-8247-0482-7.

Hutchinson, T.H. *et al.* (2006) Acute and Chronic Effects of Carrier Solvents in Aquatic Organisms: A Critical Review. *Aquat. Toxicol.* 76: 69-92.

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. Toxicology and applied pharmacology, 196(2), 191-205.

Incardona, J. P.; Day, H.L.; Collier, TK.: N. L. Toxicology and Applied Pharmacology 2006, 217, 308-321

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. Toxicology and applied pharmacology, 257(2), 242-249.

Kan, A. T., & Tomson, M. B. (2009). Ground water transport of hydrophobic organic compounds in the presence of dissolved organic matter. *Environmental toxicology and chemistry*, *9*(3), 253-263.

Kipka U, Di Toro DM. 2009. Technical basis for polar and nonpolar narcotic chemicals and polycyclic aromatic hydrocarbon criteria. III. A polyparameter model of target lipid portioning. Environ Toxicol Chem 28:1429–1438.

Korwin-Kossakowski, M., 2008. The influence of temperature during the embryonic period on larval growth and development in carp, Cyprinus carpio L., and grass carp, Ctenopharyngodon idella (Val.): theoretical and practical aspects. Arch. Polish Fish. 16, 231–314.

Latimer, J. S., Hoffman, E. J., Hoffman, G., Fasching, J. L., & Quinn, J. G. (1990). Sources of petroleum hydrocarbons in urban runoff. Water, Air, & Soil Pollution, 52(1), 1-21.

Li, D., Daler, D., 2004. Ocean pollution from land-based sources: East China Sea, China. Ambio 33, 107–113.

Livingstone, D. R. (1998). The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. Comparative Biochemistry and Physiology-Part A: Molecular & Integrative Physiology, 120(1), 43-49.

Marty, G.; Short, J.; Dambach, D.; Willits, N.; Heintz, R.; Rice, S.; Stegeman, J.; Hinton, D. *Canadian Journal of Zoology* **1997**, *75*, 989-1007.

Mayer, P. and Holmstrup, M. (2008) Passive Dosing of Soil Invertebrates with Polycyclic Aromatic Hydrocarbons: Limited Chemical Activity Explains Toxicity Cutoff. *Environ. Sci. Technol.* 42: 7516-7521

McCarty LS, Mackay D. 1993. Enhancing ecotoxicological modeling and assessment. Environ Sci Technol 27:1719–1728.

McGrath JA, Parkerton TF, Di Toro DM. 2004. Application of the narcosis target lipid model to algal toxicity and deriving predicted-no-effect-concentrations. Environ Toxicol Chem 23:2503–2517.

McGrath J, Di Toro D. 2006. Impact of low levels of residual oils on toxicity assessment of oil spills. 04-839. Final Report. Coastal Response Research Center, Durham, NH, USA.

McGrath, J.A. & Di Toro, D.M. 2009. Validation of the target lipid model for toxicity assessment of residual petroleum constituents: Monocyclic and polycyclic aromatic hydrocarbons. Environ. Toxicol. Chem. 28(6): 1130-1148.

McIntosh, S., King, T., Wu, D., & Hodson, P. V. (2010). Toxicity of dispersed weathered crude oil to early life stages of Atlantic herring (Clupea harengus). *Environmental Toxicology and Chemistry*, *29*(5), 1160-1167.

McKim, J.M., 1977. Evaluation of tests with early life stages of fish for predicting long-term toxicity. J. Fish. Res. Board. Can. 34, 1148–1154.

McKim, J.M., 1985. Early Life Stage toxicity tests. In: Rand, G.M. (Ed.), Fundamentals of Aquatic Toxicology. Taylor and Francis, London, pp. 974–1011.

Mimura J, Fujii-Kuriyama Y. Functional role of AhR in the expression of toxic effects by TCDD. Biochim. Biophys. Acta, 2003; 1619: 263–268.

Miracle, A. L., & Ankley, G. T. (2005). Ecotoxicogenomics: linkages between exposure and effects in assessing risks of aquatic contaminants to fish. Reproductive Toxicology, 19(3), 321-326.

Mitchell, K. A., & Elferink, C. J. (2009). Timing is everything: consequences of transient and sustained AhR activity. Biochemical pharmacology, 77(6), 947-956.

National Research Council, 2003. Oil in the Sea III: Inputs, Fates, and Effects. National Academies Press, Washington, DC, p. 446.

Neff, J. M., Stout, S. A., & Gunster, D. G. (2005). Ecological risk assessment of polycyclic aromatic hydrocarbons in sediments: identifying sources and ecological hazard. Integrated Environmental Assessment and Management, 1(1), 22-33.

Neilson, A. H. In *Organic Chemicals: An Environmental Perspective*; CRC Press LLC: Boca Raton, 2000.

NLM (Content Source) Encyclopedia of Earth. Eds. Cutler J. Cleveland (Washington, D.C, National Council for Science and the Environment). [First published in the Encyclopedia of Earth April 20, 2010; Last revised Date February 22, 2012; Retrieved February 12, 2013 <http://www.eoearth.org/article/Biotransformation?topic=58074

Norcross, B., Hose, J., Frandsen, M., and Brown, E. (1996). Distribution, abundance, morphological condition, and cytogenetic abnormalities of larval herring in Prince William Sound, Alaska, following the *Exxon <aldez* oil spill. *Can*. *J*. *Fish*. *Aquat*. *Sci*. 53, 2376}2387.

OECD, 1992.OECD Guidelines for the Testing of Chemicals. Fish Early Life Stage Toxicity Test No. 210: Fish, Juvenile Growth Test. Organization for Economic Cooperation and Development, Paris, France.

OECD, 2006. Draft Proposal for a New Guideline, Fish Embryo Toxicity (FET) Test. Test No. 203.

Prince, R. C., Parkerton, T. F., & Lee, C. (2007). The primary aerobic biodegradation of gasoline hydrocarbons. *Environmental science & technology*, *41*(9), 3316-3321.

Raimondo, S., Montague, B. J., & Barron, M. G. (2007). Determinants of variability in acute to chronic toxicity ratios for aquatic invertebrates and fish. Environmental Toxicology and chemistry, 26(9), 2019-2023.

Redman AD, Parkerton TF, McGrath JA, Di Toro DM. 2012. PETROTOX: an aquatic toxicity model for petroleum substances. ETC 31(11):2498-2506

Roex, E.W.M., van Langen, M.C.T., van Gestel, C.A.M., 2002. Acute toxicity of two compounds with different modes of action to the zebrafish, Danio rerio. Bull. Environ. Contam. Toxicol. 68, 269–274.

Rowlands JC and Gustafsson JA (1997) Aryl hydrocarbon receptor-mediated signal transduction. Crit Rev Toxicol 27: 109-134.

Schimmel, S.C., Hansen, D.J., and Forester, J., Transactions of the American Fisheries Society, Vol. 104, No. 3, 1974 pp.582-586.

Scott, J. A., Incardona, J. P., Pelkki, K., Shepardson, S., & Hodson, P. V. (2011). AhR2-mediated, CYP1A-independent cardiovascular toxicity in zebrafish (< i> Danio rerio</i>) embryos exposed to retene. Aquatic Toxicology, 101(1), 165-174.

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. Chemical research in toxicology, 23(1), 55-65.

Smith, K.E.C. *et al.* (2010) Passive Dosing for Producing Defined and Constant Exposure of Hydrophobic Organic Compounds during *in Vitro* Toxicity Tests. *Chem. Res. Toxicol.* 23: 55-65.

Speight, J (2006), *The Chemistry and Technology of Petroleum*, 4th Edition, CRC Press, ISBN 0-8493-9067-2.

Tuvikene, A. (1995). Responses of fish to polycyclic aromatic hydrocarbons (PAHs). In *Annales Zoologici Fennici* (Vol. 32, No. 3, pp. 295-309). Helsinki: Suomen Biologian Seura Vanamo, 1964-.

Van Leeuwen, C.J., Adema, D.M.M., Hermens, J., 1990. Quantitative

structure-activity relationships for fish early life stage toxicity.Aquat. Toxicol. 16, 321–334.

Ward, G. S. and Parrish, P.R., “Evaluation of Early LifeStage Toxicity Tests with Embryos and Juvaniles of Sheepshead Minnows (Cyprinidon variegates),” Aquatic Toxicology, ASTM STP 707, J.G.Eaton, P,R. Parrish, and A. C. Hendricks, Eds., American Society for Testing and Materials, 1980, pp. 243-247.

Westerfield, M., 2001. The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio). University of Oregon Press.

Woltering, D. M. (1984). The growth response in fish chronic and early life stage toxicity tests: A critical review. Aquatic Toxicology, 5(1), 1-21.

Yu, H. Environmental carcinogenic polycyclic aromatic hydrocarbons: photochemistry and phototoxicity; Journel of Environmental Science and Health Part C 2002, 20, 149-183.

REFERENCES CHAPTER 2

American Public Health Association, American Water Works Association and Water Environment Federation. 2005. Method 8010E (Table 8010-I). Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health Association, Washington, D.C.

Barron, M.G., Carls, M.G., Heintz, R. & Rice, S.D. 2004. Evaluation of fish early life stage toxicity models of chronic embryonic exposures to complex polycyclic aromatic hydrocarbon mixtures. Toxicological Sciences 78(1): 60-67.

Basu, N., Billiard, S., Fragoso, N., Omoike, A., Tabash, S., Brown, S. & Hodson, P. 2001. Ethoxyresorufin-O-deethylase induction in trout exposed to mixtures of polycyclic aromatic hydrocarbons. Environ Toxicol Chem 20: 1244–1251.

Belanger, S.E., Balon, E.K. & Rawlings, J.M., 2010. Saltatory ontogeny of fishes and sensitive early life stages for ecotoxicology tests. Aquat. Toxicol 97: 88– 95.

Bland, M.J. 1995. Multiple significance tests: the Bonferroni method. British Medical Journal 310: 170.

Brown, R., Akhtar, P., Akerman, J., Hampel, L., Kozin, I., Villerius, L. & Klamer, H. 2001. Partition controlled delivery of hydrophobic substances in toxicity tests using poly (dimethylsiloxane) (PDMS) films. Environ Sci Technol 35: 4097–4102.

Bucheli, T.D. & Fent, K. 1995. Induction of Cytrochrome P450 as a Biomarker for Environmental contamination in aquatic ecosystems. Critical Reviews in Environmental Science and Technology 25(3): 201-268.

Butler, J.D., Parkerton, T.F., Letinski, D.J., Bragin, G.E., Lampi, M.A., Cooper, K.R., 2013. A Novel Passive Dosing System for Determining the Toxicity of Phenanthrene to Early Life Stages of Zebrafish. *Science of The Total Environment, Volumes 463–464, 1 October 2013, Pages 952-958*

Call, D.J., Brooke, L.T., Harting, S.L., Poirer, S.H. & McCauley, D.J. 1986. Toxicity of phenanthrene to several freshwater species. *Final Report. U.S. Environmental Protection Agency, Washington, DC.*

Finney, D.J. 1948. The Fisher-Yates Test of Significance in 2X2 Contingency Tables. Biometrika 35: 145-156.

Finney, D.J. 1971. Probit Analysis, 3rd Edition. Cambridge University Press, London, England.

Foster K, Mackay D, Parkerton T, Webster E, Milford L. 2005. Five-stage environmental exposure assessment strategy for mixtures: Gasoline as a case study. *Environ Sci Technol* 39:2711–2718

French-McCay, D.P. 2002. Development and Application of an Oil Toxicity and Exposure Model, OilToxEx. Environmental Toxicology and Chemistry 21(10): 2080-2094.

Hooftman, R.N. & Evers-de Ruiter, A. 1992. Investigations into the aquatic toxicity of phenanthrene (cover-report for reproduction tests with the waterflea *Daphnia magna* and an Early Life Stage (ELS) test with the zebra fish *Brachydanio rerio*. Delft, the Netherlands: TNO Environmental and Energy Research, TNO Institute of Environmental Sciences. TNO-report R 92/290.

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. *Toxicology and Applied Pharmacology* 196: 191-205.

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. *Toxicology and Applied Pharmacology* 217(3): 308-321.

King, D.J., Lyne, R.L., Girling, A., Peterson, D.R., Stephenson, R. & Short, D. 1996. Environmental risk assessment of petroleum substances: the hydrocarbon block method, CONCAWE Report No. 96/52, Brussels, Belgium, 23 pp.

Kiparissis, Y., Akhtar, P., Hodson, P. & Brown, R. 2003. Partition-controlled delivery of toxicants: a novel in vivo approach for embryo toxicity testing. Environ Sci Technol 37: 2262–2266.

Mayer, P. & Holmstrup, M. 2008. Passive dosing of soil invertebrates with polycyclic aromatic hydrocarbons: limited chemical activity explains toxicity cutoff. Environ. Sci. Technol. 42: 7516-7521.

McElroy, A.E., Barron, M.G., Beckvar, N., Driscoll, S.B.K., Meador, J.P., Parkerton, T.F., Preuss, T.G. & Steevens, J.A. 2011. A review of the tissue residue approach for organic and organometallic compounds in aquatic organisms. Integrated Environmental Assessment and Management 7(1): 50–74.

McGrath, J.A. & Di Toro, D.M. 2009. Validation of the target lipid model for toxicity assessment of residual petroleum constituents: Monocyclic and polycyclic aromatic hydrocarbons. Environ. Toxicol. Chem. 28(6): 1130-1148.

OECD 2006. Draft Proposal for a New Guideline, Fish Embryo Toxicity (FET) Test. OECD Guideline for the Testing of Chemicals. Organization for Economic Cooperation and Development, Paris, France.

OECD 1992. OECD Guidelines for the Testing of Chemicals. Section 2: Effects on Biotic Systems Test No. 210: Fish, Early-Life Stage Toxicity Test. Organization for Economic Cooperation and Development, Paris, France.

Passino-Reader et al., 1995. Chronic Bioassays of Rainbow Trout Fry with Compounds Representative of Contaminants in Great Lakes Fish. J Gt Lakes Res 21: 373-383.

Pearson, E.S. & Hartley, T.O. 1962. Biometrika Tables for Statisticians. Vol. 1. Cambridge University Press, London, England. p. 65-70.

Pearson, E.S. & Hartley, T.O. 1962. Biometrika Tables for Statisticians. Vol. 1. Cambridge University Press, London, England. p. 65-70.

Redman, A.D., Parkerton, T.F., McGrath, J.A. & Di Toro, D.M. 2012. An aquatic toxicity model for complex petroleum substances, accepted by Environ Toxicol Chem.

Rhodes, S.M., Farwell, A., Hewitt, L.M., MacKinnon, M.D. & Dixon, D.G. 2005. The effects of dimethylated and alkylated polycyclic aromatic hydrocarbons on the embryonic development of the Japanese medaka. Ecotoxicol Environ Saf 60(3): 247–258.

Rhodes, S.M., Farwell, A., Hewitt, L.M., MacKinnon, M.D. & Dixon, D.G. 2005. The effects of dimethylated and alkylated polycyclic aromatic hydrocarbons on the embryonic development of the Japanese medaka. Ecotoxicol Environ Saf 60(3): 247–258.

Roex, E.W.M., van Langen, M.C.T. & van Gestel, C.A.M. 2002. Acute toxicity of two compounds with different modes of action to the zebrafish, Danio rerio. Bulletin of Environmental Contamination and Toxicology 68(2): 269–274.

SAS Version 9.2. Copyright© 2002-2008 by SAS Institute Inc., Cary, NC, USA.

Smith, K., Dom, N., Blust, R. & Mayer, P. 2010. Controlling and maintaining exposure of hydrophobic organic compounds in aquatic toxicity tests by passive dosing. Aquatic Toxicology. 98: 15–24.

Turcotte, D., Akhtar, P., Bowerman, M., Kiparissis, Y., Brown, R. & Hodson, P. V. 2011. Measuring the toxicity of alkyl-phenanthrenes to early life stages of medaka (*Oryzias latipes*) using partition-controlled delivery. Environmental Toxicology and Chemistry, 487-495.

United States Environmental Protection Agency (USEPA). 2010. Benchmark Dose software, V2.1.2.

Van de Meent, D., Hollander A., Comber, M. & Parkerton, T. 2010. Environmental fate factors and human intake fractions for risk assessment of petroleum products, Integrated Environmental Assessment & Management, 6: 135-144.

Wolińska, L., Brzuzan, P., Woźny, M., Góra, M., Łuczyński, M. K., Podlasz, P., ... & Piasecka, A. (2011). Preliminary study on adverse effects of phenanthrene and its methyl and phenyl derivatives in larval zebrafish, Danio rerio. *Environmental Biotechnology Selected full texts*, *7*(1), 26-33.

WEST Inc. and Gulley, D. D. 1994. TOXSTAT, V.3.4. Western EcoSystems Technology, Inc. Cheyenne, WY.

REFERENCES CHAPTER 3

American Public Health Association, American Water Works Association and Water Environment Federation. 2005. Method 8010E (Table 8010-I). Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health Association, Washington, D.C.

Belanger, S.E., Balon, E.K. & Rawlings, J.M., 2010. Saltatory ontogeny of fishes and sensitive early life stages for ecotoxicology tests. Aquat. Toxicol 97: 88– 95.

Butler, J.D., Parkerton, T.F., Letinski, D.J., Bragin, G.E., Lampi, M.A., Cooper, K.R., 2013. A Novel Passive Dosing System for Determining the Toxicity of Phenanthrene to Early Life Stages of Zebrafish. Science of the Total Environment.

Di Toro DM, McGrath JA, Hansen DJ. 2000. Technical basis for narcotic chemicals and polycyclic aromatic hydrocarbon criteria.I. Water and tissue. *Environ Toxicol Chem* 19:1951–1970.

Finney, D.J. 1971. Probit Analysis, 3rd Edition. Cambridge University Press, London, England.

Fraunhofer-Institut fur Umweltchemie und Okotoxikologie. 1979. Test Report.

Garay V, Roman G,

Isnard P. 2000. Evaluation of PNEC values: Extrapolation from Microtoxt, algae, daphnid, and fish data to HC5. *Chemosphere* 40:267–273.

Hamilton, M. A., Russo, R. C., Thurston R. V. 1977. Trimmed Spearman-Karber Method for Estimating Median Lethal Effect Concentrations in Bioassays. Fisheries Bioassay Laboratory, Montana State University, Bozeman, MT.

McGrath JA, Parkerton TF, Di Toro DM. 2004. Application of the narcosis target lipid model to algal toxicity and deriving predicted-no-effect concentrations. *Environ Toxicol Chem* 23:2503–2517.

McGrath, J.A. & Di Toro, D.M. 2009. Validation of the target lipid model for toxicity assessment of residual petroleum constituents: Monocyclic and polycyclic aromatic hydrocarbons. Environ. Toxicol. Chem. 28(6): 1130-1148.

OECD 1992. OECD Guidelines for the Testing of Chemicals. Section 2: Effects on Biotic Systems Test No. 210: Fish, Early-Life Stage Toxicity Test. Organization for Economic Cooperation and Development, Paris, France.

OECD 2006. Draft Proposal for a New Guideline, Fish Embryo Toxicity (FET) Test. OECD Guideline for the Testing of Chemicals. Organization for Economic Cooperation and Development, Paris, France.

SAS Version 9.2. Copyright© 2002-2008 by SAS Institute Inc., Cary, NC, USA.

Slooff, W. 1979. Detection Limits of a Biological Monitoring System Based on Fish Respiration. Bulletin of Environmental Contamination and Toxicology. 23: 517-523

Smith, K., Dom, N., Blust, R. & Mayer, P. 2010. Controlling and maintaining exposure of hydrophobic organic compounds in aquatic toxicity tests by passive dosing. Aquatic Toxicology. 98: 15–24.

K.E.C. Smith, A. Rein, S. Trapp, P. Mayer, U. Gosewinkel Karlson, Dynamic passive dosing for studying the biotransformation of hydrophobic organic chemicals: microbial degradation as an example, Environ. Sci. Technol. 46 (2012)4852–4860.

Van den Brink PJ, Brock TCM, Posthuma L. 2002. The value of the species-sensitivity distribution concept for predicting field.

Versteeg DJ, Belanger SE, Carr GJ. 1999. Understanding single species and model ecosystem sensitivity: Data-based comparison. *Environ Toxicol Chem* 18:1329–1346.

Westerfield, M. 2001 The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio). University of Oregon Press.

http://europa.eu.int/rapid/pressReleasesAction.do?
reference=MEMO/03/213&format=HTML & aged=0&language= EN &
guiLanguage=en. Accessed on: 27 Apr 2005.

REFERENCES CHAPTER 4

American Public Health Association, American Water Works Association and Water Environment Federation. 2005. Method 8010E (Table 8010-I). Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health Association, Washington, D.C.

Barron, M.G., Carls, M.G., Heintz, R. & Rice, S.D. 2004. Evaluation of fish early life stage toxicity models of chronic embryonic exposures to complex polycyclic aromatic hydrocarbon mixtures. Toxicological Sciences 78(1): 60-67.

Belanger, S.E., Balon, E.K. & Rawlings, J.M., 2010. Saltatory ontogeny of fishes and sensitive early life stages for ecotoxicology tests. Aquat. Toxicol 97: 88– 95.

Bland, M.J. 1995. Multiple significance tests: the Bonferroni method. British Medical Journal 310: 170.

Brown, R., Akhtar, P., Akerman, J., Hampel, L., Kozin, I., Villerius, L. & Klamer, H. 2001. Partition controlled delivery of hydrophobic substances in toxicity tests using poly (dimethylsiloxane) (PDMS) films. Environ Sci Technol 35: 4097–4102.

Butler, J.D., Parkerton, T.F., Letinski, D.J., Bragin, G.E., Lampi, M.A., Cooper, K.R., 2013. A Novel Passive Dosing System for Determining the Toxicity of Phenanthrene to Early Life Stages of Zebrafish. Science of the Total Environment.

DiToro DM, McGrath JA, Hansen DJ. 2000. Technical basis for narcotic chemicals and polycyclic aromatic hydrocarbons criteria. I. Water and tissue. Environ

Toxicol Chem 19:1951–1970.

Dyer S, WarneMJ, Meyer JS, Leslie HA, Escher BI. 2011. Tissue residue approach for chemical mixtures. Integr Environ Assess Manag 7:99–115.

Engraff, M., Solere, C., Smith, K. E., Mayer, P., & Dahllöf, I. (2011). Aquatic toxicity of PAHs and PAH mixtures at saturation to benthic amphipods: Linking toxic effects to chemical activity. Aquatic Toxicology, 102(3), 142-149.

Finney, D.J. 1971. Probit Analysis, 3rd Edition. Cambridge University Press, London, England.

Finney, D.J. 1948. The Fisher-Yates Test of Significance in 2X2 Contingency Tables. Biometrika 35: 145-156.

Incardona, J. P., Collier, T. K., and 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.

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. Toxicology and Applied Pharmacology 217(3): 308-321.

Kiparissis, Y., Akhtar, P., Hodson, P. & Brown, R. 2003. Partition-controlled delivery of toxicants: a novel in vivo approach for embryo toxicity testing. Environ Sci Technol 37: 2262–2266.

Kortenkamp A, Backhaus T, Faust M. 2009. State of the art report on mixture toxicity. Brussels: European Commission, DG Environment. 391 p.

Landrum, P. F., Chapman, P. M., Neff, J., & Page, D. S. (2012). Evaluating the aquatic toxicity of complex organic chemical mixtures: Lessons learned from polycyclic aromatic hydrocarbon and petroleum hydrocarbon case studies. Integrated Environmental Assessment and Management, 8(2), 217-230.

Mayer, P. & Holmstrup, M. 2008. Passive dosing of soil invertebrates with polycyclic aromatic hydrocarbons: limited chemical activity explains toxicity cutoff. Environ. Sci. Technol. 42: 7516-7521.

McCarty LS, Landrum PF, Luoma SN, Meador JP, Merten AA, Shephard BK, van Wezel AP. 2011. Advancing environmental toxicology through chemical dosimetry: External exposures versus tissue residues. Integr Environ Assess Manag 7:7–27.

McGrath, J. A., Parkerton, T. F., & Di Toro, D. M. (2004). Application of the narcosis target lipid model to algal toxicity and deriving predicted-no-effect concentrations. Environmental toxicology and chemistry, 23(10), 2503-2517.

McGrath, J.A. & Di Toro, D.M. 2009. Validation of the target lipid model for toxicity assessment of residual petroleum constituents: Monocyclic and polycyclic aromatic hydrocarbons. Environ. Toxicol. Chem. 28(6): 1130-1148.

OECD 2006. Draft Proposal for a New Guideline, Fish Embryo Toxicity (FET) Test guideline 203. OECD Guideline for the Testing of Chemicals. Organization for Economic Cooperation and Development, Paris, France.

OECD 1992. OECD Guidelines for the Testing of Chemicals. Section 2: Effects on Biotic Systems Test No. 210: Fish, Early-Life Stage Toxicity Test. Organization for Economic Cooperation and Development, Paris, France.

Pearson, E.S. & Hartley, T.O. 1962. Biometrika Tables for Statisticians. Vol. 1. Cambridge University Press, London, England. p. 65-70.

SAS Version 9.2. Copyright© 2002-2008 by SAS Institute Inc., Cary, NC, USA.

Smith, K., Dom, N., Blust, R. & Mayer, P. 2010. Controlling and maintaining exposure of hydrophobic organic compounds in aquatic toxicity tests by passive dosing. Aquatic Toxicology. 98: 15–24.

Turcotte, D., Akhtar, P., Bowerman, M., Kiparissis, Y., Brown, R. & Hodson, P. V. (2011). Measuring the toxicity of alkyl-phenanthrenes to early life stages of medaka (Oryzias latipes) using partition-controlled delivery. Environmental Toxicology and Chemistry, 487-495.

Westerfield, M. 2001 The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio). University of Oregon Press.

SAS Version 9.2. Copyright© 2002-2008 by SAS Institute Inc., Cary, NC, USA.

United States Environmental Protection Agency (USEPA). 2010. Benchmark Dose software, V2.1.2.

WEST Inc. and Gulley, D. D. 1994. TOXSTAT, V.3.4. Western EcoSystems Technology, Inc. Cheyenne, WY.

REFERENCES CHAPTER 5

Belanger, S.E., Balon, E.K. & Rawlings, J.M., 2010. Saltatory ontogeny of fishes and sensitive early life stages for ecotoxicology tests. Aquat. Toxicol 97: 88– 95.

Bland, M.J. 1995. Multiple significance tests: the Bonferroni method. British Medical Journal 310: 170.

Bertilsson, S., & Widenfalk, A. (2002). Photochemical degradation of PAHs in freshwaters and their impact on bacterial growth–influence of water chemistry. *Hydrobiologia*, *469*(1-3), 23-32.

Call, D.J., Brooke, L.T., Harting, S.L., Poirer, S.H. & McCauley, D.J. 1986. Toxicity of phenanthrene to several freshwater species. *Final Report. U.S. Environmental Protection Agency, Washington, DC.*

Di Toro DM, McGrath JA, Hansen DJ. 2000. Technical basis for narcotic chemicals and polycyclic aromatic hydrocarbon criteria.I. Water and tissue. *Environ Toxicol Chem* 19:1951–1970.

Hooftman, R.N. & Evers-de Ruiter, A. 1992. Investigations into the aquatic toxicity of phenanthrene (cover-report for reproduction tests with the waterflea Daphnia magna and an Early Life Stage (ELS) test with the zebra fish Brachydanio rerio. Delft, the

Kiparissis, Y., Akhtar, P., Hodson, P. & Brown, R. 2003. Partition-controlled delivery of toxicants: a novel in vivo approach for embryo toxicity testing. Environ Sci Technol 37: 2262–2266.

McGrath, J.A. & Di Toro, D.M. 2009. Validation of the target lipid model for toxicity assessment of residual petroleum constituents: Monocyclic and polycyclic aromatic hydrocarbons. Environ. Toxicol. Chem. 28(6): 1130-1148.

Netherlands: TNO Environmental and Energy Research, TNO Institute of Environmental Sciences. TNO-report R 92/290.

Passino-Reader et al., 1995. Chronic Bioassays of Rainbow Trout Fry with Compounds Representative of Contaminants in Great Lakes Fish. J Gt Lakes Res 21: 373-383.

Pearson, E.S. & Hartley, T.O. 1962. Biometrika Tables for Statisticians. Vol. 1. Cambridge University Press, London, England. p. 65-70.

Fu, P. P., Xia, Q., Sun, X., & Yu, H. (2012). Phototoxicity and Environmental Transformation of Polycyclic Aromatic Hydrocarbons (PAHs)—Light-Induced Reactive Oxygen Species, Lipid Peroxidation, and DNA Damage. *Journal of Environmental Science and Health, Part C, 30*(1), 1-41.

Rhodes, S.M., Farwell, A., Hewitt, L.M., MacKinnon, M.D. & Dixon, D.G. 2005. The effects of dimethylated and alkylated polycyclic aromatic hydrocarbons on the embryonic development of the Japanese medaka. Ecotoxicol Environ Saf 60(3): 247–258.

Roex, E.W.M., van Langen, M.C.T. & van Gestel, C.A.M. 2002. Acute toxicity of two compounds with different modes of action to the zebrafish, Danio rerio. Bulletin of Environmental Contamination and Toxicology 68(2): 269–274.

Salihoglu, N. K., Karaca, G., Salihoglu, G., & Tasdemir, Y. (2012). Removal of polycyclic aromatic hydrocarbons from municipal sludge using UV light. *Desalination and Water Treatment*, *44*(1-3), 324-333.

Strähle, U., Scholz, S., Geisler, R., Greiner, P., Hollert, H., Rastegar, S., ... & Braunbeck, T. (2012). Zebrafish embryos as an alternative to animal experiments—A commentary on the definition of the onset of protected life stages in animal welfare regulations. Reproductive Toxicology, 33(2), 128-132.

Turcotte, D., Akhtar, P., Bowerman, M., Kiparissis, Y., Brown, R. & Hodson, P. V. 2011. Measuring the toxicity of alkyl-phenanthrenes to early life stages of medaka (*Oryzias latipes*) using partition-controlled delivery. Environmental Toxicology and Chemistry, 487-495.

Wolińska, L., Brzuzan, P., Woźny, M., Góra, M., Łuczyński, M. K., Podlasz, P., ... & Piasecka, A. (2011). Preliminary study on adverse effects of phenanthrene and its methyl and phenyl derivatives in larval zebrafish, Danio rerio. *Environmental Biotechnology Selected full texts*, *7*(1), 26-33.

BIBLIOGRAPHY

EDUCATION

Rutgers University, New Brunswick, NJ

Doctor of Philosophy in Environmental Science (focus in environmental toxicology), Defended July 11th 2013

> *Dissertation*:The application of a passive dosing system for determining zebrafish early life stage toxicity of hydrocarbons for use in calibration a predictive model to acute and chronic endpoints

East Stroudsburg University, East Stroudsburg, PA

Master of Science, *cum laude,* in Biology, January 2009

> *Thesis:* Teratogenic, sub-lethal and lethal effects of 3, 4 Dichloroaniline on zebrafish embryos and research towards an alternative to fish acute toxicity testing

Bachelor of Science, in Parks and Recreation Management (environmental focused) May 2007

Community College of Baltimore County (Essex)

A.S. (general education) May, 2005

EXPERIENCE

ENVIRONMENTAL TOXICOLOGIST (ExxonMobil Biomedical Sciences Inc.)

(2008 – present)

Duties include performing fresh and salt-water acute and chronic fish, fish embryo, invertebrate and algal toxicity tests, as well as, terrestrial springtail, earthworm and plant toxicity tests. Have a working knowledge of bio-molecular mechanisms and modes of toxic action for various compound classes. Have an in depth understanding of analytical techniques used to quantify toxic compounds (GC-FID-HS, GC-MS, HPLC, TOC, UV-Vis etc…). Work with internal and external colleagues to publish, prepare and present data generated to various regulatory agencies as well as separate entities within the scientific community.

FISHERIES BIOLOGIST (New Jersey Division of Fish &Wildlife) (Summer 2007)

Duties included the identification of numerous species of aquatic organisms. Familiar with aquatic organism sampling techniques (seining, kick-netting etc…). Assisted in the discovery of numerous thermoclines of New Jersey lakes as well as analyzing their water for pH, alkalinity/hardness, conductivity, dissolved oxygen and temperature. Prepared written reports for biologist at the laboratory and developed a good working knowledge of GPS/GIS and topographical maps.

PROFESSIONAL ORGANIZATIONS

SOCIETY OF ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY (SETAC), NA

SOCIETY OF ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY (SETAC), HDC
-*Society of Environmental Toxicology and Chemistry Board of Directors (2011-present)*
Organizing and executing successful bi-annual meetings in the Hudson-Delaware area.

HEALTH AND ENVIRONMENTAL SCIENCES INSTITUTE (HESI)
-Health and Environmental Sciences Institute Animal Alternative Committee
Organizing strategic meetings between EU, China, Canada, Japan, and the US to discuss and extrapolate data which will enable an alternative movement in toxicity testing.

PUBLICATIONS

Available upon request, and included in CV (below).

INTERESTS

Outdoor recreation, volunteering, reading, learning